INHIBITION OF REOVIRUS MESSENGER RNA METHYLATION IN EXTRACTS OF INTERFERON-TREATED EHRLICH ASCITES TUMOR CELLS

G. C. Sen[+], B. Lebleu[+], G. E. Brown[+], M. A. Rebello[+], Y. Furuichi[°], M. Morgan[°], A. J. Shatkin[°] and P. Lengyel[+]

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Conn. 06520[+] and Department of Cell Biology, Roche Institute of Molecular Biology, Nutley, New Jersey 07110[°]

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SUMMARY. The methylation of (unmethylated) reovirus mRNA is impaired in extracts of interferon-treated Ehrlich ascites tumor cells. The impairment is due to one or more inhibitors. Its extent decreases with an increasing concentration of reovirus mRNA in the reaction mixture. The impairment is apparently not a consequence of the degradation of reovirus mRNA. Since methylation of reovirus mRNA is apparently required for translation, it is conceivable that the impairment of methylation may be part of the antiviral activities of interferons.

Interferons are macromolecules, probably glycoproteins, whose synthesis is induced in a variety of vertebrate cells upon viral infection or some other stimuli. They are released from the producing cells, interact with other cells and make these inefficient in supporting the growth of a broad range of viruses. The mechanism of the inhibition of virus replication in cells treated with interferon is under investigation (1). In the case of reovirus the absorption, penetration into cells, and the partial uncoating of the virus are apparently not inhibited, whereas the accumulation of virus-specific RNAs and proteins is (Galster et al, unpublished results, ref. 2). It remains to be established if the decrease in the accumulation of virus-specific macromolecules in interferon-treated cells is a consequence of the impairment of viral RNA metabolism (e.g. transcription or processing) or of the translation of viral mRNA or of both. In extracts of interferon-treated cells ($S30_{INT}$) the translation of exogenous viral mRNA is impaired (2-6). In Sephadex-treated extracts this inhibition can be at least partially overcome by addition of crude tRNA (7,8) whereas in extracts not treated with Sephadex it cannot (Sen et al, in preparation).

Abbreviations: EAT, Ehrlich ascites tumor; Endog., endogenous; INT, interferon; reo $mRNA_U$ and reo $mRNA_M$, unmethylated and methylated reo mRNA; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine; $S30_{INT}$ and $S30_C$ extracts from cells treated with interferon and from control cells; sp. act., specific activity; m^7G, 7-methylguanosine ; Gm, 2'-O-methylguanosine; VSV, vesicular stomatitis virus.

It has been recently established that many eucaryotic viral and cellular mRNAs contain blocked and methylated 5' ends (9-12). Thus the 5' ends of reovirus mRNAs consist of $m^7G5'ppp5'Gm$ sequences (12). This 5' terminal methylation of reo mRNA was shown to be required for translation in S30 extracts from cells: The translation of unmethylated reo mRNA (reo $mRNA_U$; obtained by synthesizing the RNA in the presence of an inhibitor of methylation, SAH), was blocked in cell extracts if methylation by the endogenous methylating enzymes of the extract was blocked by SAH (13).

In this communication we demonstrate that the process of methylation of (unmethylated) reo $mRNA_U$ is impaired in extracts of interferon-treated cells.

RESULTS AND DISCUSSION

We measured RNA methylation by assaying the accumulation in RNA of [^{3}H] methyl residues originating from [^{3}H] methyl-labeled SAM. Using this assay we found less methylation of RNA in extracts of INT-treated EAT cells ($S30_{INT}$) than in corresponding extracts of control cells ($S30_C$). The experiments shown

Table I. Methylation of endogenous RNA, exogenous tRNA and exogenous Reo $mRNA_U$ in different pairs of $S30_C$ and $S30_{INT}$

S30 Number	RNA Methylated	Time of Incubation: 5 min. $S30_C$	5 min. $S30_{INT}$	5 min. Inhibition	20 min. $S30_C$	20 min. $S30_{INT}$	20 min. Inhibition
		cpm x 10^{-3}/ml*		%	cpm x 10^{-3}/ml*		%
I	Endog.	85	69	19	173	163	6
	+ Reo $mRNA_U$	237	147	-	320	208	-
	+ Reo $mRNA_U$-Endog.	152	78	49	147	45	69
	+ tRNA	-	-	-	377	322	-
	+ tRNA-Endog.	-	-	-	204	159	22
II	Endog.	126	115	9	242	223	8
	+ Reo $mRNA_U$	246	170	-	331	248	-
	+ Reo $mRNA_U$-Endog.	120	55	54	89	25	72
	+ tRNA	219	204	-	419	338	-
	+ tRNA-Endog.	93	89	4	177	115	35
III	Endog.	15	12	20	28	25	11
	+ Reo $mRNA_U$	128	60	-	147	81	-
	+ Reo $mRNA_U$-Endog.	113	48	58	119	56	53
	+ tRNA	42	35	-	82	62	-
	+ tRNA-Endog.	27	23	15	54	37	32

in Table I were performed with extracts from three pairs of cell cultures grown at different times. In each case the methylation of added reo $mRNA_U$ was more impaired than that of added tRNA and that of endog. RNA was the least affected. Thus the impairment of RNA methylation is not uniform but selective.

The impairment of reo $mRNA_U$ methylation in $S30_{INT}$ is due to one or more inhibitor(s) (Table II): There was less methylation in a reaction mixture containing both $S30_C$ and $S30_{INT}$ than in one containing only $S30_C$ if the amounts of $S30_C$ in the two reaction mixtures were identical. An increase in the amount of $S30_C$ in the reaction mixture seemed to decrease the methylation of reo $mRNA_U$. This decrease may be only apparent: It might be due to the fact that the $S30_C$ had not been Sephadex-treated and thus contained endog. unlabeled SAM and furthermore SAM might have been generated during incubation at a rate increasing with the amount of $S30_C$.

The methylation of endog. RNA proceeded at a diminishing rate for at least 30 min in S30 extracts and its rate in $S30_{INT}$ was only slightly lower than that in $S30_C$ (Fig. 1). In the same conditions reo $mRNA_U$ methylation ceased

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Table 1.
Mouse interferon: The sp. act. of the partially purified preparation was 6×10^6 NIH mouse reference standard units/mg protein. This corresponds to 6×10^5 VSV plaque reduction units/mg protein. The units in this paper are VSV plaque reduction units. S30 extracts were prepared either from EAT cells in suspension culture which had been treated with 150 units/ml of INT for 18 hours ($S30_{INT}$) or from cells grown under the same conditions but not treated with INT ($S30_C$) according to the procedure in ref. 14 except that the solution with which the cells were washed was supplemented with 12 mM glucose and that the extracts were neither "preincubated" nor passed through a Sephadex column. To obtain S30 extracts of identical concentration each volume of sedimented cell pellet was homogenized in two volume of buffer. Reo mRNA was prepared by transcription on chymotrypsin-treated purified reo virions (Dearing strain type 3). For obtaining methylated reo mRNA 10 µM SAM was included in the reaction mixture, for obtaining unmethylated reo mRNA 10 µM SAH (13). tRNA was prepared from EAT cells according to a published procedure (15). Methylation of RNA: The composition of the reaction mixture (30 µl) was similar to that developed for reo mRNA translation (14) except that the concentration of methionine was 2 µM and that of the other amino acids was 50 µM each and that the reaction mixture also contained 8 µM [^{3}H] SAM (methyl labeled, from New England Nuclear, sp. act. 12.6 Ci/mmole neutralized just before using it with 2 M tris base from Sigma.) Incubation was at 37°. The reaction was stopped by addition of 0.7 ml of buffer A (10 mM tris HCl (pH 7.5), 100 mM NaCl, 5 mM EDTA, 0.5% w/v SDS) and the reaction mixture was extracted with 0.7 ml of phenol saturated with buffer A. To 0.5 ml of the resulting aqueous layer 200 µg of carrier yeast tRNA was added as well as 10 ml of 10% trichloroacetic acid. The precipitate (mainly RNA) was filtered through a nitrocellulose filter (Millipore) and its radioactivity was determined by counting in a toluene based scintillator (13). In the experiments of Table I the reaction mixtures contained either 15 µl of S30 (in the case of S30 Numbers I and II) or 5 µl of S30 (in the case of S30 Number III). The concentration of reo $mRNA_U$ and tRNA were 100 µg/ml and 250 µg/ml respectively. * [^{3}H] methyl residues incorporated into RNA.

Table II. Dominance of the inhibition of methylation by factor(s) in $S30_{INT}$

S30 Number I Type	Amount	RNA Methylated Endog.	+ Reo $mRNA_U$	+ Reo $mRNA_U$-Endog.	Inhibition
	µl		cpm x 10^{-3}/ml*		%
C	5	33	249	216	-
C	10	65	243	178	-
C	15	82	248	166	-
INT	5	33	167	134	38
INT	10	59	139	80	55
INT	15	84	137	53	68
C + INT	5 + 10	86	159	73	56
C + INT	10 + 5	90	187	97	42

Each reaction mixture (30µl) contained the type and amount of S30 indicated. The concentration of reo $mRNA_U$ was 100µg/ml. For calculating the inhibition in the reaction mixtures containing both $S30_C$ and $S30_{INT}$ the methylation of reo $mRNA_U$ in 15µl of $S30_C$ was taken as 100%. The reaction mixtures were incubated for 10 min. For further details see the legend to Table I.

* [^{3}H] methyl residues incorporated into RNA.

after 5 min or less. In line with the results in Table I the methylation of reo $mRNA_U$ was much more impaired in $S30_{INT}$ than that of endog. RNA.

The impairment of reo $mRNA_U$ methylation in $S30_{INT}$ (i.e. the ratio, methylation in $S30_C$ - methylation in $S30_{INT}$/methylation in $S30_C$) diminished with increasing concentrations of reo $mRNA_U$ (Fig. 2). This result may indicate that reo $mRNA_U$ present in excess can overcome the action of the inhibitor(s) of methylation.

The fact that methylated reo $mRNA_U$ was stable in $S30_{INT}$ (actually more so than in $S30_C$)(Table III) seems to indicate that the impairment of reo $mRNA_U$ methylation is not due to a faster degradation of the product of the methylation in $S30_{INT}$ than in $S30_C$.* Moreover results of preliminary experiments indicate that the impairment is not a consequence of either the degradation of SAM or

* The assay for RNA degradation was based on the determination of cold acid-insoluble, labeled material. Thus cleavage into large RNA segments which are insoluble in cold acid would not have been detected.

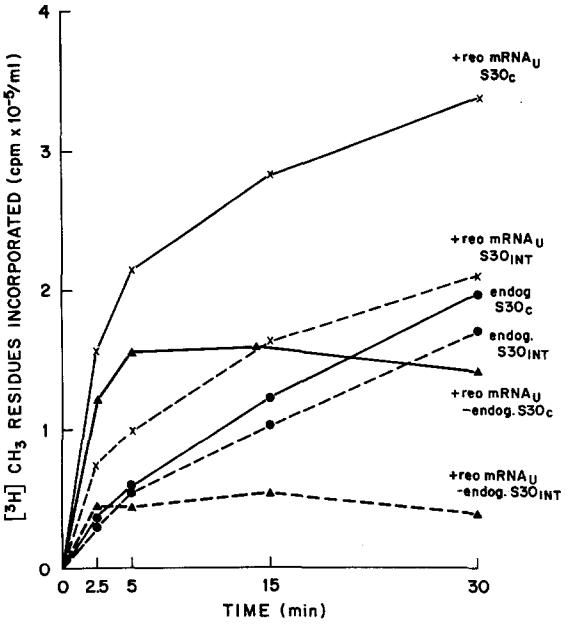

Fig. 1. Methylation of endogenous RNA, exogenous tRNA and exogenous reo $mRNA_U$ in $S30_C$ and $S30_{INT}$: Kinetics. Each reaction mixture (30 µl) contained 15 µl of S30 Number I. The concentration of reo $mRNA_U$ was 100 µg/ml. For further details see the legend to Table I.

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of the irreversible inactivation of the substrate i.e. reo $mRNA_U$ (data to be published elsewhere).

As expected, there was less methylation in both $S30_C$ and $S30_{INT}$ of reo $mRNA_M$ (i.e. reo mRNA synthesized in the presence of SAM) than of reo $mRNA_U$ (Table IV). The impairment of reo mRNA methylation in $S30_{INT}$ may be site specific: The methylation of reo $mRNA_M$ was impaired much less (if at all) than that of reo $mRNA_U$.

It should be noted that the reo $mRNA_U$ used as a methyl residue acceptor in these studies might be an unnatural substrate: The transcription of the reo genome by virion-associated enzymes results in reo $mRNA_M$, and the cellular methylating enzymes have not yet been shown to be involved in reo mRNA methylation.

Further studies are needed for establishing if the impairment of RNA methylation in $S30_{INT}$ is due to INT action and relevant to the antiviral effect of INT. Along these lines we already established that a 1.5 hour exposure of the cell culture to our INT preparation does not elicit the impairment of

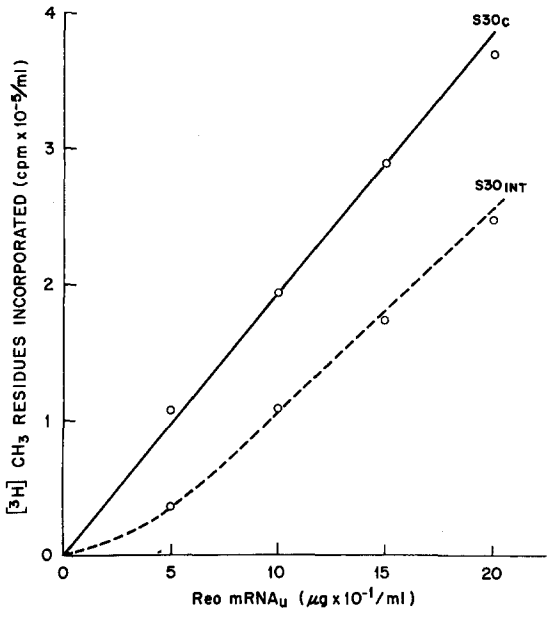

Fig. 2. Methylation of reo $mRNA_U$ in $S30_C$ and $S30_{INT}$: Dependence on the concentration of reo $mRNA_U$. Each reaction mixture (30 µl) contained 5 µl of S30 Number I. The reaction mixtures were incubated for 15 min. The labeling due to the methylation of endog. RNA (0.43×10^5 cpm/ml in $S30_C$ and 0.44×10^5 cpm/ml in $S30_{INT}$) was subtracted from the experimental values. For further details see the legend to Table I.

Table III. Stability of methylated Reo $mRNA_U$ in $S30_C$ and $S30_{INT}$

S30 Number III Type	RNA Methylated	Time of Incubation 5 min.	10 min.	20 min.
		cpm x 10^{-3}/ml*		
C	Endog.	13	14	15
C	+ Reo $mRNA_U$	107	104	95
C	+ Reo $mRNA_U$-Endog.	94	90	80
INT	Endog.	13	12	11
INT	+ Reo $mRNA_U$	42	41	41
INT	+ Reo $mRNA_U$-Endog.	29	29	30

Each reaction mixture (30µl) contained 5µl of S30. The concentration of reo $mRNA_U$ was 100µg/ml. After a 3 min. incubation in the presence of [^{3}H]-SAM under the conditions of methylation, 800 M SAH was added to each reaction mixture, and the incubation was continued until the time indicated. For further details see the legend to Table I.

* [^{3}H] methyl residues incorporated into RNA.

Table IV. Methylation of Reo $mRNA_U$ and Reo $mRNA_M$ in $S30_C$ and $S30_{INT}$

RNA Methylated	$S30_C$	$S30_{INT}$	Inhibition
	cpm x 10^{-3}/ml*		%
Endog.	33	28	15
+ Reo $mRNA_U$	139	98	-
+ Reo $mRNA_U$-Endog.	106	70	34
+ Reo $mRNA_M$	57	50	-
+ Reo $mRNA_M$-Endog.	24	22	8

Each reaction mixture (30µl) contained 5µl of S30 (Number I). The concentration of reo mRNA was 100µg/ml. The reaction mixtures were incubated for 15 min. For further details see the legend to Table I.

* [^{3}H] methyl residues incorporated into RNA.

methylation in the cell extract; this impairment apparently requires a longer incubation of the cells after exposing them to INT (data not shown). This requirement is similar to that needed for induction of the antiviral state by INT.

Obviously much remains to be learned about the above phenomena. Nevertheless the need for methylation of the 5' end of eucyarotic mRNAs for translation on the one hand and the impairment of this process in extracts of INT-treated cells on the other hand makes one wonder if the impairment of viral mRNA methylation is not part of the antiviral activities of interferons.

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