DIFFERENCE IN 5' TERMINAL STRUCTURE BETWEEN THE mRNA AND THE DOUBLE-STRANDED VIRION RNA OF REOVIRUS

R. C. Desrosiers, G. C. Sen and P. Lengyel
Department of Molecular Biophysics and Biochemistry
Yale University, New Haven, Conn. 06520

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SUMMARY. In the course of reovirus replication the double-stranded virion RNA serves as a template for reovirus mRNA (+ strand) formation. The reovirus mRNA in turn serves as a template for the synthesis of the complementary (-) strand. The latter remains associated with the template, thus forming double-stranded virion RNA. The virion associated enzymes can be activated *in vitro* to synthesize reovirus mRNAs with an $m^7G(5')ppp(5')GmpCp...$ 5' terminal structure (cap 1 structure). We find that about 50% of reovirus mRNAs formed in L cells (between 5 and 11 hours after infection) have $m^7G(5')ppp(5')GmpCmp...$ as their 5' terminal structure (cap 2 structure); the rest have a cap 1 structure. Interestingly, the large majority (over 95%) of the + strands in reovirion double-stranded RNA have cap 1 structures at their 5' termini. These observations may indicate that reo mRNAs serve as a template in double-stranded RNA formation before their cap 1 termini become converted to cap 2 termini. It is also possible, however, that mRNAs with cap 1 type 5' termini are preferred templates for double-stranded RNA formation over those with cap 2 type 5' termini.

INTRODUCTION

Many eucaryotic cellular and viral mRNAs have modified 5' terminal structures (1). This type of structure has been designated as a cap (2). In cap 1 structures (general formula $m^7G(5')ppp(5')NmpNp...$) the 5' hydroxyl of 7-methylguanosine is linked by a 5'-5' triphosphate bridge to the 5' hydroxyl of a 2'-O-methylnucleotide. Cap 2 structures (general formula $m^7G(5')ppp(5')NmpNmpNp...$ differ from cap 1 structures by having the third nucleotide also 2'-O-methylated.

Much of our knowledge about modified 5' termini of mRNA has been obtained from studies with reovirus (1). The reovirions contain ten discrete segments of ds RNA. Purified reovirions contain enzymes that synthesize, cap and methylate reo mRNAs. The activation of these enzymes requires cleavage or removal of some of the viral coat proteins. This takes place in infected cells. It can also be achieved *in vitro* by treatment of the virions with chymotrypsin (1,3). The incubation of such activated virions (designated as cores) with the 4 ribonucleoside triphosphates and the methyl donor S-adenosyl-methionine results in the synthesis of the 10 capped and methylated reo mRNAs (+ strands). Each of these + strands is transcribed from a different ds RNA segment. Moreover, each

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Abbreviations: reo, reovirus, ss, single-stranded, ds, double-stranded; hpi, hours postinfection; m^7G, 7-methylguanosine; Gm, 2'-O-methylguanosine; Cm, 2'-O-methylcytidine; m^6A, 6-methyladenosine; m^5C, 5-methylcytidine.

has the same cap 1 structure ($m^7G(5')ppp(5')GmpCp...$) at its 5' terminus (4). In the course of reovirus replication, the viral mRNA (+ strand) serves as template for the formation of a complementary strand (- strand) which is neither capped nor methylated. The - strand remains associated with the + strand, thus forming the ds virion RNA (1). The presence of a cap 1 structure in ds reovirion RNA was reported (5).

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MATERIALS AND METHODS

Isolation of labeled reo mRNA and labeled cytoplasmic reo ds RNA from infected L cells

Mouse L929 fibroblasts (L cells) were grown in suspension culture and infected with reovirus (type 3, the Dearing strain) at a multiplicity of infection of 10 plaque forming units/cell according to published procedures (6). At 2.5 hpi, 1 µg/ml of actinomycin D was added to decrease host RNA synthesis (7). At 5 hpi, the cells were sedimented by centrifugation and resuspended in fresh medium which was supplemented with 1 µg/ml of actinomycin D, 5 µM [^{3}H] methyl-labeled methionine (5 mCi/100 ml medium), 0.03 µM [^{14}C] uridine (1.1 µCi/100 ml medium), as well as 20 mM formate, 20 µM adenosine and 20 µM guanosine. The last three compounds were added to decrease the labeling of the purine rings (8). At 11 hpi, the cells were sedimented by centrifugation, washed with a buffer solution (10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 140 mM NaCl, 2.6 mM KCl) and stored at -80°. Total cytoplasmic RNA was isolated from the postnuclear supernatant fraction (9) according to published procedures (10). This was fractionated into ss RNA and ds RNA by repeated precipitation with 2 M LiCl at 4° (11). To separate the reo mRNA from other cytoplasmic ss RNAs, the cytoplasmic ss RNA preparation was hybridized to ds reovirion RNA (12). After ethanol precipitation, the RNA was treated with 1 µg/ml of pancreatic RNase A in 0.02 M Tris Cl (pH 7.2), 0.3 M NaCl at 37° for 0.5 h to hydrolyze the unhybridized RNA. The unhydrolyzed RNA was precipitated with ethanol. The precipitate was dissolved in 20 mM Tris Cl (pH 7.4), 0.3 M NaCl and fractionated by centrifugation through a 5-20% (w/w) sucrose gradient in 10 mM Tris Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA at 3° and 39,000 rpm in the SB283 rotor in the IEC B60 ultracentrifuge for 19 h. The fractions containing material sedimenting between 6S and 14S were pooled and the RNA in these was precipitated with ethanol (reo mRNA).

Cytoplasmic reo ds RNA was precipitated from the supernatant fractions of LiCl precipitation (see above) by the addition of 2 volumes of ethanol. The precipitate was dissolved and fractionated by sedimentation in a 5-20% sucrose gradient as described above. The fractions containing material sedimenting between 7S and 14S were pooled and the RNA in these was precipitated with ethanol. The RNA was dissolved and refractionated by another sucrose gradient sedimentation. The material sedimenting in the 7S to 14S region of the gradient was isolated (cytoplasmic reo ds RNA).

Isolation of labeled ds reovirion RNA from infected L cells

ds reovirion RNA was labeled as reo mRNA and cytoplasmic reo ds RNA except that 1) no labeled uridine was added, 2) the concentration of methionine was 10 µM and 3) the culture was labeled until 18 hpi. ds reovirion RNA was prepared (12) from isolated virions (6).

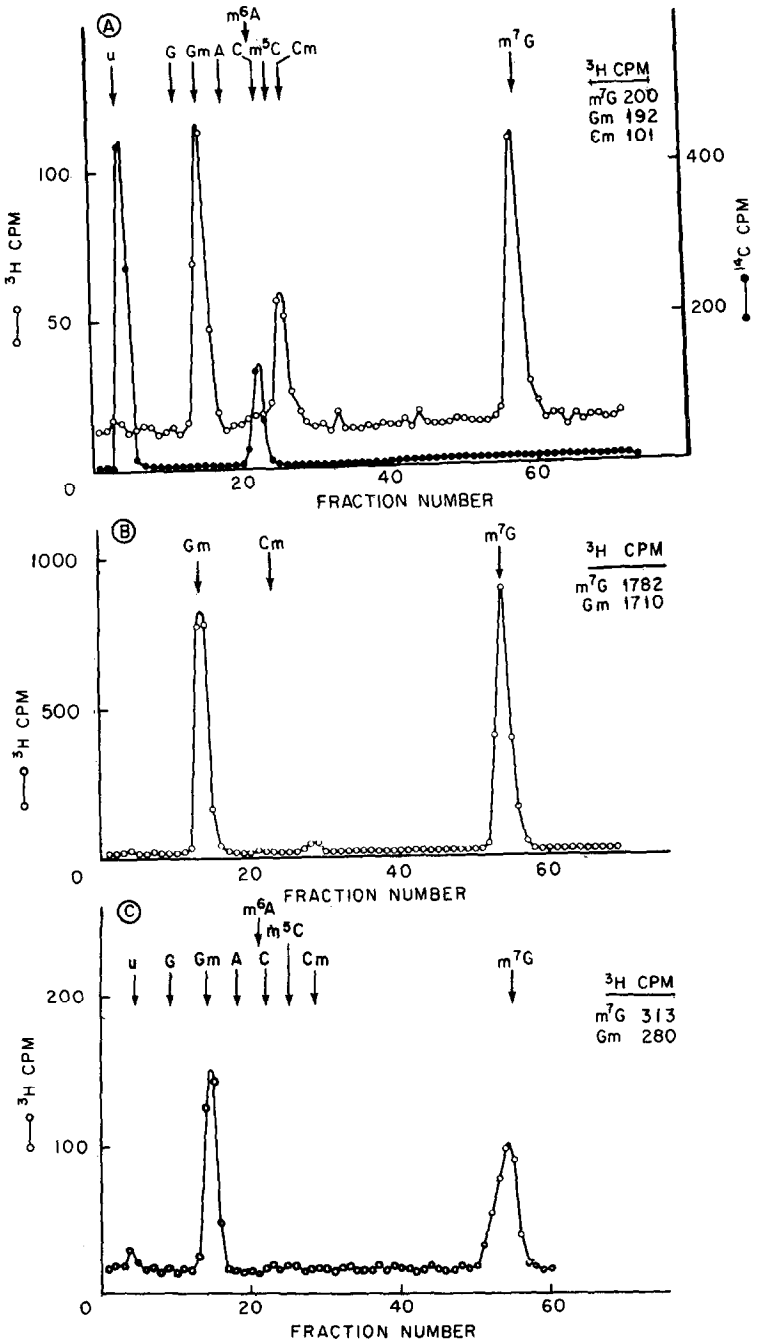

Fig. 1. Analysis by liquid chromatography of methylnucleosides in the enzymatic digests of A) reo mRNA, B) cytoplasmic reo ds RNA and C) ds reovirion RNA. Labeled reo mRNA, cytoplasmic reo ds RNA and ds reovirion RNA were denatured by heating in dimethylsulfoxide and precipitated with ethanol (5). The RNAs were digested with P1 RNase at pH 5.3 and 37° for 2 h (16). Thereafter, the reaction mixtures were incubated at 85° for 2 min, cooled to 37°, supplemented

RESULTS

Difference in methylnucleoside composition between reo mRNA and ds reovirion RNA

Reo mRNA isolated from L cells (which had been labeled with [^{14}C]-uridine and [^{3}H]-methyl methionine between 5 and 11 hpi) was hydrolyzed to nucleosides. The nucleosides were analyzed by chromatography on Aminex-A5 (Fig. 1A). The majority of [^{14}C]-labeled material was eluted together with uridine and cytidine markers. Approximately equal amounts of [^{3}H]-methyl-labeled material eluted in the position of marker Gm (40% of the [^{3}H] label) and marker m^{7}G (also 40%) and about half as much with marker Cm (20%) (Fig. 1A). The labeled material which was eluted from Aminex-A5 in the position of Cm in these experiments was also eluted together with Cm in an experiment with an ammonium formate-ethylene glycol buffer (13) (not shown).

Equal amounts of labeled Gm and m^{7}G but no detectable Cm (less than 2%) was found in reo mRNA synthesized by reovirus cores *in vitro*. This result confirms those of Furuichi *et al.* (4).

The methylnucleoside composition of ds reo RNA from two sources was analyzed: 1) Cytoplasmic reo ds RNA (Fig. 1B) was isolated from the same infected cell culture as reo mRNA. Part of this presumably originates from virion precursors in the cytoplasm and part from virions in the cytoplasm. 2) ds reovirion RNA (Fig. 1C) was isolated from virions purified from infected L cells labeled between 5 and 18 hpi. In both ds reo RNA preparations the amount of label in m^{7}G was similar to that in Gm. The amount of label in Cm was below detectable levels (less than 2%).

Difference in cap structure between reo mRNA and ds reovirion RNA

If the Cm residues found in the RNA digests originate from the cap structures then about half of the reo mRNA molecules but less than 2% of ds reovirion RNA molecules should have cap 2 type 5' termini.

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with additional P1 RNase and incubated for 2 more h (5). After adjusting the pH to 7.8 the RNAs were further hydrolyzed to nucleosides by added nucleotide pyrophosphatase (Sigma) and bacterial alkaline phosphatase (Worthington)(16). The reaction mixtures were supplemented with unlabeled methylnucleoside markers (Gm, m^{6}A, m^{5}C, Cm and m^{7}G) and injected into a 50 cm long Aminex A-5 column. This was eluted with 0.4 M ammonium formate (pH 4.55)(10,13). 15 drop fractions (about 0.5 ml) were collected until fraction 40 in A, fraction 38 in B and fraction 30 in C and thereafter 30 drop fractions (about 1 ml). The locations of the marker nucleosides in the elution patterns are indicated in the figure by arrows. These locations were previously determined by chromatography of the nucleosides individually and in various combinations and by monitoring the absorbancy of the eluates at 254 nm. The positions and amounts of U and C (^{14}C-labeled material) and of the methylnucleosides (^{3}H-labeled material) were determined by counting the fractions in ACS (Amersham Searle). The amounts of label in the various methylnucleosides are indicated in the figure.

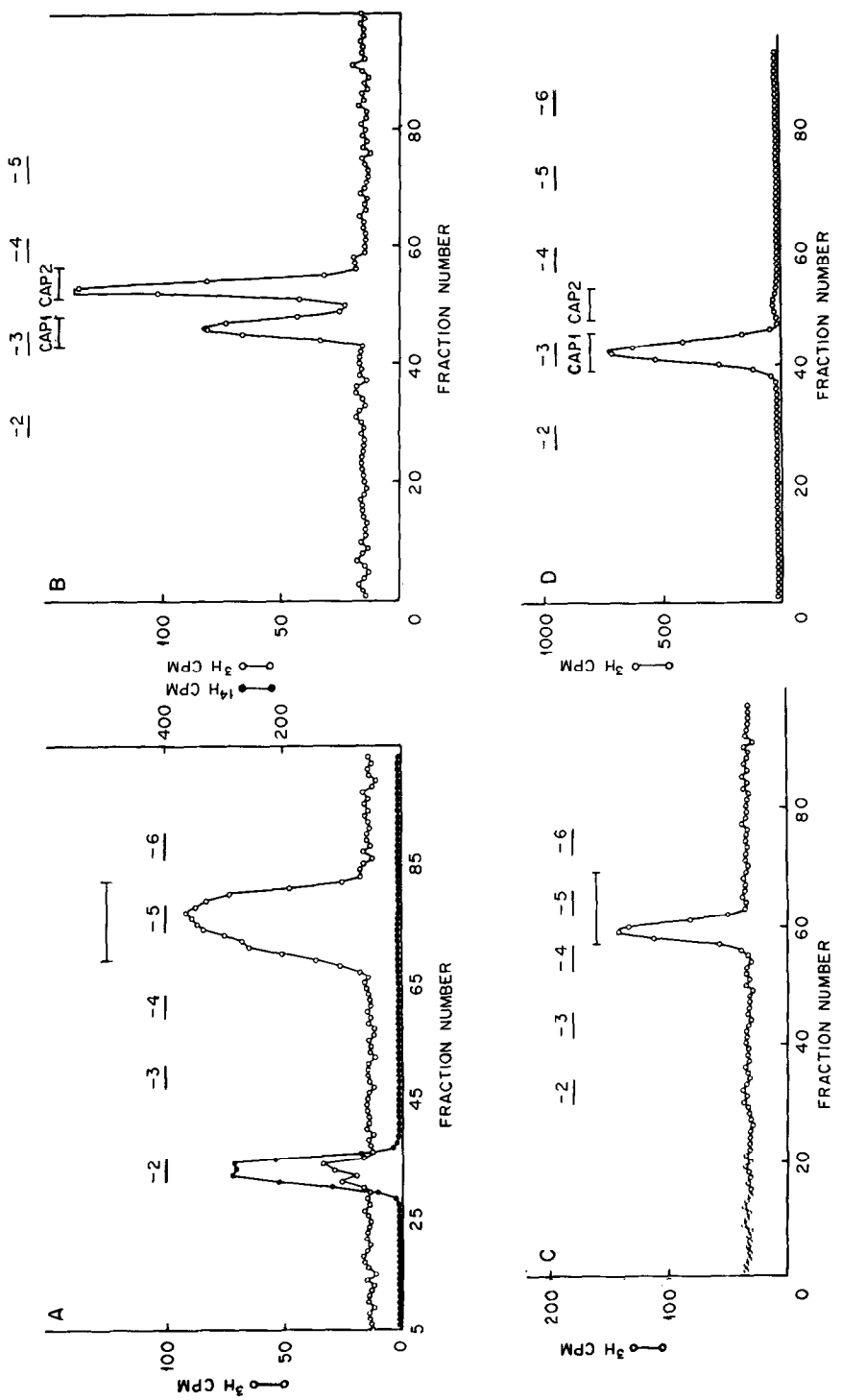
A
B
C
D
CAP1
CAP2
-2
-3
-4
-5
-6
^{3}H CPM
^{14}H CPM
FRACTION NUMBER

Fig. 2. Analysis of the cap structures of reo mRNA and ds reovirion RNA by chromatography on DEAE-cellulose. Chromatography A) of RNase T2 digest of reo mRNA, B) of alkaline phosphatase-treated cap structures of reo mRNA, C) of a RNase T2 digest of ds reovirion RNA and D) of alkaline phosphatase-treated cap structures of ds reovirion RNA.

Digestion with RNase T2 and analysis of the products. Labeled reo mRNA (in A) and ds reovirion RNA (in C) were denatured by heating in dimethylsulfoxide and precipitated with ethanol (5). The RNAs were digested in 0.01 M sodium acetate buffer (pH 4.6) with 60 units/ml of RNase T2 at 37° for 3 h. Thereafter the reaction mixtures were incubated at 85° for 2 min, cooled to 37°, supplemented with a further 30 units/ml of RNase T2 and incubated for 3 more h (5). The reaction mixtures were diluted 10 fold with buffer A (0.05 M Tris Cl (pH 7.6), 7 M urea), mixed with 10 A_{260} units of a pancreatic RNase digest of yeast RNA (serving as a source for marker oligonucleotides) and were applied to a 30 cm x 0.8 cm DEAE-cellulose column which had been equilibrated with buffer A containing 0.05 M NaCl. The column was developed with a 0.05 M to 0.25 M NaCl gradient in buffer A (total volume 100 ml). 27 drop fractions (about 1 ml) were collected. The amount of radioactivity in each fraction was determined by counting in ACS.

Alkaline phosphatase treatment of cap structures and analysis of the products. The fractions containing the cap structures from the RNase T2 digest of reo mRNA (indicated in A by a horizontal bar) and of ds reovirion RNA (indicated in C by a horizontal bar) were pooled and freed of salt and urea by dilution with H_2O, application to a DEAE-cellulose column, washing of the column with 0.01 M NH_4HCO_3 (saturated with CO_2), elution with 1 M NH_4HCO_3 (saturated with CO_2) and repeated lyophilization from H_2O (14). The products were dissolved in 0.02 M Tris Cl (pH 7.8) and treated with 8 units/ml of alkaline phosphatase at 37° for 4 h. The digests were applied to a DEAE-cellulose column and were fractionated as described above in the section on "Digestion with RNase T2 and analysis of the products" except that the NaCl gradient was from 0.05 M to 0.225 M. The underlined numbers indicate the location and the net charge of the oligonucleotide markers.

To characterize the cap structures, [^{14}C] uridine and [^{3}H] methyl-labeled reo mRNA was digested with RNase T2. This treatment produces $m^7G(5')ppp(5')GmpCp$ from reo RNA with cap 1 termini and $m^7G(5')ppp(5')GmpCmpNp$ from reo RNA with cap 2 termini. Both of these products are eluted from a DEAE-cellulose column in 7 M urea together with marker molecules carrying between -4 and -6 charges (Fig. 2A). (The source of the small amount (~3%) of [^{3}H] label eluted in the position of markers having -2 charge is unclear.) The material in the broad peak below the bar in Fig. 2A was isolated and treated with alkaline phosphatase to remove the 3' terminal phosphate. Analysis of the product by chromatography on DEAE cellulose in 7 M urea revealed two narrow peaks, one eluting in the position of cap 1 structures and the other in the position of cap 2 structures (14). The proportion of label in the two was 2:3 (Fig. 2B). This is as expected from the ratio of Cm to m^7G in reo mRNA (see Fig. 1A).

Chromatography of an RNase T2 digest of [^{3}H]-methyl-labeled ds reovirion RNA in the same conditions gave one narrow peak which was eluted between markers with -4 and -5 charges (Fig. 2C). Chromatographic analysis of the material isolated from the fractions below the bar in Fig. 2C after treatment with alkaline phosphatase revealed that about 98% consisted of products from the cleavage of RNA with cap 1 termini.

DISCUSSION

The results presented indicate that a large portion of the reo mRNA in infected L cells contains 2'-O-methylcytidine as part of cap 2 termini. The existence in infected L cells of reo mRNA with cap 2 termini was also noted by Shatkin and Both (1). So far no viral methylase has been described forming cap 2 structures. Thus the enzyme methylating in infected L cells the 2'-O-position of the C residue in the reovirus cap structure is probably of cellular origin (15).

The proportion of cap 2 structures (and of 2'-O-methylcytidine) in ds reovirion RNA is very low (2% or less).

The difference in proportion of cap 2 structures between reo mRNA and ds reovirion RNA may indicate that reo mRNAs serve as a template in ds RNA formation before their cap 1 termini become converted to cap 2 termini. It is also possible, however, that mRNAs with cap 1 termini are preferred as templates for ds RNA formation over those with cap 2 termini.

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