Cycloleucine Blocks 5′-Terminal and Internal Methylations of Avian Sarcoma Virus Genome RNA†

Kenneth Dimock and C. Martin Stoltzfus*,‡

ABSTRACT: Cycloleucine, a competitive inhibitor of ATP:L-methionine *S*-adenosyltransferase in vitro, has been used to reduce intracellular concentrations of *S*-adenosylmethionine and by this means to inhibit virion RNA methylation in chicken embryo cells that are infected with B77 avian sarcoma virus. Under conditions of cycloleucine treatment, where virus production as measured by incorporation of radioactive precursors or by number of infectious particles is not significantly affected, the internal m^6A methylations of the avian sarcoma virus genome RNA are inhibited greater than 90%. The predominant 5′-terminal structure in viral RNA produced by treated cells is $m^7G(5')pppG$ (cap zero) rather than $m^7G(5')pppG^m$ (cap 1). It appears from these results that internal m^6A and penultimate ribose methylations are not required for avian sarcoma RNA synthesis and function. Furthermore, these methylations are apparently not required for transport of genome RNA to virus assembly sites. The insensitivity of the 5′-terminal m^7G methylation to inhibition by cycloleucine suggests that the affinity of *S*-adenosylmethionine for 7-methylguanosine methyltransferase is significantly greater than for the 2′-*O*-methyltransferases or the N^6-methyltransferases.

Cellular and viral messenger RNAs contain a number of methylated nucleosides. Those at the 5′ ends of the molecules are in capped terminal structures $m^7G(5')pppXpYp$[1] where the penultimate nucleoside X usually contains a 2′-*O*-methyl group and the nucleoside Y often contains a 2′-*O*-methyl group (caps 1 and 2, respectively) (Shatkin, 1976). In addition to methylations at the 5′-termini, some viral and cellular mRNAs contain additional methylated nucleosides at internal positions (Desrosiers et al., 1974; Adams and Cory, 1975; Furuichi, et al., 1975a; Lavi and Shatkin, 1975; Perry et al., 1975; Wei et al., 1976; Sommer et al., 1976; Moss et al., 1977). Most of these methylated nucleosides are N^6-methyladenosine, although the presence of 5-methylcytidine has also been reported (Dubin and Stollar, 1975; Salditt-Georgieff et al., 1976).

The functional role of some mRNA methylations has been inferred from in vitro translation or ribosome-binding experiments using viral mRNAs and ribopolymers which either contain or lack a particular modified nucleoside. In this way, it has been shown that the terminal m^7G residue is required for efficient binding of a number of mRNAs to ribosomes (Both et al., 1975; Muthukrishnan et al., 1976b; Kozak and Shatkin, 1976). It has been further shown that methylation of the penultimate nucleoside residue plays a positive role in the binding of ribopolymers to the 40S ribosomal subunit, although this effect is much less pronounced than for the m^7G methylation (Muthukrishnan et al., 1976a). The functional role of internal m^6A is not yet known. Most cellular mRNAs and those viral mRNAs which are formed in the nucleus contain internal m^6A residues (Lavi and Shatkin, 1975; Sommer et al., 1976; Moss et al., 1977). This suggests that, although these modifications are probably not required for protein synthesis, they may be involved in mRNA processing or transport of mRNA from the nucleus to the cytoplasm.

The 38S genome RNA subunits of oncornaviruses possess structural and functional properties of eukaryotic mRNA molecules. The viral RNA subunits from avian sarcoma viruses are methylated both at their 5′ termini [$m^7G(5')pppG^m$] and internally where 10 to 15 m^6A residues are present (Keith and Fraenkel-Conrat, 1975; Furuichi et al., 1975b; Stoltzfus and Dimock, 1976; Beemon and Keith, 1977). The location of these internal methylated bases is not random; m^6A appears only in the sequences G-m^6A-C and A-m^6A-C (Dimock and Stoltzfus, 1977). This sequence specificity is identical to that of the bulk mRNA from HeLa and L cells (Wei et al., 1976; Schibler et al., 1977; Wei and Moss, 1977). In addition, a majority of the internal methylations are contained in the 3′ half of the RNA molecule (Beemon and Keith, 1977; K. Dimock, unpublished observations). Only partial translation of this RNA has been observed. In in vitro translation systems the major product is a 76 000-dalton *gag* precursor protein which is thought to be specified by the 5′-terminal part of the genome RNA (von der Helm and Duesberg, 1975; Pawson et al., 1976). It appears that in vivo avian sarcoma virus mRNA consists of 38S RNA and smaller subgenomic mRNA species (Bishop et al., 1976; Brugge et al., 1977; Hayward, 1977). A 20–24S viral-specific RNA species has been shown to direct translation of viral envelope protein sequences (Pawson et al., 1977; Stacey et al., 1977) and also hybridizes to cDNA complementary to the 3′ half of the genome RNA (Hayward, 1977). Consequently, there may be processing of the 38S RNA to form these smaller mRNA species. It is of interest that most of the m^6A methylations are in sequences of the 38S genome RNA that are present in smaller subgenomic mRNAs. This suggests that internal methylations might play a role in the processing of viral RNA.

In order to elucidate the functional role of internal methylations, it would be advantageous to define experimental conditions where such methylations are inhibited. In the studies described below, we have determined the effect on the methylation of B77 avian sarcoma virus genome RNA of cycloleucine, a competitive inhibitor of rat liver *S*-adenosylmethionine synthetase (ATP:L-methionine *S*-adenosyltransferase;

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[1] Abbreviations used are: m^6A, N^6-methyladenosine; m^7G, 7-methylguanosine; G^m, 2′-*O*-methylguanosine; met, methionine; cLeu, cycloleucine; X, any ribonucleoside; Y, any ribonucleoside; mRNA, messenger RNA; SAM, *S*-adenosylmethionine.

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TABLE I: Effects of Cycloleucine on Production of B77 Virus.

expt	conditions	amt of radioact. virus (cpm)[a] [^{3}H]Ado	[^{3}H]Met[b]	virus titer (FFU/mL)[c]
1	5 μM Met	2.1×10^6		1.4×10^6
	5 μM Met + 4 mM cLeu	3.0×10^6		
	5 μM Met + 40 mM cLeu	2.3×10^6		0.9×10^6
2	5 μM Met		1.4×10^7	
	5 μM Met + 40 mM cLeu		1.1×10^7	
3	11.4 μM Met		6.6×10^6	
	11.4 μM Met + 40 mM cLeu		5.8×10^6	
4	5 μM Met			2.4×10^6
	5 μM Met + 40 mM cLeu			1.7×10^6

[a] Virus was purified by sedimentation in continuous sucrose gradients. Fractions were collected and aliquots were counted. Total amount of radioactivity in virus peak was determined. [b] Approximately 99% of the [^{3}H]methionine radioactivity is contained in virion polypeptides. [c] Focus assays on culture medium were carried out as described under Experimental Procedures.

EC 2.5.1.6) in vitro (Lombardini et al., 1973). We have found that cycloleucine effectively blocks both internal methylations and 5′-terminal methylations of the viral RNA.

Experimental Procedure

Propagation and Purification of Virus. Bratislava 77 (B77) avian sarcoma virus was propagated in secondary cultures of leukosis-free chicken embryo fibroblasts (Spafas, Inc., Roanoke, Ill.) as previously detailed (Stoltzfus and Snyder, 1975). Following removal of cell debris, virus was concentrated from the culture medium using the poly(ethylene glycol)–Pronase procedure (Bronson et al., 1975, 1976) and purified on sucrose gradients (Stoltzfus and Snyder, 1975).

Radioactive Labeling of Virus. Confluent monolayers of chicken embryo fibroblasts producing B77 sarcoma virus in tissue culture plates or roller bottles were washed and then incubated for 3 h prior to labeling with Eagle's minimal essential medium supplemented with 10% calf serum containing various concentrations of methionine or cycloleucine. Labeling conditions have been described previously (Stoltzfus and Dimock, 1976; Dimock and Stoltzfus, 1977). The cultures were labeled with either (1) 50–100 μCi/mL L-[*methyl*-^{3}H]methionine and 0.2 μCi/mL [2-^{14}C]uridine or (2) 50–100 μCi/mL [2,8-^{3}H]adenosine for 12–24 h.

Focus Assay. Focus assays were carried out essentially according to published techniques (Vogt, 1969). Cells were plated at a concentration of 2×10^6 cells per plate in 60-mm petri dishes in the presence of 2 μg/mL polybrene (Aldrich Chemical Co., Milwaukee, Wis.) After a 1-h incubation at 38 °C, cells were infected with appropriate dilutions of virus. The next day the medium was removed and the cells were overlaid with 0.8% Bacto-agar (Difco Laboratories, Detroit, Mich.) in culture medium supplemented with 2% beef embryo extract. Foci were counted 1 week later.

Isolation and Purification of RNA. Methods of isolation and purification of B77 sarcoma virus RNA subunits have been previously described (Stoltzfus and Snyder, 1975; Stoltzfus and Dimock, 1976). In brief, viral RNA was isolated by chloroform–phenol extraction in the presence of 0.5% sodium dodecyl sulfate and sedimented on glycerol gradients to recover native 70S viral RNA. The 70S RNA was denatured to 38S RNA subunits which were sedimented on sucrose gradients containing 85% formamide.

Enzymatic Digestion. The conditions for digestion of viral 38S RNA subunits with RNase T_2, *Penicillium* nuclease (P_1), nucleotide pyrophosphatase, and bacterial alkaline phosphatase have all been described (Dimock and Stoltzfus, 1977).

Chromatography and Electrophoresis. Descending paper chromatography was carried out in 2-propanol–water–ammonium hydroxide (7:2:1, v/v). High-voltage paper electrophoresis was performed in pyridine–acetic acid–water (1:10:89, v/v), pH 3.5. Whatman 3 MM paper was used in both cases.

Materials. L-[*methyl*-^{3}H]Methionine (>5 Ci/mmol) and [2-^{14}C]uridine (56 mCi/mmol) were purchased from Amersham, Arlington Heights, Ill. and [2,8-^{3}H]adenosine (48 Ci/mmol) from ICN Pharmaceuticals, Inc., Irvine, Calif. Bacterial alkaline phosphatase was obtained from Worthington, Freehold, N.J.; RNase T_2 from Calbiochem, LaJolla, Calif.; nucleotide pyrophosphatase from Sigma, St. Louis, Mo.; and *Penicillium* nucleus from Yomasa-Shoyu Co., Tokyo, Japan. Cycloleucine was purchased from Calbiochem, LaJolla, Calif., and unlabeled nucleotides and nucleosides from P-L Biochemicals, Milwaukee, Wis.

Results

Effect of Cycloleucine on Synthesis of B77 Sarcoma Virus. In order to test the effect of cycloleucine on the methylation of viral RNA in chicken embryo cells it was necessary to establish conditions of treatment where synthesis of viral RNA and proteins is not severely depressed. It was shown previously that synthesis of Chinese hamster ovary (CHO) cell preribosomal 45S RNA continues at about 60% of control rates in the presence of cycloleucine (Caboche and Bachellerie, 1977). Protein synthesis was also shown to proceed at a somewhat reduced rate in CHO cells at cycloleucine levels up to 40 mM at a methionine concentration of 10 μM. Therefore, similar conditions were employed to test the effect of cycloleucine on the growth of B77 avian sarcoma virus in chicken embryo fibroblasts.

Infected chicken embryo cells producing B77 sarcoma virus were treated with cycloleucine at various concentrations and labeled in the presence of [^{3}H]adenosine. After a 24-h period, virus-containing medium was collected from treated and untreated cells, the virus was purified, and the total amount of radioactivity in purified virus was determined (Table I, experiment 1). There appears to be little or no inhibition of synthesis of radioactive RNA in virions under conditions of cycloleucine treatment.

In order to confirm that virus synthesis is not inhibited in the presence of cycloleucine, we compared the total amount of virus obtained from infected cells labeled with [*methyl*-^{3}H]methionine for 24 h in the presence or absence of cycloleucine. As can be seen in Table I, experiments 2 and 3, a 10 to 20% inhibition was observed. No qualitative or quantitative differences between virions produced in cycloleucine-treated or control cells were observed when the [*methyl*-^{3}H]methionine-labeled polypeptide chains were analyzed by gel electrophoresis (J. Allan, unpublished observations). Thus, cycloleucine treatment does not result in a significant inhibition of synthesis of radioactive protein in virions.

Finally, we measured the amounts of infectious virus produced under both sets of conditions as determined by focus-forming activity (Table I, experiments 1 and 4). In both ex-

TABLE II: Inhibition of Internal Methylation in [^{3}H]Adenosine-Labeled B77 38S RNA by Cycloleucine.[a]

conditions	% m^{6}Ap[b]	m^{6}Ap/genome[c]	% inhibition
100 μM Met	0.49	10.9	
5 μM Met	0.44	9.8	10
5 μM Met + 4 mM cLeu	0.05	1.1	90
5 μM Met + 40 mM cLeu	0.02	0.4	96

[a] Data from Figure 1. [b] % m^{6}Ap = (m^{6}Ap cpm)/(m^{6}Ap + Ap cpm) × % Ap, where % Ap = 24.8%. ^{32}P-labeled B77 38S RNA was digested to completion with RNase T_2. The mononucleotides were separated by high-voltage paper electrophoresis. % Ap was determined from Ap cpm/total cpm. [c] Assuming 9000 nucleotides per genome (Jacobson and Bromley, 1975).

periments, a decrease in infectious titer of the medium obtained from cycloleucine-treated infected cells was observed. However, these differences are not large and do not exceed the limits of error of the infectivity assay. We conclude, therefore, that incubation of infected chick embryo fibroblasts in the presence of 40 mM cycloleucine causes little or no change in the amounts of virions produced as determined by viral RNA, protein, and infectious particles. Cells treated with cycloleucine for 24 h, as in the experiments described above, remain 85% viable, as indicated by exclusion of trypan blue stain. This value is identical to that of the control cells in medium containing normal or lowered concentrations of methionine (data not shown).

Effect of Cycloleucine on Internal Methylation of B77 RNA. We next determined if the RNA isolated from virus produced in the presence of cycloleucine contained normal amounts of internal N^6-methyladenosine. Cells infected and producing virus were incubated in medium containing (a) normal concentrations of methionine (100 μM), (b) reduced concentrations of methionine (5 μM), and (c) reduced concentrations of methionine plus various concentrations of cycloleucine. RNA was isolated from [^{3}H]adenosine-labeled virus produced under these conditions and digested with T_2 ribonuclease, and the resulting 3′-nucleoside monophosphates were separated by paper chromatography as described under Experimental Procedure. Under these conditions, Ap can easily be separated from m^{6}Ap. The results of these analyses are presented in Figure 1 and Table II. Note that reducing the amount of methionine in the medium appears to have only a slight effect on the extent of internal methylation of the virus RNA (~10%). However, when cycloleucine is present in the low methionine medium, the amount of m^{6}Ap in viral RNA is reduced to less than one residue per RNA subunit, a greater than 90% inhibition of methylation. We conclude that cycloleucine is effective in blocking internal methylation of viral RNA. It is also apparent from the results given in Figure 1 that conversion of the adenosine label to guanosine is significantly reduced in the presence of cycloleucine. We do not yet understand the reasons for this inhibition; however, a similar effect was noted with another inhibitor of RNA methylation, ethionine (K. Dimock, unpublished observations).

Effect of Cycloleucine on 5′-Terminal Methylations of B77 RNA. From the results of the previous experiment, we cannot determine whether there is an effect of cycloleucine on the methylation of the 5′-terminal capped structure that is present in each of the genome RNA subunits of the virus. Consequently, another type of experiment was carried out. Virus was isolated from cells which were labeled for 24 h with both

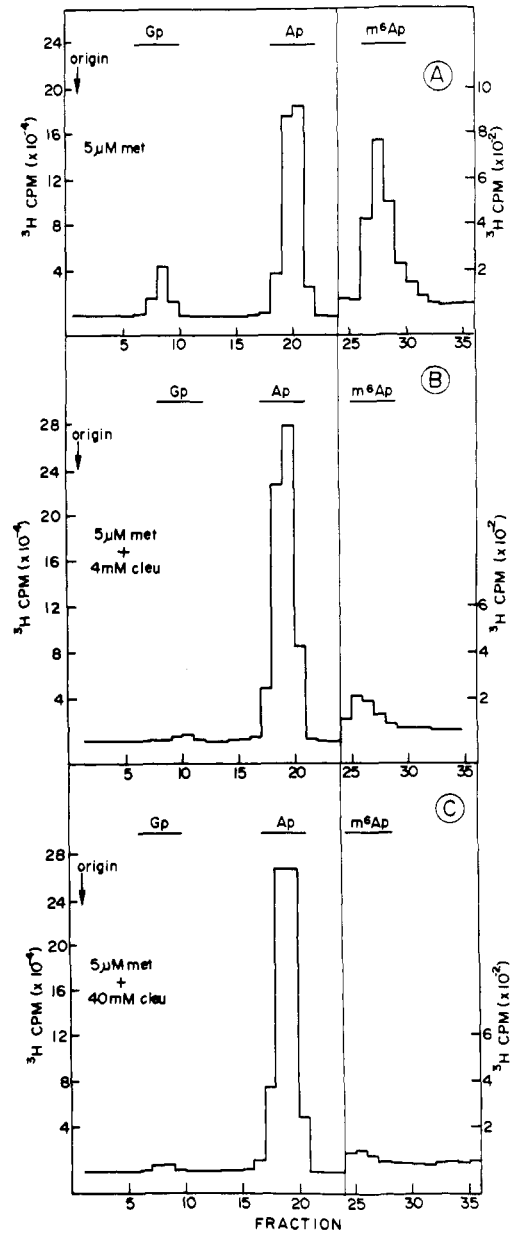

FIGURE 1: Paper chromatography of RNase T_2 digests of [^{3}H]adenosine-labeled B77 38S RNA. B77 transformed chick embryo fibroblasts were incubated in culture medium containing (A) 5 μM methionine, (B) 5 μM methionine + 4 mM cycloleucine, or (C) 5 μM methionine + 40 mM cycloleucine 3 h prior to labeling with [^{3}H]adenosine. Virus was isolated and RNA extracted. The 35S RNA was digested with RNase T_2 and analyzed by descending paper chromatography (72 h). The chromatogram was dried, 1.0-cm strips were cut, soaked 2 h in 0.5 mL of water, and counted for radioactivity in 5 mL of Aquasol. Bars represent nonradioactive markers located by UV light.

[^{14}C]uridine and [*methyl*-^{3}H]methionine in the presence and absence of 40 mM cycloleucine. The viral 38S RNA was purified and digested with *Penicillium* nuclease and bacterial alkaline phosphatase. The expected products of such a digestion are nucleosides and the 5′-terminal blocked and methylated structure, m^{7}G(5′)pppG^m, which is resistant to the enzymatic treatment. Following paper electrophoresis of the hydrolysates, the radioactive profiles illustrated in Figure 2 were obtained.

The [^{3}H]methyl-labeled peak which migrates toward the anode represents capped termini. This material was eluted and digested with nucleotide pyrophosphatase and bacterial alkaline phosphatase. The 5′-terminal structure of B77 38S

TABLE III: Inhibition of Methylation in [^{3}H]Methyl-Labeled B77 38S RNA by Cycloleucine.

conditions	[^{14}C]U (cpm)[a]	[^{3}H]m^{6}A (cpm)[a]	^{3}H 5′-terminal cpm			m^{6}A cpm/ m^{7}G cpm	G^m cpm/ m^{7}G cpm	m^{7}G cpm/ [^{14}C]U cpm
			total[a]	m^{7}G[c]	G^m[b]			
5 μM Met	1870	301	65	35	30	8.62 ±0.86	0.86 ±0.09	0.019 ±0.004
5 μM Met + 40 mM cLeu	2120	35	55	51	4	0.69 ±0.07	0.08 ±0.08	0.024 ±0.004
% inhibit. rel to m^{7}G						92.0	90.7	

[a] Data from Figure 2. [b] m^{7}G (or G^m) cpm = % m^{7}G (or G^m) × total 5′-terminal cpm, where % m^{7}G (or G^m) is determined from the data in Figure 3 and total 5′-terminal cpm is determined from data in Figure 2. [c] Errors reflect counting statistics.

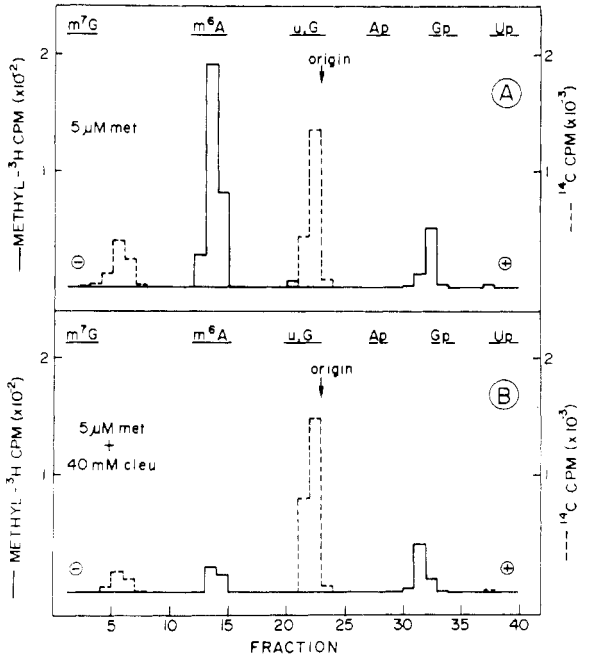

FIGURE 2: High-voltage paper electrophoresis of P_1 nuclease and bacterial alkaline phosphatase digests of B77 38S RNA. [^{14}C]Uridine- and [*methyl*-^{3}H]methionine-labeled 38S RNA was isolated from virus grown in cells incubated in culture medium containing (A) 5 μM [*methyl*-^{3}H]methionine. (B) 5 μM [*methyl*-^{3}H]methionine and 40 mM cycloleucine. The RNA was digested with P_1 nuclease and bacterial alkaline phosphatase and analyzed by paper electrophoresis at 2500 V for 2.5 h. The electropherogram was dried and 1.0-cm strips were cut and counted for radioactivity in 5 mL of toluene-based scintillation fluid. Counting efficiency on paper strips in toluene-based cocktail is approximately 15–20% of that in Aquasol.

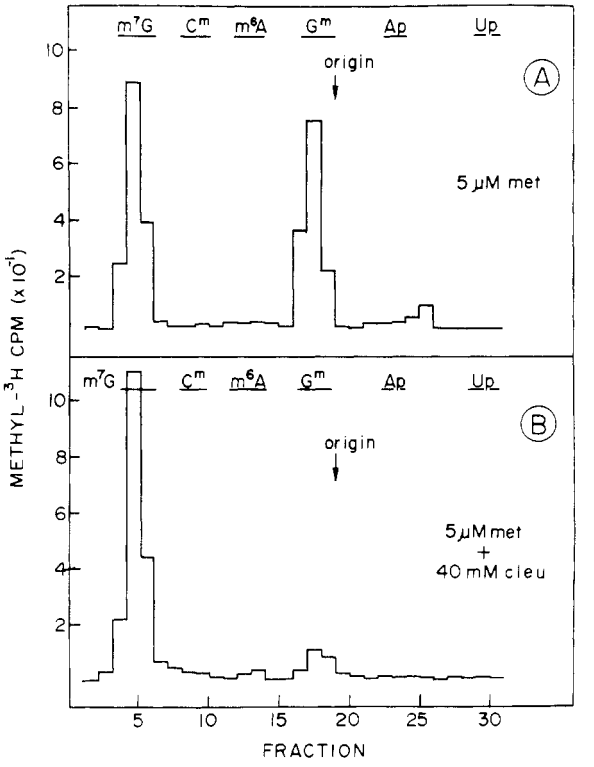

FIGURE 3: High voltage paper electrophoresis of nucleotide pyrophosphatase and bacterial alkaline phosphatase digests of 5′-terminal structures. The "cap" peaks in Figures (A) 2A and (B) 2B were eluted from the appropriate paper strips with water following three washes in toluene and drying. The samples were evaporated to dryness, dissolved in buffer, and digested with nucleotide pyrophosphatase and bacterial alkaline phosphatase. The hydrolysates were analyzed by paper electrophoresis at 2500 V for 2 h. The electropherogram was dried, 1.0-cm strips were cut and soaked for 2 h in 0.5 mL water, and then counted for radioactivity in 5 mL of Aquasol. The anode is on the right.

RNA, under normal labeling conditions, is m^{7}G(5′)pppG^m; therefore, the expected digestion products are m^{7}G and G^m, which can be separated by paper electrophoresis as shown in Figure 3A. The distribution of radioactivity in various peaks is summarized in Table III. It is indicated from the data that methylation of adenosine residues in B77 RNA subunits from virus produced in the presence of cycloleucine is inhibited by 92%. This value agrees favorably with the results obtained in the experiments described in the previous section using [^{3}H]adenosine-labeled RNA. Note that, under conditions of cycloleucine treatment, the methylation of the penultimate guanosine of the cap is reduced 91% relative to the terminal m^{7}G methylation (Figure 3B and Table III). It appears, therefore, that the major methylated 5′-terminal structure of viral genome RNA subunits produced in the presence of cycloleucine is m^{7}G(5′)pppG, a cap zero structure.

It is also indicated from the data given in Table III that the ratios of [^{3}H]methyl-labeled m^{7}G to [^{14}C]uridine from viral RNA produced in cycloleucine-treated and control cells are not significantly different. This suggests that most of the viral RNA molecules produced in cycloleucine-treated cells contain a 5′-terminal m^{7}G residue. We cannot be certain from these data that there are no RNA molecules which lack a methylated 5′-terminus, since the specific activities of the [^{3}H]methyl or the [^{14}C]uridine label in the RNA from treated and control cells have not been directly compared. The following observations, however, suggest that the specific activities are not significantly different. [^{3}H]Adenosine incorporation into m^{6}A provides a direct indication of inhibition of adenosine methylation independent of assumptions concerning the relative specific activities (Table II). Because the inhibition of the [^{3}H]methyl label in m^{6}A is comparable to the inhibition of the [^{3}H]adenosine label in m^{6}A, and since the same pool of

[^{3}H]methyl is presumably used in the formation of m^{7}G and m^{6}A, then the [^{3}H]methyl specific activities of m^{7}G in the RNA from treated and control cells do not appear to be significantly different. Furthermore, the amounts of virus produced by treated and control cells as determined by [^{14}C]uridine, [^{3}H]methionine, [^{3}H]adenosine and infectivity assays are similar (Tables I and III).

In summary, 40 mM cycloleucine blocks the formation of both m^{6}A and the penultimate G^m in B77 38S RNA subunits by greater than 90%. On the average, less than 0.5 m^{6}A residue and 0.1 G^m per subunit molecule remain of the 9–10 m^{6}A and 1 G^m per subunit which are present under normal conditions. The biosynthesis of 5′-terminal m^{7}G, on the other hand, does not appear to be affected under conditions of cycloleucine treatment.

Discussion

We have shown that treatment of B77 sarcoma virus infected cells with cycloleucine results in inhibition of both internal adenosine methylations and 5′-terminal ribose methylations of the genome RNA. It is possible by this means to isolate virus containing undermethylated RNA in order to test whether these modifications alter the ability of the RNA to serve as a primer-template in a reverse transcriptase reaction or as mRNA in in vitro translation systems. It will also be possible to prepare undermethylated RNA for use as substrate in the isolation of cellular mRNA methylating enzymes.

It appears from the experiments described in this paper that internal adenosine methylations and 5′-terminal ribose methylations of viral RNA are not essential for the production of similar amounts of virus as determined by [^{3}H]adenosine and [^{3}H]methionine incorporation into virions (Table I). This suggests that viral RNA and protein synthesis proceed in cells where internal and ribose methylations of viral genome RNA are reduced to levels of less than one residue per molecule. It remains to be shown whether methylation of intracellular viral-specific RNA is affected by cycloleucine to the same extent. Our results indicate that production of infectious virus is inhibited to a small extent in the presence of cycloleucine. However, the differences are not large. Further work is required to determine whether or not these small differences are significant.

It has been reported previously that an analogue of *S*-adenosylhomocysteine, 5′-deoxy-5′-isobutyladenosine (SIBA), causes a greater than 90% reduction in production of the Schmidt–Ruppin strain of Rous sarcoma virus by infected chick embryo fibroblasts (Robert-Géro et al., 1975). It was speculated that this inhibition might be due to interference by SIBA of viral RNA methylation. Since we observed little or no inhibition of virus replication when methylation of viral RNA was reduced by greater than 90%, it suggests either that SIBA is interfering with another step of virus replication or that, in contrast to cycloleucine, is preventing m^{7}G methylation and that this methylation is required for virus replication. Indeed, it has been reported (Jacquemont and Huppert, 1977) that SIBA inhibits m^{7}G methylation of mRNA, whereas m^{6}A methylation is not significantly affected in herpes simplex virus type 1 infected cells.

In studies to be presented elsewhere (Dimock and Stoltzfus, manuscript in preparation), we have confirmed the observation that inhibition of ribosomal RNA methylation by cycloleucine results in an accumulation of ribosomal RNA precursors (Caboche and Bachellerie, 1977). Since methylation affects the processing of ribosomal RNA, it was anticipated that undermethylation of precursors to mRNA might affect the processing of such precursors. The existence in infected cells of viral-specific RNA molecules larger than the 38S virion genome RNA has been reported, and these RNAs may serve as precursors to 38S RNA (Bishop et al., 1976). Methylation of these potential precursors is apparently not required, however, to convert them to 38S RNA. Also, transport of the undermethylated viral RNA to virus assembly sites appears to be normal, since normal amounts of virus are produced (see Table I).

Our results suggest that the m^{7}G 5′-terminal methylations of the virion RNAs are not significantly affected in the presence of cycloleucine. This is in contrast to methylations of internal adenosine and 5′-terminal ribose which are strongly inhibited. Recently, it was reported that a small fraction of 5′ termini with cap zero structures are present in poly(A)-containing RNA isolated from the cytoplasm of Novikoff hepatoma cells exposed to another inhibitor of RNA methylation, *S*-tubercidinylhomocysteine (STH) (Kaehler et al., 1977). It appears, therefore, that m^{7}G methylations of mRNA from both Novikoff hepatoma cells and sarcoma virus RNA in transformed chick embryo fibroblasts proceed under conditions where other RNA methylations are inhibited. This suggests that there may be a priority in the type of methylations which occur under conditions where methylation inhibitors are present. It should be pointed out, however, that, in spite of similarities in their effects, these two inhibitors probably act at different steps of the RNA methylation process. Cycloleucine has been shown to be a competitive inhibitor of methionine in the SAM-synthetase reaction (Lombardini et al., 1973), whereas STH is an analogue of *S*-adenosylhomocysteine. Cycloleucine acts by reducing the intracellular concentration of SAM (Caboche, 1977), whereas STH probably acts as an inhibitor in the enzymatic transfer of methyl groups from SAM to the RNA.

The reason for the priority of m^{7}G methylations will require further studies of the properties of the purified methylation enzymes. One possibility, however, is that the m^{7}G methyltransferase possesses a significantly greater affinity for SAM than does the 2′-*O*-methyltransferase or *N*^{6}-methyltransferase. Although the effects of SAM concentrations on the activities of cellular methyltransferases have not yet been determined, similar studies carried out with enzymes in purified virions have been done. Purified vaccinia virions and vesicular stomatitis virions contain both m^{7}G methyltransferase and 2′-*O*-methyltransferase activities. It has been shown that cap zero structures predominate in mRNA molecules synthesized in vitro by detergent-disrupted vaccinia virions under conditions where SAM concentrations are limiting, whereas only cap 1 structures are present under conditions where SAM is at saturating concentrations (Moss et al., 1976). In contrast, 5′-terminal structures of the type G(5′)pppA^m are present in mRNA molecules synthesized by detergent-disrupted vesicular stomatitis virions under conditions of limiting SAM concentrations (Testa and Banerjee, 1977). Thus, in the case of the vaccinia enzymes, the m^{7}G methyltransferase possesses a greater affinity for SAM than the 2′-*O*-methyltransferase, whereas the reverse is true for the vesicular stomatitis virus enzymes. It appears that in this respect the cellular enzymes resemble the vaccinia enzymes rather than the vesicular stomatitis virus enzymes.

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