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Biosynthesis and utilization of extensively undermethylated poly(A)$^+$ RNA in CHO cells during a cycloleucine treatment

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ABSTRACT

The role of RNA methylations in the control of mRNA maturation and incorporation into polysomes has been investigated through a study of the effects in vivo of cycloleucine, a specific inhibitor of S-adenosyl-methionine mediated methylation.

During the cycloleucine treatment, the rate of biosynthesis of hnRNA and its subsequent polyadenylation were only slightly reduced as compared with untreated cells. However a significant lag-time in the cytoplasmic appearance of poly(A)$^+$ undermethylated molecules was observed, in parallel with a transient shift in the average size of hnRNA towards higher molecular weight. Nevertheless, the total amount of pulse-labelled poly(A)$^+$ mRNA transferred to cytoplasm after a long chase time (3 h.) was approximatively the same for both cycloleucine-treated and control cells. Extensively undermethylated poly(A)$^+$ cytoplasmic RNAs, possessing a 5' terminal cap were incorporated into polysomes in proportions very similar to control messenger molecules.

These results suggest that a normal level of methylation is not stringently required for the production of functional mRNA molecules although it appears to be of importance for the kinetics of the maturational process.

INTRODUCTION

Most of eucaryotic RNA species are not directly synthesized as functional molecules but are processed from primary transcripts by a variety of molecular modifications [1]. Among these post-transcriptional events, the addition of methyl groups at definite sites within RNA molecule is a process of general occurence (concerning altogether ribosomal transfer and messenger RNAs) that could play a prominent role either in the control of RNA processing or in the functional properties of mature RNAs [1-5]. We have approached this problem by analyzing the effects of a selective inhibition of nucleic acid methylations on the biosynthesis of functional rRNA and tRNAs, during cycloleucine treatments of Chinese Hamster Ovary (CHO) cells [6,7].

In mammalian cells, the majority of mRNA molecules, as well as a fraction of hnRNA, contains methyl groups in two general types of structures : 5' terminal caps and in internal m^6A. The 5' terminal caps can be either

m^7GpppX_mY (cap 1) or $m^7GpppX_mY_m$ (cap 2) in which a 7-methyl guanosine group is linked to a 2'O methylated nucleotide X_m by a 5'-5' triphosphate bond, with a possible additional 2'O methylation on the penultimate nucleotide Y [8]. The internal m^6A content of mRNA corresponds to about one residue per molecule whereas hnRNA, longer than mRNA contains about 4-6 times as many m^6A per chain [5]. The similarity of methylated sequences in mRNA and hnRNA [9] and the conservative flow of methyl labelling in both 5' terminal caps [10] and internal m^6A [9] from nucleus to cytoplasm strongly suggest a prominent role for methyl groups in the control of pre-mRNA processing. On the other hand, a series of in vitro translation studies have shown that terminal caps facilitate translational initiation of viral mRNAs [8,11].

In the present study, the characteristics of the production of polysomal poly(A)$^+$ mRNAs as well as of presumptive pre-messenger poly(A)$^+$ hnRNA were analyzed during an inhibition of RNA methylations. The rate of production of poly(A)$^+$ hnRNA was only slightly reduced during a cycloleucine treatment. Conversely, undermethylated poly(A)$^+$ mRNA reached the cytoplasm in amounts very similar to control but with a longer lag-time after transcription. These molecules, possessing a non-methylated 5' terminal cap, were integrated into polysomes in proportions analogous to normally methylated messenger RNA.

MATERIALS AND METHODS

The material and methods used here are the same as described previously [6,7] with the following additions and modifications.

^{32}P labelling conditions : Cells exponentially growing in monolayer were washed with a prewarmed phosphate free Hanks basal saline solution and incubated for 2 hours in prewarmed phosphate-free low methionine medium. Cells were labelled for 4 hours in the same prewarmed medium containing ^{32}P phosphate (140 µCi/ml; 50 Ci/mg, Amersham England). Cycloleucine treatments (2 mg/ml) were begun 30 min before labelling.

Cell fractionation : Cells in monolayer were immediately detached with glass beads (Ø 0.25 - 0.30 mm; B. Braun Melsungen) in R buffer (40 mM Tris-HCl pH 7.7, 25 mM KCl, 4 mM $MgCl_2$ and 10 µg/ml polyvinylsulfate), in the presence of 0.5 % (v/v) cemulsol NPT 6 and 500 µg/ml collagenase. After 2 min hand-shaking in the cold (4°C), glass beads were discarded by decantation and the suspension containing the detached cells submitted to the action of an Ultra-Turrax to prepare nuclei as previously described [12]. After elimination of mitochondria, the supernatant was added with sodium deoxycholate 1 % (w/v)

and layered on a 30 % sucrose cushion in the same medium (1/3 v/ 2/3 v) and centrifuged 100 min at 50,000 rev/min in a Spinco Ti 50 rotor. The resulting pellet was essentially composed of polysomes + ribosomes. The supernatant was called soluble fraction.

RNA extraction and analysis : Ribosomal pellets were resuspended at 4°C in Tris 50 mM (pH 7.4) - EDTA 10 mM -1 % SDS and the solution incubated 1 hour at 20°C in presence of 200 µg/ml Proteinase K (Merck). An equal volume of water satured phenol-chloroform (1 v/v) was added and the mixture vigourously shaken for 10 min at 20°C. After centrifugation, the aqueous phase was re-extracted one more time with phenol-chloroform. After addition of NaCl (0.2 M final) the RNA was precipitated with 2 volumes of cold ethanol and allowed to stand overnight at - 20°C before analysis. RNA was then fractionated into poly(A)$^+$ and poly(A)$^-$ RNA by two consecutive passages through oligo (dT)-cellulose columns [13], one of which after heat denaturation of RNA 5 min at 70°C at low ionic strength.

Nuclear RNA was first extracted from pelleted nuclei as described above for polysomal RNA. After ethanol precipitation and redissolution in TMK buffer, deproteinized nuclear RNA was treated by 100 µg/ml pancreatic DNase I (Worthington), 1 h. at 0°C, then once more extracted by phenol-chloroform after proteinase K treatment in the presence of 1 % SDS. After ethanol precipitation and redissolution in 0.12 M NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.4, nuclear RNA was analyzed onto oligo(dT)-cellulose as described above.

Analysis of poly(A)$^+$ RNA by sucrose gradient was performed according to Perry et al. [14]. RNA was heat denatured 2 min at 60°C in 80 % DMSO and sedimented through a 15-30 % sucrose gradient 15 h at 25.000 rpm in a SW 41 Spinco rotor. Fractions were collected and counted in a Packard Tricarb liquid scintillation spectrometer.

Isolation and titration of 5' terminal cap structures was performed by chromatography on a DBAE cellulose according to Schibler and Perry [15]. ^{32}P-labelled polysomal poly(A)$^+$ RNA was exhaustively hydrolyzed with RNase T_2 (40 units in 0.3 ml 0.1 M NaAc (pH 4.5), 8 h. at 37°C), the digest chromatographied on DBAE-cellulose [16] and elution of caps was carried out with 1 M sorbitol [15]. Radioactivity was determined in both mononucleotides and caps.

RESULTS

Cycloleucine effects on poly(A)$^+$ RNA biosynthesis and methylation

Cycloleucine a competitive inhibitor *in vitro* of methionine adenosyl transferase, rapidly blocks the synthesis of S-adenosyl-methionine *in vivo* without drastic effects on protein or nucleic acid biosynthesis and acts as a selective potent and reversible inhibitor or nucleic acid methylations [6].

In a preliminary experiment, the overall extent of RNA methylation in the absence or presence of cycloleucine was determined through pulse-labelling with (methyl-^{3}H) methionine. As shown in Table 1, the amount of (methyl-^{3}H) labelling recovered in extensively purified total cellular RNA of cycloleucine treated CHO cells corresponded only to about 5 % of the control level, while the rate of (^{3}H) uridine incorporation was only slightly reduced (70 % of control cells).

However, this result did not give an insight on mRNA methylation since we previously observed that most of RNA methyl groups of CHO cells were located either in rRNA or in tRNA molecules. Consequently, a direct titration of methylation was performed on total poly(A)$^+$ RNA. Highly purified RNA was fractionated into poly(A)$^+$ and poly(A)$^-$ RNAs by two consecutive runs on oligo(dT) cellulose column. Our experimental procedure involved a denaturation step at low ionic strength before chromatography and allowed the obtention of poly(A)$^+$ RNA devoid of any detectable rRNA contamination. As shown

TABLE 1 - Cycloleucine effects on total RNA biosynthesis and methylation

	(^{3}H) Uridine counts incorporated		(^{3}H) Methionine counts incorporated	
	Control cells	Cycloleucine treated cells	Control cells	Cycloleucine treated cells
Total RNA	$7.7\ 10^6$ (100 %)	$5.3\ 10^6$ (69 %)	$3.1\ 10^6$ (100 %)	$1.6\ 10^5$ (5 %)
poly(A)$^-$ RNA	$7.2\ 10^6$ (100 %)	$5.1\ 10^6$ (71 %)	$2.9\ 10^6$ (100 %)	$1.2\ 10^5$ (4 %)
poly(A)$^+$ RNA	$1.2\ 10^5$ (100 %)	$8.4\ 10^4$ (70 %)	$1.3\ 10^4$ (100 %)	$4.8\ 10^2$ (3.7 %)

Four identical cultures in Falcon flasks (containing 10^7 cells) were pulse-labelled for 30 min with (^{3}H) uridine (40 µCi/ml; 40 Ci/mmol) or for 20 min with (methyl-^{3}H) methionine (25 µCi/ml; 5 Ci/mmol) either in the absence or presence of 2 mg/ml cycloleucine. Total cellular RNA were extracted, purified and separated into poly(A)$^+$ and poly(A)$^-$ fractions by oligo(dT) cellulose chromatography. The radioactivity recovered in the various fractions was normalized for identical number of cells and corrected for slight variations in the yield of RNA recovery through purification procedure, by 260 nm O.D. determinations before oligo(dT) fractionation.

in Table 1, the extent of methylation inhibition induced by a cycloleucine treatment was very similar for poly(A)$^+$ and total cellular RNAs (95 %). Both RNA classes behaved similarly concerning their biosynthetic rates in presence of cycloleucine : the incorporation of (^{3}H) uridine corresponded to about 70 % of control values.

Fate of nuclear poly(A)$^+$ RNA synthezised and processed in presence of cycloleucine

In a second set of experiments, we have analyzed the variations in poly(A)$^+$ RNA nuclear content, during an inhibition of RNA methylations, this RNA species being assumed to represent precursors to cytoplasmic mRNA.

After a 30 min pulse with (^{3}H) uridine, 68 % of total cellular poly(A)$^+$ RNA labelling was located in isolated nuclei of cycloleucine-treated cells, a value slightly but significantly higher than for control cells (62 %). The amount of radioactivity recovered in poly(A)$^+$ RNA per nucleus in treated cells was not very different from untreated cells (85 % of the control).

We studied the fate of these labelled molecules during a chase experiment performed in the presence of cold uridine. As previously reported [6], an efficient blocking of (^{3}H) uridine incorporation into RNA was achieved during the chase following a 30 min pulse with the radioactive precursor. Poly(A)$^+$ and poly(A)$^-$ RNA labellings were mesured for varying chase times. For both nuclear RNA classes, a decrease in radioactivity was observed in cycloleucine treated and control cells (Fig. 1). In control cells, the 50 % decay for poly(A)$^-$ nuclear RNA was reached after about 120 min (Fig 1a) : it corresponded mainly to the maturation and export of ribosomal RNA to cytoplasm. In treated cells, the decay of poly(A)$^-$ nuclear RNA was markedly reduced, in good agreement with the previously reported [6] inhibitory effects of cycloleucine on ribosomal RNA maturation. Similarly, poly(A)$^+$ hnRNA (Fig. 1b) decayed more slowly in cycloleucine treated than in control cells, with an average half-life of about 210 min (instead of 90 min in the absence of the drug). This relative accumulation of poly(A)$^+$ hnRNA during an inhibition of RNA methylation was quantitatively confirmed in another series of experiment : cycloleucine-treated and control cells were pulse-chased with (^{3}H) uridine or (^{14}C) uridine respectively. Batches of (^{3}H) and (^{14}C) labelled cells corresponding to the same chase-time were assayed for poly(A)$^+$ RNA distribution among nuclear and cytoplasmic fraction, after a prior mixing of the cells before fractionation, in order to eliminate possible errors originating from slight variations in the nuclear RNA recovery between different samples.

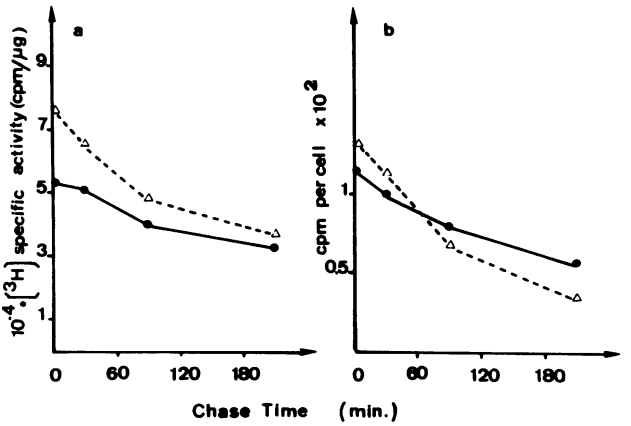

FIGURE 1 - Decay of pulse-labelled nuclear RNA during a cycloleucine treatment

Exponentially growing cells in Falcon flasks were pulsed for 30 min with (^{3}H) uridine (40 µCi/ml, 40 Ci/mmol) and chased in cold medium containing 200 µM unlabelled uridine. Different times after the beginning of the chase (0,30 min, 90 min, 210 min) incubation was stopped and cells harvested; the RNA was extracted from isolated nuclei and fractionated in poly(A)$^+$ and poly(A)$^-$ RNA as described in Materials and Methods.

The specific radioactivity of poly(A)$^-$ RNA (a) was determined after 260 nm O.D. measurements on the oligo(dT) cellulose unretained fraction. For poly(A)$^+$ RNA (b) retained counts per nucleus was plotted instead of a specific radioactivity (in cpm/µg) difficult to determine, due to the low content of poly(A)$^+$ RNA in nuclei.

(△– –△) Control cells; (●——●) Cells pulsed chased in presence of cycloleucine (2 mg/ml).

The size distribution of poly(A)$^+$ hnRNA labelled during a one hour (^{3}H) uridine pulse was analyzed by sucrose gradient sedimentation under denaturing conditions (Fig. 2). No dramatic differences between cycloleucine treated and control cells poly(A)$^+$ RNA size spectrum were revealed by analysis of the radioactivity profiles; however, a significant shift towards the high molecular weights was clearly seen when RNA methylation was inhibited. Conversely, the size distribution of both treated and control cell poly(A)$^+$ nuclear RNA became perfectly superimposable, when the one-hour pulse label with (^{3}H) uridine was chased for two hours in the presence of unlabelled precursor (result not shown).

Effects of cycloleucine on mRNA integration into polysomes

In a preliminary experiment (results not shown), we observed that neither the cytoplasmic ribosome content nor the size distribution of polysomes were significantly modified by cycloleucine, at least during the first 3 hours of treatment : two identical batches of cells were labelled with equal

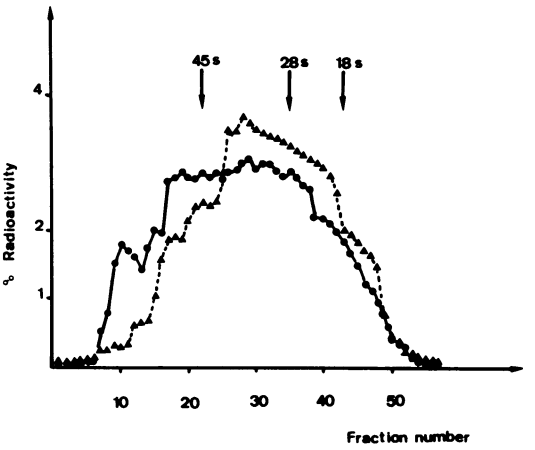

FIGURE 2 - Size analysis of poly(A)$^+$ hnRNA synthesized during a cycloleucine treatment

Cells were pulsed for one hour with (^{3}H) uridine (20 µC/ml, 20 Ci/mmol); nuclei were isolated, RNA extracted and poly(A)$^+$ hnRNA purified by chromatography on oligo(dT) cellulose column. Before sucrose gradient analysis (see Materials and Methods), poly(A)$^+$ RNA was denatured with 80 % DMSO at 60°C. For a better comparison, results were plotted for each fraction as percentage of total counts of the gradient.
(▲- - -▲) Control cells, (●——●) Cycloleucine treated cells.

molar concentrations of either (^{3}H) uridine or (^{14}C) uridine for 18 hours and chased in cold medium for two hours before a further 3 hours incubation in the presence or absence of cycloleucine. Harvested cells were mixed and processed for cytoplasmic polysomes isolation, which were analyzed onto sucrose gradient: almost superimposable radioactivity profiles were obtained for both isotopes.

In a second set of experiments,we have examined the cytoplasmic appearance of labelled poly(A)$^+$ RNA during a chase following a 30 min pulse with (^{3}H) uridine. In Fig. 3b, we have plotted the poly(A)$^+$ RNA radioactivity recovered in ribosomal (from monomer to polysomes) fraction; nearly all the radioactive cytoplasmic poly(A)$^+$ RNA was located in this fraction for both treated and control cells. After a 30 min. pulse, the amount of cytoplasmic poly(A)$^+$ RNA labelling in cycloleucine-treated cells corresponded to about 40 % of the control level. However, an important recovery of this inhibition was observed for longer chase time : a value exceeding 80 % of the control was reached after 210 min of chase. It is worth mentioning that throughout the chase experiment, the distribution of cytoplasmic poly(A)$^+$ RNA between ribosomal and "soluble" fractions was not modified, the content of the latter fraction being almost negligible.

As previously reported [6], a dramatic decrease in the cytoplasmic appearance of poly(A)$^-$ RNA (that is mainly rRNA) was observed during the cycloleucine treatment (fig. 3a).

From the data of fig. 1b and 3b, the proportion of poly(A)$^+$ RNA radioactivity in cycloleucine treated cells relative to control cells has been calculated. Results corresponding to nuclei, polysomes and total cell respectively were plotted in fig. 4. An analogous evolution was observed for both subcellular fractions during the chase time, with a continuous increase in the relative content of poly(A)$^+$ RNA. However, a significant shift was observed between curves corresponding to nuclear and cytoplasmic RNAs, suggesting that newly synthesized poly(A)$^+$ nuclear RNA was exported to cytoplasm with a delay during an inhibition of RNA methylations. It must be noted that the deficiency of whole cycloleucine-treated cell in poly(A)$^+$ RNA gradually disappeared during the pulse-chase experiment to reach control value after 210 min.

In the above described experiments, RNA was extracted from a total ribosomal pellet containing the most part of free mono-ribosomes in addition

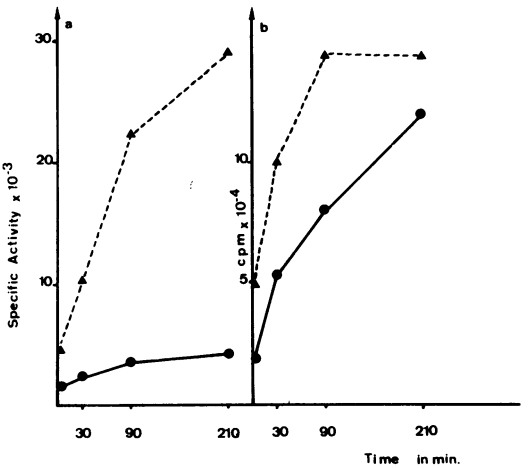

FIGURE 3 - Cycloleucine effects on labelled RNA appearance into polysomal fraction during a pulse-chase

Same experimental procedure as in Fig. 1, RNA was extracted from the ribosomal pellet and analyzed as described in Materials and Methods.

a) poly(A)$^-$ RNA. The amount of RNA present in each sample has been determined by optical density measurement and the RNA specific activity (cpm/µg) plotted.

b) poly(A)$^+$ RNA. Total (^{3}H) counts in RNA of each sample were counted. Radioactivity has been normalized for sample size variations between time points.

(▲- - -▲) Control cells, (●——●) Cycloleucine treated cells.

to polysomes. Consequently, it was not possible in these conditions to discriminate between poly(A)$^+$ RNA integrated into large polysomes and molecules not actually engaged in the translational process, i.e. present either in fast-sedimenting (> 50S) free mRNPs or in blocked initiation complex (bound with only one or two mono-ribosomes). In order to clear up this point, we have examined whether the distribution of newly synthesized poly(A)$^+$ RNA among cytoplasmic elements of increasing S values was modified by the cycloleucine treatment. The results of this experiment are depicted in Fig. 5 and Table 2.

The amount of poly(A)$^+$ RNA radioactivity was measured in the 3 "pooled" areas termed A, B and C, containing respectively polysomes, disomes + monosomes, and elements sedimenting at less than 50S. The results of Table 2 clearly show that the distribution of poly(A)$^+$ RNA among these areas was unaffected by the cycloleucine treatment. For both treated and control cells, it is worth observing that the major part of poly(A)$^+$ RNA was actually recovered in the "A" (polysomes) fraction, for the two chase times studied. In another experiment (results not shown), the "A" area was further fractionated into

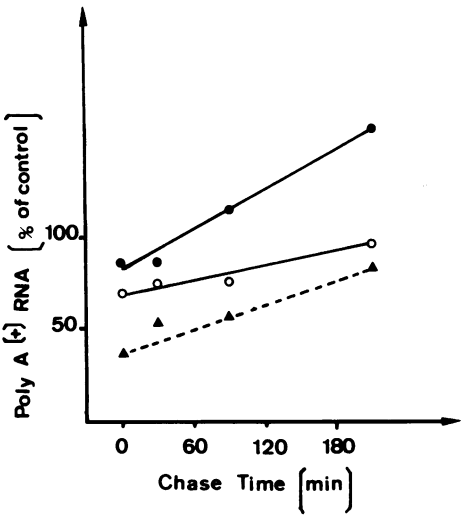

FIGURE 4 - Relative content in poly(A)$^+$ RNA of cycloleucine-treated versus control cells.

Measurements from data of figures 1 and 3. For each sample, the poly(A)$^+$ RNA content in cycloleucine-treated cells has been expressed as percentages of control, in total cell, nuclear and cytoplasmic fractions respectively.

(▲- - -▲) Cytoplasmic poly(A)$^+$ RNA, (●——●) Nuclear poly(A)$^+$ RNA (O——O) Total cellular poly(A)$^+$ RNA.

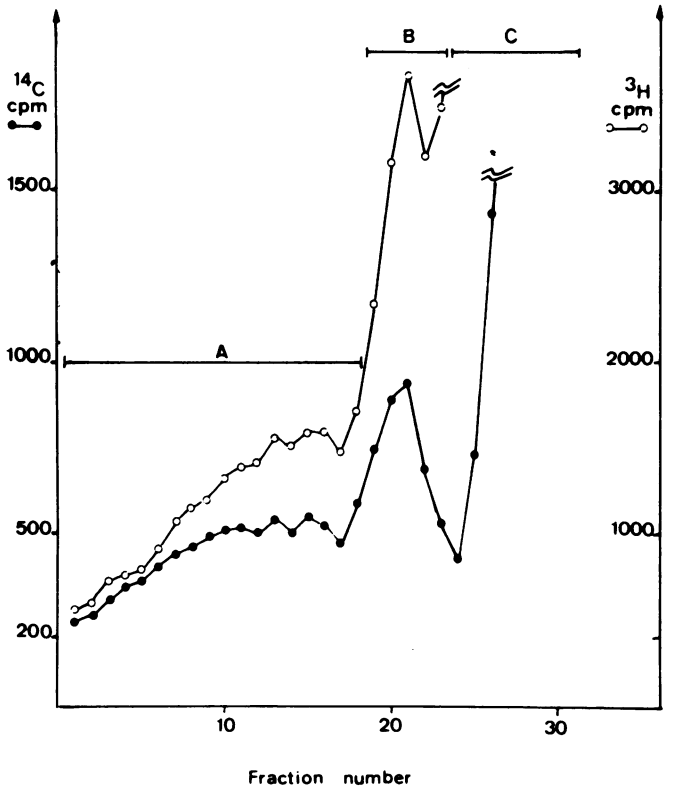

FIGURE 5 - Polysomes distribution after a cycloleucine treatment

Cells were prelabelled for 16 hours with (^{14}C) uridine (0.03 µCi/ml, 0.4 Ci/mmol). Thirty minutes after cycloleucine addition (2 mg/ml), cells were pulsed for 1 hour with (^{3}H) uridine (20 µCi/mmol) and chased for 30 min in the presence of unlabelled uridine. Cells were harvested and a post-mitochondrial supernatant (see Materials and Methods) was isolated, added with DOC (1 % final) and analyzed onto a 15 - 30 % sucrose gradient (in TKM buffer), by a 30 min run at 49,000 RPM (rotor Spinco SW 50.1). Aliquots of the collected fractions were assayed for radioactivity content. S values were determined from a run of rat liver ribosomal subunits in a parallel gradient.

two sub-regions corresponding to elements sedimenting faster or slower than 160S. In this case again, no differences were observed in the quantitative repartition of labelled poly(A)$^{+}$ RNA.

Finally, it must be pointed out that the cycloleucine treatment resulted in a significantly increased proportion of oligo(dT)-bound (^{3}H) RNA in the "A" and "B" fractions of the polysomes gradient, due to the drastic inhibition on the appearance of poly(A)$^{-}$ RNA (mainly rRNA), as elsewhere described [6].

TABLE 2 - Distribution of poly(A)$^+$ RNA in cytoplasmic fractions.

Fraction of the sucrose gradient	(^{3}H) cpm in poly(A)$^+$ RNA			
	1 hour pulse + 30 min. chase		1 hour pulse + 2 hours chase	
	Control cells	Cycloleucine treated cells	Control cells	Cycloleucine treated cells
A	32,700 (69)	17,600 (77)	49,500 (59)	36,200 (55)
B	8,000 (17)	3,230 (14)	21,300 (25)	16,000 (24)
C	6,850 (14)	1,910 (9)	13,500 (16)	14,300 (21)
Σgradient	47,550 (100)	22,740 (100)	84,300 (100)	66,500 (100)

Same experiment as described in Fig. 5. After the one-hour pulse with (^{3}H) uridine, cells were chased for 30 min or 2 hours. Control cells were similarly pulse-chased in the absence of cycloleucine. Fractions from the sucrose gradients were pooled in three batches, A (large polysomes), B (disomes + monosomes), and C (soluble fractions) as shown in fig. 5. After a proteinase K treatment and a phenol-chloroform extraction, RNA was precipitated by ethanol. RNA was then fractionated in poly(A)$^+$ and poly(A)$^-$ RNAs by chromatography on oligo(dT) cellulose column. The (^{14}C) uniform prelabelling of RNA has permitted corrections for slight variations in the RNA recovery between different samples during all fractionation and RNA purification. Values into brackets are, for each fraction of the sucrose gradient, the percentages of total input poly(A)$^+$ (^{3}H) RNA.

Methylation of poly(A)$^+$ RNA

Most of the methyl groups on messenger RNA molecules, i.e. 5' terminal caps and internal m^{6}A, are added at the nuclear level. However, an additional methylation takes place in the cytoplasm [17] in a 2'O position of the penultimate Y nucleotide, corresponding to the transformation of cap 1 into cap 2.

The extent of poly(A)$^+$ RNA methylation during a cycloleucine treatment was determined after a 20 min pulse labelling of CHO cells with (methyl ^{3}H) methionine as described in Table 3. In these conditions, one must expect that an important fraction of the poly(A)$^+$ RNA labelling in cytoplasm corresponds in fact to the late 2'-O methylation in Y position [10, 17]. As it is shown in table 3, poly(A)$^+$ RNA methylations were extensively inhibited in the

TABLE 3 - Cycloleucine effects on poly(A)$^+$ RNA methylation

(^{3}H) cpm in	Nucleus		Cytoplasm	
	Poly(A)$^-$	Poly(A)$^+$	Poly(A)$^-$	Poly(A)$^+$
Control cells	2.7 10^6	8,600	4.2 10^5	4,201
Cycloleucine treated cells	1.1 10^5 (4.1)	325 (3.8)	3.3 10^4 (7.8)	122 (2.9)

10^7 cells were pulse labelled 20 min with (^{3}H) methionine (25 µCi/ml; 5 Ci/mmol) RNAs were extracted from nuclei and ribosomes highly purified, then fractionated into poly(A)$^-$ and poly(A)$^+$ RNA. Radioactivity has been normalized for identical number of cells taking into account slight variations in the RNA recovery. A special care has been used for radioactivity determination due to the low level of poly(A)$^+$ RNA labelling. The sample counting time was long enough to register at least 10^4 desintegrations. For each vial actual background was determined by precounting.

presence of cycloleucine in both subcellular compartments : only 3-4 % residual labelling were detected in nuclear or cytoplasmic poly(A)$^+$ RNA. It thus appears that all types of methyl additions on mRNA molecules, including late cytoplasmic methylations, were equally inhibited by cycloleucine.

Analysis of 5' terminal caps in polysomal poly(A)$^+$ RNA

Since the addition of methyl groups on poly(A)$^+$ hnRNA and cytoplasmic poly(A)$^+$ mRNA was almost suppressed in the presence of cycloleucine, it could be asked whether the 5' terminal blocked "caps" were still present on undermethylated polysomal poly(A)$^+$ mRNA. This question was examined by an analysis of an RNase T2 digest of (^{32}P) long labelled polysomal poly(A)$^+$ RNA on a DBAE-cellulose column, a procedure previously described by Furuichi [18] for the separation of the capped ends from the bulk of internal nucleotides and phosphorylated 5' non-capped termini ; this method depends on the selective affinity for DBAE of cis 2'-3' diols of the terminal (m^7)G. The RNase T2 treatment should give rise to terminal fragments m^7GpppX_mpYp and $m^7GpppX_mpY_mpZp$, originating from normally methylated cap 1 and cap 2 respectively. In the case of unmethylated mRNA, GpppXp only would be generated from capped ends. Thus, since most of poly(A)$^+$ RNA molecules in cytoplasm have a cap [5], one should expect to recover in the DBAE retained fractions on the average 5-6 phosphate groups per normally methylated capped messenger molecule, 4 phosphate groups per unmethylated capped molecule and no phosphate at all for uncapped poly(A)$^+$ RNA. Taken together with an average size of

1800 nucleotides for polysomal poly(A)$^+$ RNA of control CHO cells (result not shown), this would respectively result in 0.30 % 0.22 % and 0 % of retained counts, since we observed that the size distribution of polysomal poly(A)$^+$ RNA synthesized and processed during the cycloleucine treatment was very similar to control.

Our experimental data (Table 4) corresponded to 0.24 $\pm$ 0.05 % of DBAE-retained counts for polysomal poly(A)$^+$ RNA of control cells, in good agreement with the predicted value (0.30 %). The value obtained for polysomal poly(A)$^+$ RNA of cycloleucine-treated cells (0.25 $\pm$ 0.07 %) is also consistent with the presence of blocked terminal structures at the 5' end of these molecules. This result and the data shown in the above section indicate that unmethylated 5' terminal caps can be added to mRNA molecules during an inhibition of RNA methylations.

DISCUSSION

In vitro studies [8,17] have provided valuable informations on both the enzymatic process of cap formation in viral mRNAs and the role(s) of blocked 5' terminal structures at the translation level in acellular systems.

On the other hand, an estimation of the potential relationship between methylation and mRNA processing, recently suggested by various studies of methyl groups in hnRNA and mRNA[1,5,9] would be greatly improved by the use of an *in vivo* specific inhibitor of RNA methylations. Several authors have recently described the utilization of various inhibitors of RNA methylases *in vivo* either for tRNA [19] or mRNA [20]. However, in these experiments, only a partial inhibition of RNA methylation could be achieved which hampered a straightforward interpretation of the results. In the present study, we performed an extensive *in vivo* inhibition of RNA methylation by means of cycloleucine treatments.

Cycloleucine, a competitive inhibitor of ATP : L-methionine-S-adenosyl transferase *in vitro*, induces *in vivo* a rapid decrease of the intracellular pool of S-adenosyl methionine, the donor of methyl groups for nucleic acid methylations and consequently acts as a potent and reversible inhibitor of RNA methylations, as shown in previous papers [6, 7]. The extent of RNA methylation was appreciated through pulse-labellings with (methyl-^{3}H) methionine. The validity of this approach was inferred, firstly, from the unmodified cellular uptake of labelled methionine in the presence of cycloleucine and, secondly, from the rapid equilibration of the intracellular pool of S-adeno-

TABLE 4 - Cycloleucine effects on poly(A)$^+$ polysomal mRNA 5' end structure

cpm in	Nucleotides	Caps
Control cells	$5.85\ 10^4$	138 (0.24 ± 0.05)
Cycloleucine treated cells	$4.1\ 10^4$	101 (0.25 ± 0.07)

CHO cells were labelled for 4 hours with (^{32}P) orthophosphate (140 µCi/ml; 50 Ci/mg) in the presence or absence of cycloleucine (2 mg/ml). Polysomes were isolated, RNA extracted and poly(A)$^+$ RNA purified as described in Materials and Methods. Nearly equal amounts of poly(A)$^+$ RNA were digested exhaustively with ribonuclease T2. The digest was then diluted with 10 vol DBAE application buffer [15] and applied to a 0.4 x 4 cm acetylated-DBAE cellulose column. After washing of the column with application buffer (60 ml), caps were eluted with 1 M sorbitol in the same buffer. Total counts in nucleotides and caps were determined. Values into brackets are percent of total counts recovered into caps.

syl-methionine (half time of less than 5 min). It is worth mentioning that direct titration of alkali-resistant 2'O methylated dinucleotides in rRNA gave identical results to those obtained by means of (methyl-^{3}H) labellings.

The level of both nuclear and late cytoplasmic poly(A)$^+$ RNA methylations, estimated through a 20 min pulse labelling with (methyl-^{3}H) methionine, was considerably inhibited (more than 95 %) during the cycloleucine treatment, thus indicating that all types of methyl additions onto poly(A)$^+$-RNA were equally affected in our conditions. This result is only in apparent contrast with a recent report of Kaehler et al [20], using the analogue S-tubercidinylhomocysteine (STH) as an *in vivo* inhibitor of mRNA methylases, on Novikoff hepatoma cells. These authors observed little or no inhibition of methylation at the 7-position of terminal G in cytoplasmic poly(A)$^+$ RNA whereas 2'O methylations were suppressed. This result could be interpretated in terms of a selection of m^{7}G capped molecules for cytoplasmic export when the overall RNA methylations are only moderately inhibited. However, this differential response does not seem to operate in our conditions, since the extensive inhibition of methyl groups incorporation is correlated to only a moderately reduced flow of poly(A)$^+$ RNA into cytoplasm. Taken together with the presence of a maximum of four methyl groups per mRNA molecule (2 or 3 in the 5' terminal cap and one internal N^6-adenylate residue), our results indicate that most of these mRNA molecules are completely devoid of any methyl residue.

The functional activity of the unmethylated poly(A)$^+$ RNAs which had been synthesized and exported to cytoplasm in the presence of cycloleucine was examined in another set of experiments, taking polysomal location as a criterion of operational messenger. The cycloleucine treatment does not modify the size spectrum of preexisting polysomes nor the quantitative distribution of newly synthesized poly(A)$^+$ mRNA among polysomal fractions : unmethylated poly(A)$^+$ RNA are normally integrated into large polysomes, in proportion very similar to control and without any lag for the utilization of these cytoplasmic unmethylated molecules for the polysome assembly, since none cytoplasmic accumulation as free cytoplasmic mRNP was observed. This result must be put together with recent reports of in vitro studies focused on the role of the 5' terminal cap in the translation process [11, 21, 22]. Using all free extracts from wheat germ, the translational efficiency of viral mRNAs was studied in correlation with modification of its 5' terminal cap [21]. These results suggested an obligatory role of the m^{7}G for translation of eukaryotic mRNA. They were subsequently reevaluated by further comparative studies of wheat germ and reticulocyte lysate systems [11]. In this latter case, the 5' terminal m^{7}G of VSVmRNA was found to have a much less important role in recognition by translational components. One could imagine that the discrepancy between the results on the wheat germ system and, on the other hand, the reticulocyte system and our in vivo results, depends on the absence or presence of appropriate factors which could interact with m^{7}G at the time of protein synthesis initiation, as for example a cap binding protein [23].

Our results clearly establish that none of the 3 or 4 methyl groups generally found in eucaryotic messenger RNA is stringently required for the production of functional mRNA. However the lack of methylation is correlated to obvious perturbations in mRNA processing. As for the transcription level, a precise determination of the rate of synthesis of poly(A)$^-$ hnRNA during the cycloleucine treatment is difficult to obtain, due to the superimposition of pre-rRNA molecules,as we have settled not to perform a selective block by low doses of Actinomycin D which could result in feed-back alterations in the level of hnRNA production [24]. However, we did not observe significant variations in the labelling of nuclear RNA molecules larger than 45S when CHO cells were pulsed for short periods with (^{3}H) uridine in the presence of cycloleucine, which seems to indicate that the undermethylation does not induce drastic modifications in the biosynthesis rate of hnRNA.

As for the post-synthetic modifications of the pre-mRNA transcript in eucaryotic cells, one still lacks a detailed knowledge of the actual se-

quence of maturational events(methylations, cleavage and splicing [25], polyadenylation [26] and protein binding). Anyhow, our results clearly establish that hnRNA methylation is not necessary for its polyadenylation, even if a light initial decrease in newly synthesized poly(A)$^+$ hnRNA appearance was observed (15 %) in presence of cycloleucine. In mammalian cells, hnRNA maturation is accompagnied by a decrease in the average size of precursor molecules [1].This processus, still unclear, was correlated to the reduction of the internal m^6A group number per molecule [5]. Routinely, we observed that the average size of total poly(A)$^+$ hnRNA was,for short given labelling time, higher for undermethylated molecules than for normally methylated RNAs. It must be pointed out that the size distribution of both types of molecules became almost superimposable for prolonged periods of chase. Put together with the initial lag-time observed for the cytoplasmic appearance of unmethylated poly(A)$^+$ RNA, these results could indicate that if m^6A in hnRNA are important for the kinetics ofits processing, the presence of the methyl group does not play a key-role in the site specificity of the maturational cleavages. Obviously, a more precise evaluation of the interaction between methylation and mRNA processing is difficult to be drawn from our present study performed on a complex population of messenger molecules. In order to gain a further insight onto this problem, we are now studying the production of a specific mRNA during an inhibition of RNA methylation by cycloleucine in Friend cells committed to differentiate.

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