Volume 8 Number 2 1980 Nucleic Acids Research

Assignment of reovirus mRNA ribosome binding sites to virion genome segments by nucleotide sequence analyses

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Received 21 August 1979

SUMMARY

All ten reovirus genome RNA segments were radiolabeled at their 3'-termini by incubation with RNA ligase and ^{32}pCp. The extent of radiolabeling was similar for each of the double-stranded RNAs in the genome segment mixture. Radioactivity was equally distributed between the separated plus and minus strands indicating that the 5'-cap in plus strands did not block 3'-end-labeling of minus strands. The 3'-termini of the four S and three M segments included the common sequences: ...U-A-G-C in minus strands and ...U-C-A-U-C in plus strands. By comparing the minus strand 3'-sequences with 5'-sequences of reovirus mRNAs, small-size genome segments S2, S3 and S4 were correlated with the previously sequenced initiation fragments s46, s45 and s54 derived from small class mRNAs. Medium-size genome segments M1, M2 and M3 similarly were correlated with fragments m30, m52 and m44, respectively. The N-terminal amino acid sequences deduced from the mRNA nucleotide sequences can now be assigned to the nascent chains of particular reovirus proteins.

INTRODUCTION

Human reovirus type 3 is the prototype of many different double-stranded RNA-containing viruses that infect a wide variety of animals and plants (1-3) as well as some unicellular organisms including yeasts and fungi (4) and bacteria (5). Extensive studies on the molecular biology of reoviruses have revealed a number of striking features, many of them characteristic of eukaryotic viral and cellular systems. The genome of reovirus, like other double-stranded and some single-stranded RNA viruses, is segmented (6). Although all ten reovirus genome segments are required for infectivity (7), the mechanisms for their correct assembly into virions during morphogenesis remains an intriguing unknown. In addition to the genome RNA, reovirions contain RNA polymerase that transcribes one strand of each of the duplex segments to form the viral messenger RNAs (8-10). Nucleotide phosphohydrolase, guanylyl transferase and methyl transferases, enzymes that modify the 5'-ends of nascent viral mRNAs, are also packaged within purified reovirions (11). Similar transcriptase and RNA-modifying activities have been found in both DNA and RNA

viruses (12). Consequently, reovirus mRNAs (13) and other viral mRNAs synthesized in the presence of the methyl donor, S-adenosylmethionine, have a methylated, "capped" 5'-terminal structure, $m^7G(5')ppp(5')N$ identical to the 5'-caps found in most eukaryotic viral and cellular mRNAs (14).

Several different studies with reovirus mRNA have provided evidence that the cap is involved in the initiation of protein synthesis (15-17) and formed the basis for a general model to explain how eukaryotic ribosomes may select initiation regions in mRNAs (18). Ten primary viral polypeptides are synthesized in reovirus mRNA directed cell-free translating systems and in reovirus-infected mouse L cells (3,19). Eight of these products are assembled into virions, three forming the outer protein shell and five comprising the inner core particle. It remains to be determined which of the polypeptides correspond to the five enzyme activities that are also present in viral cores. The virus-specified polypeptides correspond in size to the coding capacities of the ten viral mRNAs, and recent biochemical and genetic findings have allowed the assignment of the viral polypeptides to the individual reovirus genome segments which encode them (20,21). Furthermore, the ribosome binding sites of six of the ten reovirus mRNAs, derived as 40S ribosome-protected fragments from RNase-treated initiation complexes, have been sequenced (22). For each mRNA, a single initiation fragment was obtained that included the cap and the 5'-proximal A-U-G codon (23). However, genome segment assignment of the fragments was not made.

As part of a continuing effort to understand how the genome segments of reovirus and other multisegmented viruses replicate and assemble, we have been analyzing the structures and sequences of the termini of the viral RNAs. Recently it was found that the 3'-termini of both strands of the ten reovirus genome RNAs can be labeled by RNA ligase-catalyzed addition of ^{32}pCp (24). We have used this method to prepare ^{32}P-labeled reovirus RNA for gel sequencing. By comparing the 3'-sequences of separated strands of the duplex RNAs with the mRNA 5'-terminal sequences established previously (25), we have been able to relate mRNA ribosome binding sites to individual reovirus genome segments of the S and M size classes. Taken together with the protein coding assignments (20,21), the results allow a prediction of N-terminal amino acid sequences of reovirus nascent polypeptides.

MATERIALS AND METHODS

Virus RNA.

Reovirus type 3 Dearing strain was purified from infected mouse L

cells, and the double-stranded viral genome RNA was obtained by phenol extraction and Sephadex G-100 gel filtration as detailed previously (26).

3'-End labeling of RNA and isolation of individual genome segments.

Genome RNA was ^{32}P-labeled at the 3'-termini by the method of England et al. (27). In a typical experiment a specific activity of 2 x 10^6 cpm/µg RNA, corresponding to about 24% of the theoretical maximum, was obtained by incubation of 160 µg of RNA for 16 hr in a reaction volume of 0.3 ml containing 150 units of RNA ligase (PL Biochemical) and 500 µCi ^{32}pCp (s.a. = 2,300 Ci/mmole, New England Nuclear). After purification by G-100 gel filtration and ethanol precipitation, the RNA was separated into ten segments by electrophoresis for 40 hr at 130 volts (25-30 ma) in a 30 x 14 x 0.15 cm, 5% polyacrylamide slab gel in 36 mM Tris-HCl - pH 7.4 containing 30 mM NaH_2PO_4 and 1 mM EDTA. RNA bands were located by autoradiography of the wet gel on Kodak XR-5 film. Regions of the gel containing the bands were cut from the slab, crushed by passage through an 18 gauge hypodermic needle, and the RNA was eluted by shaking at 37°C for 24 hr in 10 ml of 50 mM Tris buffer - pH 6.8 containing 0.1 M NaCl, 1 mM EDTA and 0.1% sodium dodecyl sulfate. Residual fragments of polyacrylamide were removed from the solutions of eluted RNA by centrifugation (9,000 rpm, 10 min) followed by passage through a small column of glass wool. RNA was recovered and further purified by ethanol precipitation three times, and the pellet was washed with 95% ethanol.

Separation of the plus and minus RNA strands of genome segments.

Reovirus mRNA was synthesized in vitro with viral cores and separated into the large, medium and small classes by glycerol gradient centrifugation (19,26). For the studies reported here, a purified ^{32}P-labeled individual genome RNA segment of the S or M class was mixed with a greater than ten-fold excess of the corresponding nonradioactive mRNA class ($\sim 10^7$ cpm of one genome RNA segment and 2-5 A_{260} units of small or medium class mRNAs). The RNA was denatured by incubation for 30 min at 37°C in 90% dimethyl sulfoxide in 20 mM Tris buffer - pH 7.4 containing 1 mM EDTA. The denatured RNA was ethanol-precipitated and dissolved in 0.4 ml of 10 mM Tris buffer - pH 7.4 containing 0.3 M KCl and 1 mM EDTA. After annealing for 8 hr at 68°C, samples were diluted two-fold with H_2O, and the RNA was precipitated at -20°C by addition of two volumes of ethanol. The single-stranded and double-stranded RNAs were then separated by chromatography on cellulose CF-1 (28). Because strand displacement was found to be incomplete at this stage, the annealing and CF-1 column chromatography were repeated.

Sequence analysis.

^{32}P-Labeled plus strands and duplexes with ∿95% of their ^{32}P content in minus strands were recovered from CF-1 cellulose by ethanol precipitation for subsequent analysis by partial digestion and polyacrylamide gel electrophoresis (29,30). The duplexes were denatured in dimethyl sulfoxide and reprecipitated with ethanol before digestion. RNAs were dissolved in H_2O, and 5 µl aliquots (∿20,000 cpm) were used for each partial digestion in 10 µl final volumes containing 20 µg of carrier yeast tRNA (twice phenol-extracted) and the following additions: $\bar{OH}$: 50 mM $NaHCO_3$, 1 mM EDTA - pH 9; T_1: 0.005 units RNase T_1 in 10 mM Tris buffer - pH 7.4, 1 mM EDTA; U_2: 0.025 units RNase U_2 in 10 mM NaAc buffer - pH 4.5; Phy: 0.1 µg Physarum polycephalum RNase PhyI in 10 mM NaAc buffer - pH 4.5; and Panc: 0.1 ng pancreatic RNase in 10 mM Tris buffer - pH 7.4, 1 mM EDTA. Incubations were for 15 min at 37°C except the alkaline hydrolysis which was for 30 min at 90°C. For chemical digestion of plus strands, reactions were carried out as described by Peattie (31). Digests dissolved in 2-5 µl of buffer consisting of 20 mM Tris-HCl, 1 mM EDTA, 0.05% each of xylene cyanol and bromophenol blue, and 8 M urea - pH 7.4 were loaded onto 28x37x0.03 cm, 20% polyacrylamide slab gels. Electrophoresis was in the same buffer for 5-30 hr at 1000 volts. Autoradiographs were obtained by exposure for 2-14 days to pre-flashed XR-5 film at -70°C with Dupont "Lightning Plus" intensifying screens.

Enzymes.

RNase PhyI was purified from culture fluids of P. polycephalum by ammonium sulfate precipitation and DE-52 cellulose chromatography (32). Pancreatic RNase was purchased from Worthington Biochemical Corp. and RNases T_1 and U_2 from Calbiochem.

RESULTS

3'-End labeling of genome RNA plus and minus strands.

Previous studies of reovirus genome RNA terminally labeled with γ-^{32}P-ATP and polynucleotide kinase (33) or by reduction with ^{3}H-borohydride (34) established that the ends of the two strands of each duplex segment are base-paired in the structure:

(+) 5' m^7GpppG^m-C...A-U-C 3'

(-) 3' C -G...U-A-Gpp 5'

Incubation with RNA ligase and ^{32}pCp also radiolabeled reovirus genome RNA. Consistent with 3'-terminal attachment of ^{32}pCp, the ten genome segments ranging in molecular weight from 0.6 x 10^6 to 2.7 x 10^6 (6) were radiolabeled

to a similar extent by this procedure (Fig. 1, left lane). Before separating the plus and minus strands, the ^{32}P-labeled duplex segments recovered from the gel were re-analyzed by electrophoresis under the same conditions as a test of purity. As shown in Fig. 1, each of the recovered bands consisted of a single RNA segment.

Each M and S duplex segment was separated into its constituent plus and minus strands by annealing with excess unlabeled reovirus mRNA. As shown in Fig. 2A for representative segment S3, the peak of ^{32}P-labeled plus strands (fractions 21-28) that were displaced from duplex RNA (fractions 41-48) by a single round of annealing comprised less than the expected 50% of the counts. However, after a second round of annealing 80% of the radioactivity eluted as double-stranded RNA consistent with the ^{32}P-labeled plus strand having been effectively "chased" from the duplex (Fig. 2B). The completeness of plus and minus strand separation was checked by DEAE-cellulose chromatography of the ^{32}P-labeled oligonucleotides released from the single-stranded and double-stranded RNA by RNase T_1 digestion. As shown for the representative RNA segment S3, essentially all of the radioactivity from the single-stranded RNA eluted as a single peak in the position of a long oligonucleotide of net ne-

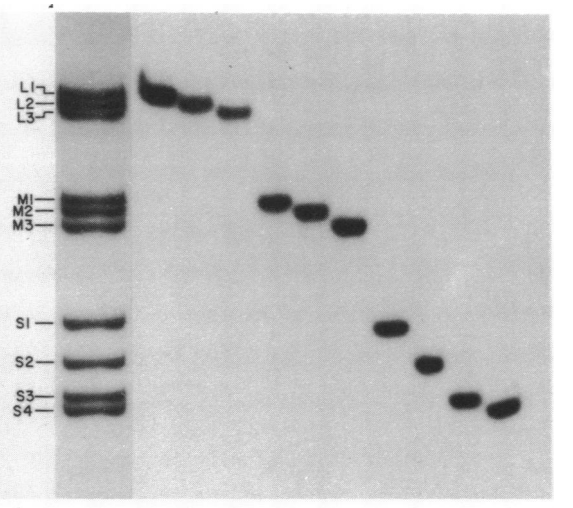

Figure 1: Polyacrylamide gel electrophoresis of ^{32}pCp-labeled reovirus genome RNA. Double-stranded RNA from purified virions was radiolabeled and analyzed as described in Materials and Methods. The ten genome segments (L1-3, M1-3, S1-4) located by autoradiography were eluted from the individual bands and separately analyzed under the same conditions.

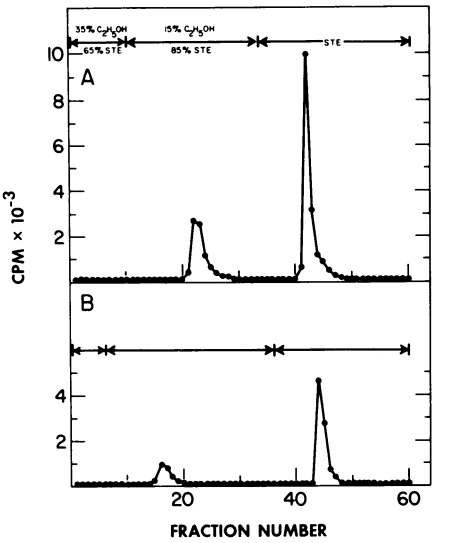

Figure 2: Separation of reovirus RNA plus strands and duplexes containing radiolabeled minus strands by chromatography on CF-1 cellulose.
A. ^{32}P-labeled double-stranded RNA segment S3 was annealed with an excess of mRNA and analyzed by CF-1 cellulose column chromatography. Aliquots of eluted fractions were counted, and the peak fractions pooled and ethanol-precipitated. Total radioactivity in plus strands (fractions 21-28) and duplex RNA (41-48) was 8.1 x 10^5 and 2.5 x 10^6 cpm, respectively.
B. The double-stranded RNA (fractions 41-48) was re-annealed with mRNA and analyzed again as in panel A. The distribution of radioactivity was 1:4 for single-stranded:double-stranded RNAs. STE = 0.1 M NaCl, 50 mM Tris-HCl - pH 7.4, 1 mM EDTA.

gative charge more than 6 (Fig. 3A). RNase T_1 digestion of the denatured double-stranded RNA, by contrast, released 95% of the radioactivity as a fragment that eluted with a net charge close to -3 (Fig. 3B). Since the 3'-terminus of the minus strands of the reovirus genome RNA is ...GpC (34), RNase T_1 digestion of ...GpC$\overset{*}{\text{p}}$Cp would be expected to release C$\overset{*}{\text{p}}$Cp of net charge of -3. The remaining radioactivity from the double-stranded RNA eluted as a longer oligonucleotide in a position consistent with its release from residual ^{32}P-labeled plus strands in the re-annealed duplexes. Thus the single-stranded plus RNA appeared to be free of ^{32}P-labeled minus strands while the minus strands in duplexes remained about 6% contaminated with ^{32}P-labeled complement even after re-annealing. From these and similar results obtained with the other segments it was calculated that more than 90% of the radioactivity in the re-annealed duplexes resided in minus strands,

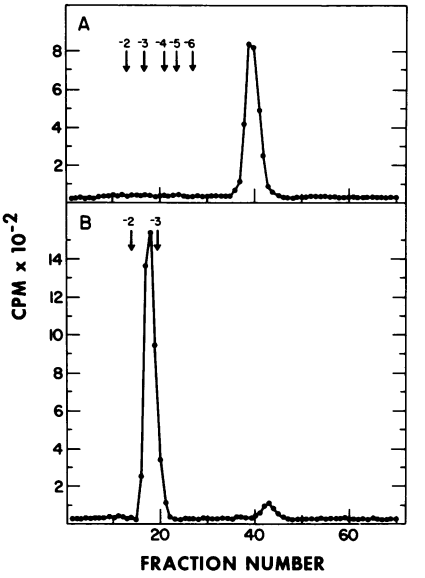

Figure 3: DEAE-cellulose chromatography of RNase T_1 digests of separated plus and minus strands of reovirus RNA. ^{32}P-Labeled genome segment S3 was separated into plus strands and duplexes containing radiolabeled minus strands as in Fig. 2. The plus strands (panel A) and minus strands (panel B) obtained by ethanol precipitation after denaturation with 90% dimethyl sulfoxide were incubated for 2 hr at 37°C in 0.2 ml of 50 mM Tris buffer - pH 7.5 containing 2 mM EDTA, 1 mg yeast tRNA, and 300 units RNase T_1 and analyzed by DEAE-cellulose column chromatography as described previously (35). The arrows indicate the elution positions of marker oligonucleotides of increasing net negative charge obtained from the A_{260} profile.

and the overall distribution of radioactivity between plus and minus strands was about equal. Thus, both basepaired 3'-ends of each of the double-stranded RNA segments were susceptible to RNA ligase-catalyzed transfer of ^{32}pCp indicating that the presence of the 5'-cap in the complementary strand (35) did not block labeling of the 3'-end of the minus strand. Furthermore, unlike the 3'-termini, the 3'-hydroxyl group in the 5'-linked m^7G was not a substrate for RNA ligase.

Minus strand 3'-terminal sequences.

Figures 4 and 5 show autoradiographs of gels of partial digests of the ^{32}P-labeled minus strands of genome segments S1-S4. The sequences are summarized in Table I. Four nucleotides were common at the minus strand 3'-ends.

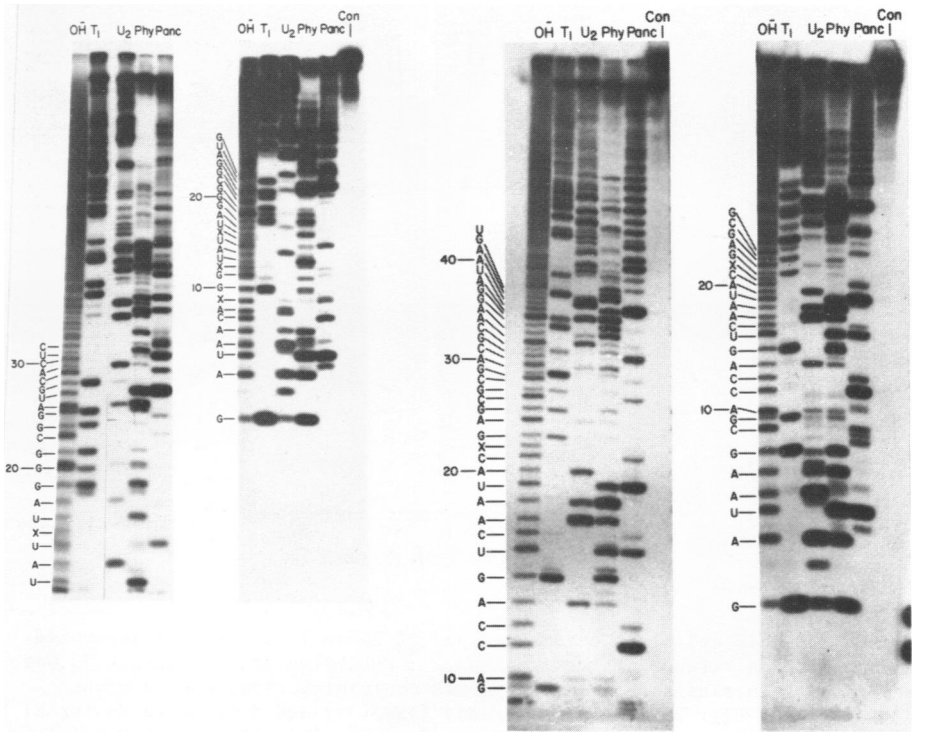

Figure 4: Analyses of segment S1 (left) and S2 (right) minus strands. Migration times for the gel profiles from right to left were about 6, 14, 5 and 15 hr.

M segment minus strands contained the same sequence, ...U-A-G-C-3' (data not shown) in agreement with the complementary 5'-sequences determined for the plus-stranded mRNAs (25).

Since the reovirus genome segments consist of plus and minus strands that are basepaired end-to-end (34) and the viral mRNAs apparently are complete copies of the plus strands (10), the 3'-terminal sequences of the reovirus genome segment minus strands should be complementary to the 5'-ends of the corresponding viral mRNAs. This comparison is shown in Table II. The 5'-terminal sequences were obtained by fingerprinting uniformly ^{32}P-labeled reovirus mRNAs synthesized *in vitro* (25); the S and M segment 5'-sequences were deduced from the experimentally determined 3'-terminal sequences of minus strands. Although many residues were not identified by the gel method, there was sufficient information to allow comparison of the genome and mes-

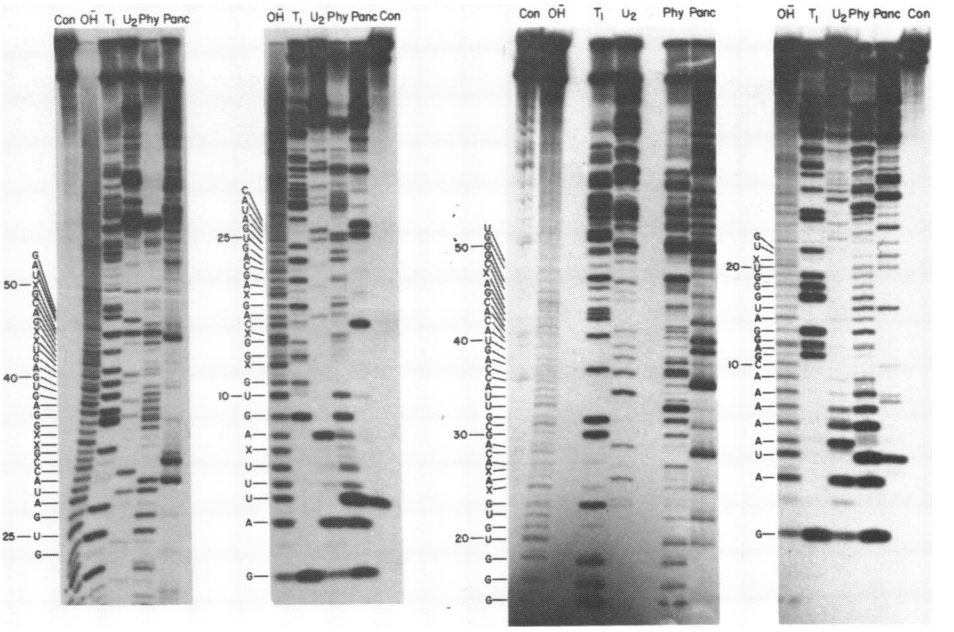

Figure 5: Analyses of segment S3 (left) and S4 (right) minus strands. Times of migration for the gel profiles shown right to left were about 6.5, 18, 7 and 20 hr.

sage sequences. The complementary sequences of genome segments S2, S3 and S4 correspond reasonably well to the three ribosome binding sites, s46, s45 and s54 that were derived previously from a mixture of reovirus s mRNAs by RNase trimming of 40S initiation complexes (25). Segments M1, M2 and M3 apparently are the templates for fragments m30, m52 and m44. [The mRNA fragment designations were based on fragment chain length, counting the cap m^7G

Table I

3'-Terminal Sequences of Reovirus Genome RNA Minus Strands

3'	10	20	30	40	50
S1	C G A U AACAXGGXUAUXUAGGGCGGAUGCACUC...				
S2	C G A U AAGCGACCAGUCAAUACXGAGCGCGACGCAAGGAUAAGU...				
S3	C G A U UUXAGUGXGGXCAGXAGCAGUGAUACCGXXGGAGUGAGUXXGACGXUAG...				
S4	C G A U AAAAACGAGGAUGGGUGUGXAXACAGCGUUACCAGUACACGAXCGGGU...				

Table II

Comparison of Sequences Complementary to the 3'-Termini of Genome Minus Strand RNAs and 5'-Termini of S and M Class mRNAs*

5'	1	10	20	30	40	50
S1	G CUAUUGUXCCXAUAXAUCCCGCCUACGUGAG...					
S2	G CUAUUCGCUGGUCAGUUAUGXCUCGCGCUGCGUUCCUAUUCA...					
mRNA (s46)	m^7GpppG^mCUAUUCGCUGGUCAGUUAUGGCUCGCUGCGCGUUCCUAUUCAAG...					
S3	G CUAAAXUCACXCCXGUCXUCGUCACUAUGGCXXCCUCACUCAX...					
mRNA (s45)	m^7GpppG^mCUAAAGUCACGCCUGUCGUCGUCACUAUGGCUUCCUCACUCAG...					
S4	G CUAUUUUUGCUCCUACCCACACXUXUGUCGCAAUGGUCAUGUGCUXGCCCA...					
mRNA (s54)	m^7GpppG^mCUAUUUU-GCCUCUUCCCAGACGUUGUCGCAAUGGAGGUGUGCUUGCCCAACG...					
M1	G CUAUUCGCG					
mRNA (m30)	m^7GpppG^mCUAUUCGCG					
M2	G CUAAUCUGCXGACCGU					
mRNA (m52)	m^7GpppG^mCUAAUCUGCUGACCGU					
M3	G CUAXXXUGACCCUGAUC					
mRNA (m44)	m^7GpppG^mCUAAAGUGACCGUGGUC					

*mRNA sequences from reference 25.

as residue number one (25).] The transcript of S1 apparently did not yield a ribosome-protected site that could be sequenced in the previous studies of reovirus mRNAs, possibly because it was present in low amounts in the mRNA mixture or was inefficiently bound to, or protected by, ribosomes.

The results provide some additional information about the ribosome binding sequences comprising the 5'-termini of reovirus mRNAs. In s46 the order of the six underlined residues was not determined by fingerprinting. The data obtained with the corresponding genome segment S2 indicate that the order is C-G-C-G-C-U. For segment S3 and the corresponding ribosome binding fragment, s45, there was good agreement between the sequences, but several of the residues in the S 3 sequence were not identified. There was some disparity between the sequence of segment S4 and its putative mRNA fragment s54,

possibly due to technical difficulties such as the presence of contaminating residual plus strands that cause artifactual bands in the gel profiles of partial digests. Finally, the gel analyses indicated the presence of five consecutive U residues at positions 5-9 of segment S4. Only four U's were reported in this region from fingerprint analyses (25,36), as indicated by the dash in s54. However, Hastings and Millward found (37) that the 5'-terminal T_1 RNase fragment of one of the mRNAs was m^7GpppG^m-CpU-A-U-U-U-U-U-Gp, consistent with the sequence deduced from the minus strand of genome segment S4 (Table II).

Plus strand 3'-terminal sequences.

Partial enzymatic digests of 3'-end-labeled plus strands of the individual S and M genome segments were analyzed by electrophoresis in polyacrylamide gels. Identification of some 3'-proximal sequences also required gel electrophoresis of chemical digests and two-dimensional analysis by the wandering spot method (38). A common sequence, ...U-C-A-U-C-3' was present in all seven plus strands, and G was absent from the first ten or more nucleotides in each RNA.

DISCUSSION

The aim of the present study was two-fold: (i) to relate the different reovirus genome segments to the corresponding mRNA 5'-terminal sequences that were shown to be ribosome binding sites in cell-free translation initiation studies (25) and (ii) to determine if, as in the case of the single-stranded genome RNAs of influenza virus (39-41), the multiple double-stranded genome RNA segments of reovirus have common terminal sequences. For these purposes RNA ligase-catalyzed transfer of ^{32}pCp (27) was used to label both 3'-termini of the genome duplexes, and the separated plus and minus strands of individual S and M class segments were sequenced by partial digestion and polyacrylamide gel electrophoresis. Extensive, complete nucleotide sequences were not obtained by the enzymatic procedure because of the presence of base-paired, enzyme-resistant sites in the RNAs and uncertainties due to sequence specific cleavages by Phy and pancreatic RNases. Another possible source of difficulty in both enzymatic and chemical digests was the presence of residual contaminating complementary strand RNA. Nevertheless, the data were sufficient to establish that the four reovirus genome S segments and three M segments contain common 3'-terminal sequences, ...U-A-G-C-3' in minus strands and ...U-C-A-U-C-3' in plus strands. In three S species and M3 plus strands

the sixth residue was U, but in segments S3, M1 and M2 a mixture of A and U residues were detected in this position, consistent with sequence heterogeneity. Further analyses of RNA from recloned virus are needed to assess the significance of this result. Common terminal sequences in genome RNA segments may be a characteristic of multi-segmented RNA viruses related to genome assembly during maturation. For reovirus the terminal sequences presumably are recognized by the viral transcriptase and replicase activities as the respective starting sites for the synthesis of mRNAs and the minus strands of genome duplex RNAs. Since the viral mRNAs are transcribed end-to-end on template genome segment RNAs and are not polyadenylated (42), the plus strand 3'-sequences also correspond to eukaryotic transcription terminators. It is of interest in this regard that the 3'-ends of the plus strands generally are rich in A and U residues and preceded by G clusters, i.e. similar to a variety of bacterial terminators (43).

Recently it was found that reovirus genome segment S1 codes for a minor component of the outer shell of virions, capsid polypeptide σ1 (20,21). It also corresponds to the viral hemagglutinin which determines host range (44). Segments S2, S3 and S4 code respectively for viral core polypeptide σ2, a non-structural protein σNS and the major outer shell polypeptide of virions, σ3 (20,21). Reovirus structural proteins, with the exception of polypeptide μ1 which is cleaved during maturation, are known to have blocked N-termini (45). However, on the basis of the genome segment assignments of the proteins (20,21), the 5'-terminal sequences of the corresponding mRNAs (25), and the assignment of mRNAs to genome segments, the amino acid sequences of nascent chains of three reovirus-specified σ proteins can be predicted as: σ2 = Met-Ala-Arg-Ala-Ala-Phe-Leu-Phe; σNS = Met-Ala-Ser-Ser-Leu; and σ3 = Met-Glu-Val-Cys-Leu-Pro-Asn. The amino acid sequences, similarly predicted from the mRNA initiation fragments (25), for minor virion polypeptide μ2, the cleaved virion structural protein μ1 and the non-structural μNS protein (coded for by segments M1, 2 and 3 respectively) are: μ2 = Met-Ala-Tyr-Ile-Ala; μ1 = Met-Gly-Asn-Ala; and μNS = Met-Ala-Ser-Phe-Lys-Gly-Phe-Ser.

ACKNOWLEDGMENTS

We thank Dr. R. Braun of the University of Bern, Switzerland for providing P. polycephalum spores and Dr. N.J. Palleroni of Hoffmann-LaRoche for growing the organism and providing culture filtrates for RNase PhyI purification. Alba LaFiandra and Maureen Morgan kindly provided reovirus genome RNA and mRNA.

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