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Impairment of Reovirus mRNA Methylation in Extracts of Interferon-Treated Ehrlich Ascites Tumor Cells: Further Characteristics of the Phenomenon

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We reported earlier that the methylation of unmethylated reovirus mRNA (reo $mRNA_U$) by the cellular methylating enzymes is impaired in extracts of uninfected, interferon-treated Ehrlich ascites tumor cells ($S30_{INT}$). We find now that after the methylation of reo $mRNA_U$ has stopped in $S30_{INT}$, the RNA can be reisolated and further methylated in an extract of control cells ($S30_C$). Thus the impairment of methylation in $S30_{INT}$ cannot be due to cleavage or irreversible inactivation of reo $mRNA_U$. Freshly added reo $mRNA_U$ can be methylated in $S30_{INT}$ in which the methylation of previously added reo $mRNA_U$ has stopped. This indicates that the impairment is not due to the depletion of *S*-adenosylmethionine (the methyl donor), the accumulation of *S*-adenosylhomocysteine (an inhibitor of methylation), or the irreversible inactivation of the methylating enzymes. It may be due, however, to the unavailability of reo $mRNA_U$ for methylation. The extent of the impairment of reo $mRNA_U$ methylation in $S30_{INT}$ decreases with an increasing concentration of reo $mRNA_U$ but is not affected by added poly(U), ribosomal RNA, or encephalomyocarditis virus RNA (an mRNA that is probably not capped or methylated at its 5′ end). The methylation of reo $mRNA_U$ is also impaired in an extract from cells that have not been treated with interferon but with the interferon inducer poly(I)·poly(C). The inhibitor is apparently a macromolecule that is inactivated during incubation. It decreases the methylation at the 7 position of the 5′ terminal guanylate residue. In vitro, the rate of reo mRNA synthesis by reovirus cores in the presence of $S30_{INT}$ is the same as in the presence of $S30_C$. However, the methylation of the de novo synthesized reo mRNA by the core-associated methylating enzyme(s) in vitro is inhibited by $S30_{INT}$ but not by $S30_C$. The relevance of these phenomena to the inhibition of reovirus replication in interferon-treated cells remains to be established.

Interferons are glycoproteins formed in a large variety of animal cells upon viral infection. They are released, interact with other cells, and inhibit in these the multiplication of various viruses (9). We have been studying the nature of the impairment of reovirus replication in interferon-treated mouse L929 fibroblasts (L cells) and Ehrlich ascites tumor (EAT) cells (11, 15, 21; see also references 12 and 29). After penetration into the cells, the reovirions are converted into subviral particles by removal and cleavage of their outer coat proteins. Each reovirion (and each subviral particle) contains 10 different genomic double-stranded RNA segments. Subviral particles isolated from infected cells and also reovirus cores produced by partial digestion of reovirions by chymotrypsin exhibit a transcriptase activity. When incubated in the presence of the four common ribonucleoside triphosphates, both types of particles synthesize reo mRNA's. Each of these mRNA's is transcribed from a different segment of the viral genome. The 10 reo mRNA's fall into three size classes according to sedimentation velocity: large (l_1, l_2, l_3), medium (m_1, m_2, m_3), and small (s_1, s_2, s_3, s_4) mRNA's (19). Reovirus cores also contain an enzyme (GTP:mRNA guanylyltransferase or capping enzyme) which attaches a guanylate residue to the 5′ end of each of the reo mRNA's. This process is called capping. Its products, the capped reo mRNA's, have a 5′ terminal G(5′)ppp(5′)Gp sequence. Other enzymes associated with reovirus cores catalyze the transfer

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of methyl residues from *S*-adenosylmethionine (SAM) to the 7 position of the above-noted 5′ terminal guanylate residue and to the 2′-*O*-position of the adjacent guanylate residue. We will refer to this process as methylation. $m^7G(5')ppp(5')GmpCp$ . . . . is the 5′ nucleotide sequence of the methylated capped reo mRNA's that are synthesized by the virion-associated enzymes (26).

Enzymes capping the 5′ termini of mRNA's and enzymes methylating the cap structure are also present in uninfected eukaryotic cells (14, 24). Many eukaryotic cellular and viral mRNA's are capped and methylated (26). The methylation (but not the capping) of mRNA in vitro by both cellular and viral core-associated enzymes can be blocked by *S*-adenosylhomocysteine (SAH). The use of this inhibitor allows the synthesis of capped but unmethylated reoviral mRNA's (reo $mRNA_U$) by the core-associated reovirion transcriptase and capping enzymes in vitro (26). Experiments involving the use of reo $mRNA_U$ and of methylated and capped reo mRNA's (reo $mRNA_M$) revealed that the presence of the methyl group at the 7 position of the 5′ terminal guanylate residue of reo mRNA's is essential for the efficient translation of the mRNA in cell-free protein-synthesizing systems from wheat germ or from mouse L cells. Without this residue the viral mRNA apparently does not form initiation complexes with wheat germ ribosomes (3, 4).

In the course of attempts to uncover the steps of reovirus replication which are impaired in interferon-treated cells, we compared the methylation of added reo $mRNA_U$ in extracts of uninfected control EAT cells ($S30_C$) with that in extracts of uninfected, interferon-treated EAT cells ($S30_{INT}$) (25). We found that the methylation of added reo $mRNA_U$ by the host enzymes in $S30_{INT}$ was impaired. The impairment was not a consequence of the cleavage of the products of methylation, and its extent decreased with an increasing concentration of reo $mRNA_U$ in the reaction mixture (21, 25).

Further characteristics of these and of related phenomena are the topic of this communication. Some of these results have been reported (S. Shaila, B. Lebleu, G. C. Sen, G. E. Brown, and P. Lengyel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S209, p. 239; B. Lebleu, G. C. Sen, S. Shaila, G. E. Brown, and P. Lengyel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S210, p. 239).

MATERIALS AND METHODS

Chemicals and enzymes. Sephadex G-25, Sephadex G-100, and DEAE-dextran were purchased from Pharmacia Fine Chemicals; poly(I)·poly(C) and poly(U) from Miles Laboratories, Inc.; DEAE-cellulose from Whatman GF/C; alkaline phosphatase (BAPF, 34 units/mg) and chymotrypsin from Worthington Biochemicals Corp.; T2 RNase (250 units/mg) from Sigma Chemical Corp.; [^{3}H]SAM (methyl labeled; specific activity, 12.6 Ci/mmol) and [8-^{3}H]GTP (specific activity, 9.35 Ci/mmol) from New England Nuclear; β,γ-[^{32}P]GTP (specific activity, 2.14 Ci/mmol) from ICN Corp; the minimal essential media (F-14 and F-15) and fetal calf serum from GIBCO; and ACS scintillation fluid from Amersham/Searle Corp.

Interferons. (i) Mouse interferon. A partially purified mouse interferon preparation was obtained by infecting EAT cells with Newcastle disease virus and purifying the interferon by chromatography on CM-Sephadex (manuscript in preparation). The preparation had a specific activity of 2×10^7 NIH mouse reference standard units per mg of protein. This corresponds to 2×10^6 vesicular stomatitis virus plaque reduction units per mg of protein. The units for mouse interferon throughout this paper are vesicular stomatitis virus plaque reduction units (28).

(ii) Human interferon. A preparation of crude human interferon (specific activity, 10^4 units/mg of protein) was obtained by the superinduction of the foreskin fibroblast strain FS-4 and assayed according to Havell and Vilček (16). The units for human interferons are NIH human interferon reference standard units (*G-0230901-52).

Cells and viruses. EAT cells (27) were grown at 37°C in suspension culture in F-14 medium supplemented with 7% fetal calf serum or in monolayers in F-15 medium supplemented with 7% fetal calf serum. Reovirus type 3 (Dearing strain) was used. Encephalomyocarditis (EMC) virus was grown, and EMC RNA was prepared according to Aviv et al. (1). Mengo virus was grown under the same conditions as EMC virus. EAT ribosomal RNA was prepared in the following way. Total cytoplasmic RNA was extracted, and RNA containing poly(A) was separated from the rest by passing through an oligo(dT) cellulose column (2). RNA lacking poly(A) was sedimented through a sucrose gradient, and RNA sedimenting at 18 and 28*S* was pooled and recovered.

Treatment of cells with interferon and the interferon inducer poly(I)·poly(C) and preparation of cell extracts (S30). Growing suspension cultures of cells were diluted to a density of 4×10^5 cells/ml and treated with 60 units of interferon per ml for 18 h. Treatment with interferon at this concentration caused over 95% decrease in the yield of vesicular stomatitis virus in a single growth cycle. S30 from interferon-treated cells ($S30_{INT}$) and from untreated cells ($S30_C$) were prepared as described earlier for L cells (13), except that the solution in which the cells were washed was supplemented with 12 mM glucose and dithiothreitol was not included in the buffer. Unless otherwise indicated, the S30s were not preincubated and not Sephadex treated (i.e., the last step of the preparation was centrifugation at 30,000 × *g* for 15 min as described in reference 13). The treatment of EAT cells with the interferon inducer

poly(I)·poly(C) (10 μg/ml) in monolayers and the preparation of S30 [$S30_{poly(I)\cdot poly(C)}$] from such cells and corresponding cells not treated with the interferon inducer have been described (6). (The treatment caused over 99% reduction in the yield of vesicular stomatitis virus in a single growth cycle.)

Preparation of reovirus mRNA's. These were prepared by transcription on reovirus cores (obtained by treatment of reovirions with chymotrypsin) according to Both et al. (5). The reaction mixtures for the preparation of methylated reovirus mRNA (reo $mRNA_M$) included 100 μM SAM, and those mixtures for preparing unmethylated reovirus mRNA (reo $mRNA_U$) included 100 μM SAH. Both preparations consisted of species with heterogeneous 5′ terminal structures occurring in approximately the following proportions: reo $mRNA_M$ ($m^7GpppGm$ . . . ., 75%; ppG . . . ., 25%; GpppG . . . ., 1 to 2%) and reo $mRNA_U$ (ppG . . . ., 70 to 75%; GpppG . . . ., 25 to 30%) (4). For preparing "CAP" [^{32}P]-labeled reo mRNA, β,γ-[^{32}P]GTP was included in the reaction mixture at a concentration of 1.25 mCi/ml, and the total GTP concentration was reduced to 1.0 mM.

Assay of reo $mRNA_U$ methylation. This was based on determining the amount of [^3H]CH_3 residues transferred from [^3H]CH_3-labeled SAM to RNA, i.e., cold trichloroacetic acid-insoluble material not extracted into phenol (25).

Alkaline phosphatase treatment of reo mRNA. To remove the 5′ terminal phosphate residues from the 5′ terminal ppG . . . . structure, the reo $mRNA_U$ was incubated with alkaline phosphatase (0.3 mg/ml) in 50 mM Tris-chloride (pH 8.0) at 37°C for 10 min. Alkaline phosphatase was removed by extraction with phenol, and the RNA was recovered by dialysis and precipitation with ethanol (Table 1).

Analysis of reo mRNA by centrifugation through sucrose gradients. Aqueous samples containing labeled reo mRNA were layered on 12.5-ml linear sucrose gradients (7 to 20%, wt/vol) in 100 mM NaCl, 10 mM Tris-chloride (pH 7.5), and 5 mM EDTA. The gradients were centrifuged in the SB283 rotor in an IEC B-60 centrifuge at 2°C and 39,000 rpm for 12 h. Fractions of approximately 0.4 ml were collected from the bottom and counted after addition of 10 ml of ACS scintillation fluid (Fig. 2).

Determination of the site of labeling in reo $mRNA_U$ prepared with β,γ-[^{32}P]GTP and in reo $mRNA_U$ methylated in S30 with [^3H]SAM. This was done according to the method of Furuichi et al. (10). Reo $mRNA_U$ was digested with T2 RNase (0.3 mg/ml) in 10 mM sodium acetate (pH 4.5) at 37°C for 3 h. The digest was supplemented with 13.0 units of absorbancy at 260 nm of an RNase A digest of unlabeled yeast RNA containing mono- and oligonucleotides. The reaction mixture was supplemented with urea (final concentration, 7 M) and applied to a DEAE-cellulose column (0.7 by 25 cm) which had been equilibrated with 7 M urea, 50 mM Tris-chloride (pH 7.5), and 50 mM NaCl. The column was eluted with 100 ml of a linear gradient of NaCl (50 to 250 mM) in 7 M urea and 50 mM Tris-chloride (pH 7.5). The absorbancy at 254 nm of the eluate was monitored. Each fraction (1 ml) from the eluate was supplemented with 10 ml of ACS scintillation fluid and counted. The 254-nm absorbancy peaks, resulting from the elution of unlabeled oligonucleotides of different net charges, served as internal markers for determining the net charge of the labeled component in the T2 RNase digest of the reo $mRNA_U$ (Fig. 1 and 7).

Assays of core transcription and core methylation. Reovirus cores were prepared by chymotrypsin treatment of purified reovirions according to the method of Both et al. (5), except that the transcription reaction was done at 37°C, no Macaloid was added, and GTP was added after the formation of cores (Fig. 9 and 10).

RESULTS

Impairment of reo $mRNA_U$ methylation is not due to faster cleavage of reo mRNA in $S30_{INT}$ than in $S30_C$. The 5′ terminal sequence of the molecules serving as substrates for methylation in our reo $mRNA_U$ preparation (cap-labeled, alkaline phosphatase-treated reo $mRNA_U$) was G(5′)pp*p(5′)G . . . . (Fig. 1). The methylation assay was based on the conversion of acid-soluble labeled material (methyl-labeled SAM) to acid-insoluble labeled material (methyl-labeled reo mRNA). In the experiment shown in Fig. 2A the methylation of reo $mRNA_U$ reached 50 to 60% of its final level after 1 min and stopped after 5 min in both $S30_C$ and $S30_{INT}$. The final level of methylation was about

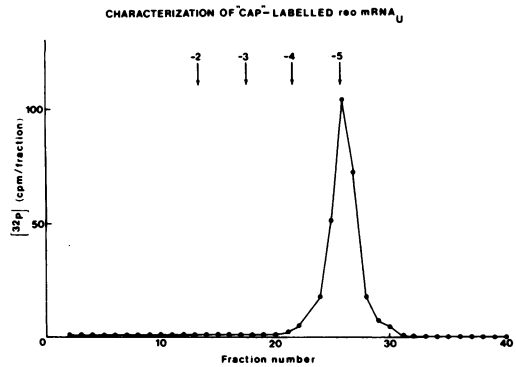

FIG. 1. *Characterization of cap-labeled reo $mRNA_U$. Reo $mRNA_U$ was synthesized by viral cores in the presence of β,γ-[^{32}P]GTP and SAH. Treatment with alkaline phosphatase was used to remove the two phosphate residues from RNA molecules with 5′ terminal p*pG. . . . moieties. (p* indicates a [^{32}P]-labeled phosphate moiety). As expected, approximately 70% of the ^{32}P radioactivity in the RNA became acid soluble upon the phosphatase treatment. The RNA was recovered thereafter, digested with T2 RNase, and analyzed by DEAE-cellulose chromatography in the presence of 7 M urea. This revealed only one labeled species with a −5 charge. This verifies that the cap structure Gpp*pG. . . . is the only site labeled in our alkaline phosphatase-treated reo $mRNA_U$ preparation.*

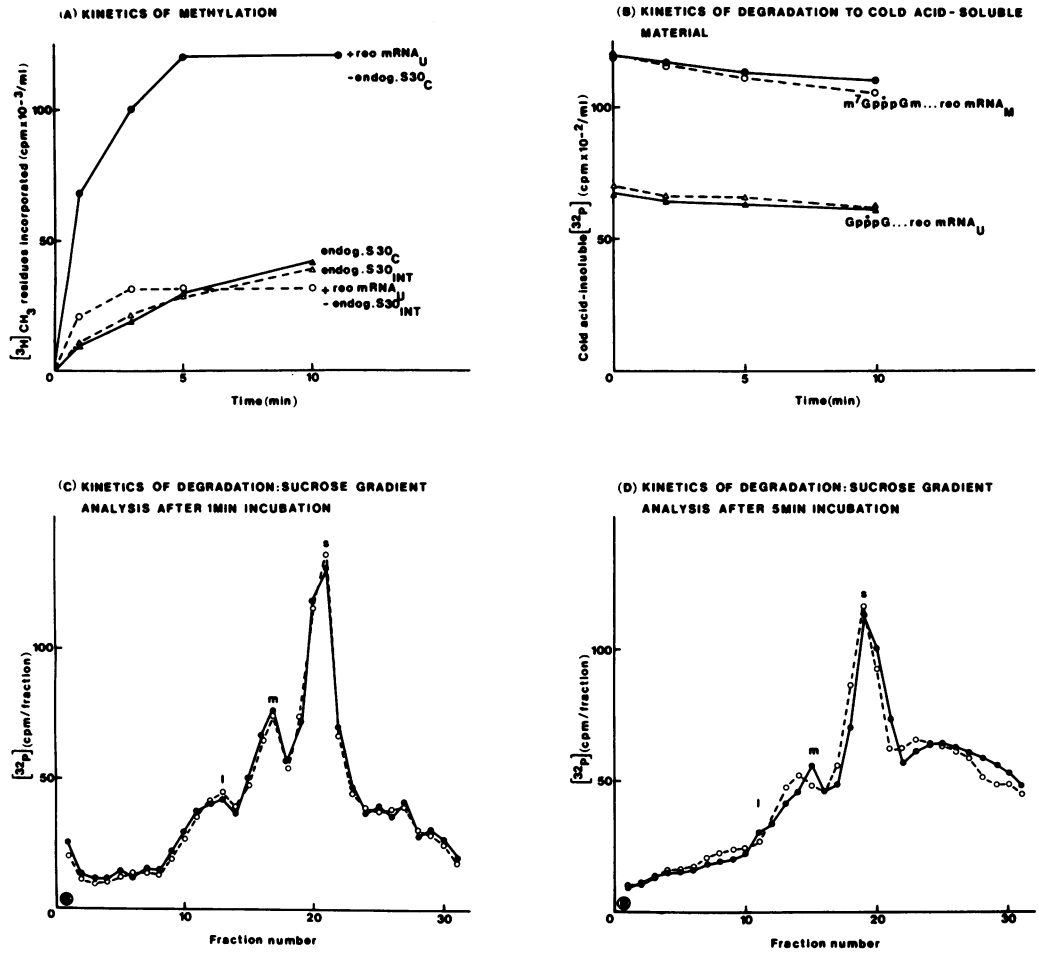

FIG. 2. *Comparison of the kinetics of methylation and degradation of CAP-labeled reo mRNA$_U$ in S30$_C$ and S30$_{INT}$. In all experiments CAP ^{32}P-labeled alkaline phosphatase-treated reo mRNA's (designated as CAP-labeled reo mRNA) were used. (For the characterization of CAP-labeled reo mRNA$_U$, see the legend to Fig. 1.) (A) Kinetics of methylation of CAP-labeled reo mRNA$_U$ in S30$_C$ and S30$_{INT}$. CAP-labeled reo mRNA$_U$ (100 μg/ml) was added to the reaction mixtures as specified in the figure. As indicated in the figure, endogenous methylation was subtracted from the methylation occurring with added reo mRNA$_U$. (B) Kinetics of degradation of CAP-labeled reo mRNA$_M$ and reo mRNA$_U$ to acid-soluble material in S30$_C$ and S30$_{INT}$. A 100-μg portion of CAP-labeled reo mRNA$_M$ or reo mRNA$_U$ per ml was incubated under methylation conditions, except that [^{3}H]SAM was absent and 800 μM SAH was added to the reaction mixtures. The processing of the incubated reaction mixtures was as described for the methylation of RNA. The continuous lines in the figure indicate incubation in S30$_C$; the discontinuous lines indicate incubation in S30$_{INT}$. (C) Kinetics of degradation of CAP-labeled reo mRNA$_U$ in S30$_C$ and S30$_{INT}$: analysis on sucrose gradients. A 100-μg portion of CAP-labeled reo mRNA$_U$ per ml was incubated under methylation conditions for 1 min, except that unlabeled SAM was substituted for [^{3}H]SAM. The incubated reaction mixtures were extracted with phenol as described for the methylation assay, and the resulting aqueous layers were applied on top of sucrose gradients and analyzed as described in Materials and Methods. The continuous lines indicate incubation in S30$_C$; discontinuous lines indicate incubation in S30$_{INT}$. The peaks of sedimentation of large, medium, and small size classes of reo mRNA's are represented as l, m, and s, respectively, in the figure. (D) Same as (C), except that the reaction mixtures were incubated for 5 min.*

70% lower in S30$_{INT}$ than in S30$_C$. Under the same conditions the rates of hydrolysis of cap-labeled, alkaline phosphatase-treated reo mRNA$_M$ and reo mRNA$_U$ into acid-soluble products in S30$_{INT}$ were indistinguishable from those in S30$_C$, and less than 10% of the label in the RNAs became acid soluble (Fig. 2B). Since the amount of acid-insoluble labeled material was the measure of methylation, these results prove that the impairment of methylation is

not a consequence of a faster cleavage of the methylation products into acid-soluble material in $S30_{INT}$ than in $S30_C$.

However, the results did not rule out the possibility that the impairment could be due to a faster degradation in $S30_{INT}$ of reo $mRNA_U$ into acid-insoluble fragments that might not be substrates for cap methylation. This possibility was made unlikely by the results in Fig. 2C and 2D. These reveal that the size distribution of cap-labeled, alkaline phosphatase-treated reo $mRNA_U$ incubated under methylation conditions in $S30_{INT}$ and $S30_C$ were indistinguishable after 1 min (by which time methylation reached only 50 to 60% of its final level) and also after 5 min (by which time methylation has reached its final level). It should be noted that the rate of cleavage of uniformly ^{32}P-labeled reo $mRNA_U$ and reo $mRNA_M$ in $S30_{INT}$ was also indistinguishable from that in $S30_C$. This conclusion is based on experiments in which the reo mRNA's were incubated under methylation conditions and their cleavage into acid-soluble material was monitored after 5, 10, 15, and 20 min of incubation by precipitation with cold trichloroacetic acid, and their cleavage into large and insoluble fragments was detected after a 15-min incubation by analysis on sucrose gradients (data not shown).

Impairment of reo $mRNA_U$ methylation in $S30_{INT}$ is not due to an irreversible inactivation of reo $mRNA_U$. The experiments outlined in Fig. 3 were performed to test if the impairment of reo $mRNA_U$ methylation in $S30_{INT}$ could be due to an irreversible inactivation of reo $mRNA_U$ as a substrate for methylation. For this purpose portions of reo $mRNA_U$ were methylated in $S30_C$ and $S30_{INT}$ until methylation stopped, and the extent of methylation in each extract was determined. Thereafter total RNA (including reo $mRNA_U$) was isolated from the reaction mixtures and portions of it were added to $S30_C$ and $S30_{INT}$ and further methylated in these. (To determine what fraction of the methylation of total RNA during the second incubation was due to reo $mRNA_U$ methylation, total RNA was also isolated from $S30_C$ and $S30_{INT}$ which had been incubated without added reo $mRNA_U$ and further methylated in $S30_C$ and $S30_{INT}$.) The data in Fig. 3 indicate that: (i) as expected, the methylation of reo $mRNA_U$ was much less in $S30_{INT}$ (13,552 cpm) than in $S30_C$ (24,976 cpm) and (ii) reo mRNA that had been incubated in $S30_{INT}$ until its methylation stopped could be reisolated and further methylated in $S30_C$. Actually such reo mRNA reisolated from reaction mixtures containing $S30_{INT}$ was a much better substrate for further methylation in $S30_C$ (18,952 cpm) than reo mRNA reisolated from reaction mixtures containing $S30_C$ (8,645 cpm). Interestingly, the total methylation of reo $mRNA_U$ first in $S30_{INT}$ and thereafter in $S30_C$ (32,504 cpm) was not very different from that of reo $mRNA_U$ methylated first in $S30_C$ and also in $S30_C$ thereafter (33,621 cpm).

These results indicate that the impairment of reo mRNA methylation in $S30_{INT}$ cannot be the consequence of the irreversible inactivation of reo $mRNA_U$ as a substrate for methylation.

Freshly added reo $mRNA_U$ can be methylated in an $S30_{INT}$-containing reaction mixture in which the methylation of previously added reo $mRNA_U$ has stopped. To eliminate the possibility that the impairment of reo $mRNA_U$ methylation in $S30_{INT}$ might be a consequence of either the depletion of SAM or the accumulation of SAH, or the irreversible inactivation of the methylating enzyme(s), the following experiment was performed (Fig. 4).

Added reo $mRNA_U$ was methylated in one set of two reaction mixtures including $S30_C$ and a second set of two reaction mixtures including $S30_{INT}$. As expected, there was less methylation of reo $mRNA_U$ in the reaction mixtures with $S30_{INT}$ than in those with $S30_C$. After the methylation of reo $mRNA_U$ had stopped in all reaction mixtures, further reo $mRNA_U$ was added to one reaction mixture from each set. This resulted in a second burst of methylation, which was again less extensive in the reaction mixture with $S30_{INT}$ than in the one with $S30_C$. The finding of the second burst of methylation in $S30_{INT}$ supplemented with further reo $mRNA_U$ seems to indicate that the impairment of methylation is not due to the lack of the methylating capacity of $S30_{INT}$ but might rather be a consequence of the unavailability of reo $mRNA_U$ for methylation.

Impaired methylation of reo $mRNA_U$ in $S30_{INT}$ is probably not a consequence of impaired capping. Only about 25 to 30% of the molecules in the reo $mRNA_U$ preparation have GpppG . . . . as their 5′ termini and are direct substrates for CAP methylation. Most of the rest have ppG at the 5′ ends and have to be capped before being methylated (4). If both types of molecules had become methylated in our experiments, then the observed impairment of methylation in $S30_{INT}$ could be: (i) due to an inhibition of the process of methylation itself, (ii) an indirect consequence of impaired capping, or (iii) a consequence of a combination of both effects.

To distinguish between these possibilities, we treated the reo $mRNA_U$ preparation with alkaline phosphatase to convert the ppG termini to G termini without affecting the GpppG termini. The molecules having G at the 5′ termini

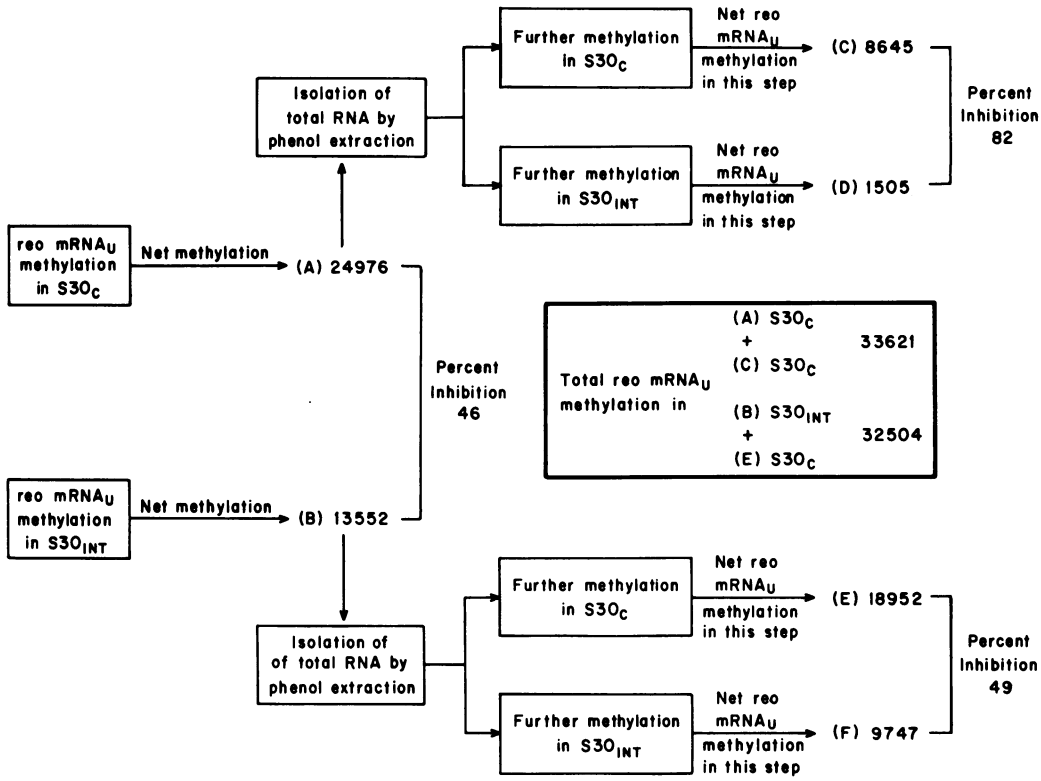

FIG. 3. *Schematic presentation of an experiment showing that reo mRNA$_U$, which had been incubated in S30$_{INT}$ until its methylation stopped, can be reisolated and further methylated by incubation in S30$_C$. Step 1: S30$_C$ or S30$_{INT}$ was incubated with [^{3}H]SAM and without or with reo mRNA$_U$ (100 μg/ml) under methylation conditions for 10 min. (By this time the methylation of reo mRNA$_U$ has stopped.) Step 2: the total RNA was isolated from each of the four reaction mixtures (from step 1) by phenol extraction, dialysis against distilled water, and lyophilization. The recovery of isolated labeled RNA was over 90%. Step 3: S30$_C$ or S30$_{INT}$ was incubated with or without [^{3}H]SAM and with or without total RNA (isolated in step 2 from step 1 reaction mixtures of the same volume as those used in step 3) under methylation conditions for 10 min. The total amount of [^{3}H]methyl residues in RNA after the specified steps of the experiment were as listed below. From step 1 incubations: (1) S30$_C$ + [^{3}H]SAM, 6,944; (2) S30$_C$ + reo mRNA$_U$ + [^{3}H]SAM, 31,920; (3) S30$_{INT}$ + [^{3}H]SAM, 5,488; (4) S30$_{INT}$ + reo mRNA$_U$ + [^{3}H]SAM, 19,040; from step 3 incubations: (5) S30$_C$ + total RNA from (1), 6,685; (6) S30$_C$ + total RNA from (2), 27,895; (7) S30$_C$ + total RNA from (3), 4,095; (8) S30$_C$ + total RNA from (4), 17,167; (9) S30$_C$ + total RNA from (1) + [^{3}H]SAM, 40,565; (10) S30$_C$ + total RNA from (2) + [^{3}H]SAM, 70,420; (11) S30$_C$ + total RNA from (3) + [^{3}H]SAM, 33,635; (12) S30$_C$ + total RNA from (4) + [^{3}H]SAM, 65,660; (13) S30$_{INT}$ + total RNA from (1) + [^{3}H]SAM, 32,935; (14) S30$_{INT}$ + total RNA from (2) + [^{3}H]SAM, 55,650; (15) S30$_{INT}$ + total RNA from (3) + [^{3}H]SAM, 27,877; and (16) S30$_{INT}$ + total RNA from (4) + [^{3}H]SAM, 50,697. The incubations without [^{3}H]SAM, i.e., (5) to (8), served to determine the amount of label from step 1 still present after steps 2 and 3. It was established that total labeled RNA was as stable during step 3 incubations in S30$_{INT}$ as in S30$_C$. The values ascribed to (A) to (F) in the figure were computed in the following way: (A) = (2) − (1); (B) = (4) − (3); (C) = (10) − (9) − (6) + (5); (D) = (14) − (13) − (6) + (5); (E) = (12) − (11) − (8) + (7); (F) = (16) − (15) − (8) + (7).*

are unlikely to be capped, since ppG (or at least pG) termini are thought to be needed for capping (22). In the experiment described in Table 1 we compared the extents of methylation of alkaline phosphatase-treated and untreated reo mRNA$_U$ in S30$_C$ and S30$_{INT}$. The data revealed that the methylation of treated reo mRNA$_U$ (which is presumably no substrate for capping) in S30$_{INT}$ is inhibited at least to the same degree as that of untreated mRNA$_U$. This seems to indicate that methylation proper is impaired in S30$_{INT}$. The finding that treated and untreated reo mRNA$_U$ are methylated to a similar extent in S30$_C$ indicates that little, if any, capping occurs under the conditions of the experiment.

Impairment of reo mRNA$_U$ methylation in S30$_{INT}$ is not affected by the addition of reovi-

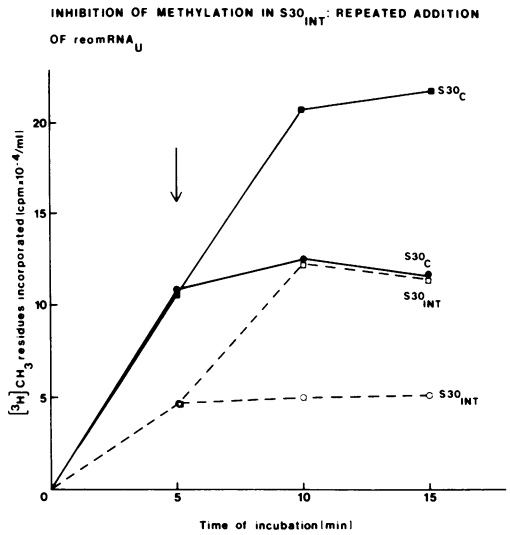

FIG. 4. *Freshly added reo $mRNA_U$ can be methylated in an $S30_{INT}$-containing reaction mixture in which the methylation of previously added reo $mRNA_U$ has stopped. Two sets of 30 μl of reaction mixtures were incubated: the first set of two tubes with $S30_C$ (● and ■); the second set of two tubes with $S30_{INT}$ (○ and □). Each tube was supplemented with 3 μg of reo $mRNA_U$ at time zero. Portions measuring 7 μl were taken from each at 5 min of incubation. At the same time (as indicated by the arrow) 3 μg of reo $mRNA_U$ in 2 μl of H_2O was added to one tube from each set (■ and □) and 2 μl of H_2O to the other tubes from each set (● and ○). The incubation of the four tubes was continued, and portions were taken from each at 10 and 15 min. The data in the figure are net methylations of reo $mRNA_U$. These were calculated by subtracting from the total methylation in the four tubes the methylation of endogenous RNA. The latter was determined in tubes incubated without added reo $mRNA_U$. The amounts subtracted (in cpm, × 10^{-4}/ml) are as follows. For $S30_C$: 2 (5 min), 4 (10 min), and 6 (15 min); for $S30_{INT}$: 2.2 (5 min), 4.4 (10 min), and 6.6 (15 min).*

rus double-stranded RNA. We have noted earlier that various exogenous RNAs, including reo $mRNA_U$, are degraded faster in $S30_{INT}$ than in $S30_C$ but only if the extracts are supplemented with double-stranded RNA (at a concentration between 0.6 and 15 μg/ml) and ATP (6; G. E. Brown, B. Lebleu, G. C. Sen, M. Kawakita, S. Shaila, and P. Lengyel, Fed. Proc. 35:1414, 1976; G. C. Sen, B. Lebleu, G. E. Brown, M. Kawakita, E. Slattery, and P. Lengyel, Nature [London], in press).

These observations prompted us to test the effect of double-stranded RNA on the impairment of methylation in $S30_{INT}$. In the experiment described in Table 2 a reo $mRNA_U$ preparation that had been freed, as far as possible, from contaminating double-stranded RNA by repeated precipitation with 2 M LiCl was used. We tested the effect of the addition of 5 μg of double-stranded reovirus RNA per ml on the methylation of the above reo $mRNA_U$ preparation in $S30_C$ and in $S30_{INT}$. The results in Table 2 indicate that the addition of double-stranded RNA had no effect on either the extent of methylation in $S30_C$ or its impairment in $S30_{INT}$ under our experimental conditions.

Effect of the addition of various single-stranded RNAs on the impairment of reo $mRNA_U$ methylation in $S30_{INT}$. We have reported earlier that the impairment of reo $mRNA_U$ methylation in $S30_{INT}$ decreases upon increasing the concentration of reo $mRNA_U$ in the reaction mixture (25). The results in Fig. 5 indicate that the inhibition can also be overcome by the addition of increasing quantities of reo $mRNA_M$. Seventy-five percent of the molecules in the reo $mRNA_M$ preparation have $m^7GpppGmp$ . . . . at their 5′ termini, and 25% have ppGp . . . (4). Whether only one of the two types of molecules or both can overcome the impairment remains to be established.

We also tested the effect on the impairment of methylation of the addition of poly(U), ribosomal RNA, and EMC RNA. None of these RNAs seemed to overcome the impairment of reo $mRNA_U$ methylation in $S30_{INT}$ (Table 3) when tested at a concentration at which reo $mRNA_U$ or reo $mRNA_M$ does partially overcome the inhibition.

Impairment of methylation in S30s prepared from cells treated with homologous or

TABLE 1. *Effect of pretreating the reo $mRNA_U$ preparation with alkaline phosphatase on the inhibition of its methylation in $S30_{INT}$*[a]

Methylated RNA	[3H]CH_3 residues incorporated (cpm, × 10^{-2}/ml) in:		% Inhibition
	$S30_C$	$S30_{INT}$	
Endogenous	428	451	0
+ Control reo $mRNA_U$ − endogenous	1,113	500	55
+ Alkaline phosphatase-treated reo $mRNA_U$ − endogenous	1,019	305	70

[a] The reo $mRNA_U$ preparation was treated with alkaline phosphatase as described in Materials and Methods. The control reo $mRNA_U$ did undergo the same treatments but in the absence of alkaline phosphatase. The concentration of reo $mRNA_U$ during methylation was 75 μg/ml, and the reaction mixtures were incubated for 10 min.

TABLE 2. *Inhibition of the methylation in $S30_{INT}$ of reo $mRNA_U$ (presumably free of double-stranded RNA) and effect of added double-stranded reovirus RNA on the inhibition*[a]

Double-stranded reovirus RNA added (μg/ml)	Methylated RNA	[^{3}H]CH_3 residues incorporated (cpm, × 10^{-2}/ml) in:		% Inhibition
		$S30_C$	$S30_{INT}$	
–	Endogenous	570	464	19
–	+ reo $mRNA_U$ – endogenous	915	277	70
5	Endogenous	597	486	19
5	+ reo $mRNA_U$ – endogenous	874	234	73

[a] The reo $mRNA_U$ transcribed by reovirus cores (see Materials and Methods) was freed from contaminating double-stranded reovirus RNA by five repeated precipitations with 2 M LiCl (18). Double-stranded reovirus RNA was isolated from reovirions according to the procedure of Ito and Joklik (18), except that the adenylate-rich oligonucleotides were removed by fractionating the RNA preparation by gel filtration on Sephadex G-100. The reo mRNA concentration during the methylation was 50 μg/ml, and the reaction mixtures were incubated for 10 min.

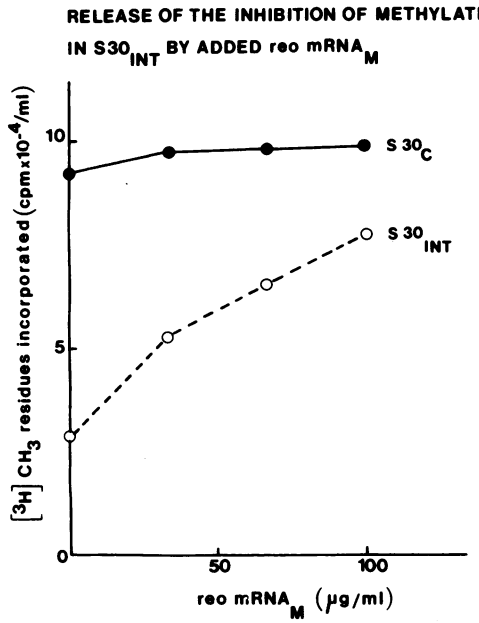

FIG. 5. *Release of the inhibition of reo $mRNA_U$ methylation in $S30_{INT}$ by added reo $mRNA_M$. The reo $mRNA_U$ concentration was 50 μg/ml, and the incubations were for 10 min. For each point shown in the figure contributions due to the methylation of the endogenous RNA and of any added reo $mRNA_M$ have been subtracted. Methylation of endogenous RNA accounted for 5 × 10^4 cpm/ml in $S30_C$ and for 4 × 10^4 cpm/ml in $S30_{INT}$. Net methylation due to the addition of 33 μg of reo $mRNA_M$ per ml was 2.6 × 10^4 cpm/ml in $S30_C$ and 2.0 × 10^4 cpm/ml in $S30_{INT}$. Symbols: ●, net methylation of reo $mRNA_U$ in $S30_C$; ○, net methylation of reo $mRNA_U$ in $S30_{INT}$.*

heterologous interferons or with the interferon inducer poly(I)·poly(C). The experiments in Table 4 were performed to test if treatments that elicit the antiviral state in cells also result in the impairment of methylation in the cell extract. The results indicate that the exposure of cells to higher interferon concentrations increases the impairment of methylation in their extracts more than exposure to lower concentrations (experiment 1). Incubating cells with interferon for a length of time (1.5 h) that is insufficient for establishing the antiviral state (20) does not impair the methylation activity in the cell extract (experiment 2). The infection of interferon-treated or untreated cells with Mengo virus has little or no effect on the impairment of methylation in the cell extract (experiment 3). Treatment of cells with a heterologous interferon does not impair the methylation in the cell extract (experiment 4). Methylation is inhibited in an extract from cells that were treated with the interferon inducer poly(I)·poly(C) (9) instead of the partially purified mouse interferon preparation (experiment 5).

Characteristics of the inhibitor. (i) Impaired methylation in Sephadex-treated $S30_{INT}$. To test whether the inhibitor is a macromolecule or not, the small molecules were removed from $S30_C$ and $S30_{INT}$ by gel filtration through Sephadex G-25. The data in Table 5 show that the impairment is manifested in Sephadex-treated $S30_{INT}$. The extent of the impairment is, however, less than in a non-Sephadex-treated extract.

(ii) Inactivation of the inhibitor during incubation. To test whether the decrease in the impairment in $S30_{INT}$ upon Sephadex treatment is due to a partial inactivation of the inhibitor during gel filtration or due to its partial removal, the following experiment was performed. We incubated portions of Sephadex-treated $S30_C$ and $S30_{INT}$ at 37°C for various lengths of time (preincubation) before testing their capacity to methylate reo $mRNA_U$. The data in Fig. 6 reveal that the extent of methyla-

TABLE 3. *Effect of the addition of poly(U), ribosomal RNA, and EMC RNA on the inhibition of reo mRNA$_U$ methylation in S30$_{INT}$*[a]

Additions	Methylated RNA	[^{3}H]CH$_3$ residues incorporated (cpm, × 10^{-2}/ml) in:		% Inhibition
		S30$_C$	S30$_{INT}$	
None	Endogenous	222	193	
	+ reo mRNA$_U$ − endogenous	293	116	60
Poly(U) (100 μg/ml)	Endogenous	231	180	
	+ reo mRNA$_U$ − endogenous	361	98	73
EAT, ribosomal RNA (100 μg/ml)	Endogenous	254	255	
	+ reo mRNA$_U$ − endogenous	355	80	77
EMC RNA (66 μg/ml)	Endogenous	218	235	
	+ reo mRNA$_U$ − endogenous	285	68	76

[a] The reo mRNA$_U$ concentration was 33 μg/ml. The reaction mixtures were incubated for 10 min.

TABLE 4. *Inhibition of reo mRNA$_U$ methylation in extracts prepared from cells that were treated with either the interferon inducer poly(I)·poly(C) or with homologous or heterologous interferon preparations*[a]

Expt no.	Cell treatment	[^{3}H]CH$_3$ residues incorporated (cpm, × 10^{-2}/ml)		% Inhibition of net reo mRNA$_U$ methylation
		Endogenous	+ reo mRNA$_U$ − endogenous	
1	None	198	811	
	Mouse interferon (20 U/ml)	105	405	50
	Mouse interferon (60 U/ml)	146	234	71
2	None	195	165	
	Mouse interferon (60 U/ml; 1.5 h)	246	221	0
	Mouse interferon (60 U/ml; 18 h)	241	82	50
3	None	534	554	
	Mouse interferon (50 U/ml)	469	338	39
	Mengo virus infection	362	734	
	Mouse interferon (50 U/ml) and Mengo virus infection	482	414	44
4	None	476	406	
	Mouse interferon (50 U/ml)	323	223	45
	Human interferon (200 U/ml)	328	394	3
5	DEAE-dextran	281	549	
	DEAE-dextran + poly(I)·poly(C)	217	281	49

[a] The reo mRNA$_U$ concentration was 100 μg/ml in all experiments, except in experiment 1 in which it was 50 μg/ml. The reaction mixtures were incubated for 10 min. In experiments 1 through 4, the concentrations of the mouse or human interferon to which the cell cultures were exposed were as indicated in parentheses in column 1. A portion of cell culture in experiment 2 was (as indicated in parentheses) exposed to interferon for 1.5 h; the interferon treatment of all other cultures lasted for the usual 18 h. In experiment 3, if so indicated, the interferon-treated or control cell cultures were infected with Mengo virus (multiplicity of infection, 10) 2 h before preparing the cell extracts. In experiment 5, for the treatment of cells with poly(I)·poly(C) and DEAE-dextran, see Materials and Methods. [DEAE-dextran enhances interferon induction by poly(I)·poly(C) but does not induce by itself.]

TABLE 5. *Effect of gel filtration through Sephadex G-25 on the inhibition of reo mRNA$_U$ methylation in S30$_{INT}$*[a]

Sephadex G-25 treatment of S30s	Methylated RNA	[^{3}H]CH$_3$ residues incorporated (cpm, × 10^{-2}/ml) in:		% Inhibition
		S30$_C$	S30$_{INT}$	
No	Endogenous	329	293	11
No	+ reo mRNA$_U$ − endogenous	299	79	74
Yes	Endogenous	136	112	18
Yes	+ reo mRNA$_U$ − endogenous	441	228	48

[a] Where indicated, S30 extracts that had been passed through a Sephadex G-25 column in TKM buffer (25 mM Tris-chloride, pH 7.5, 80 mM KCl, 4 mM magnesium acetate, and 6 mM 2-mercaptoethanol) to remove small molecules were used. The reo mRNA$_U$ concentration was 50 μg/ml, and the reaction mixtures were incubated for 10 min.

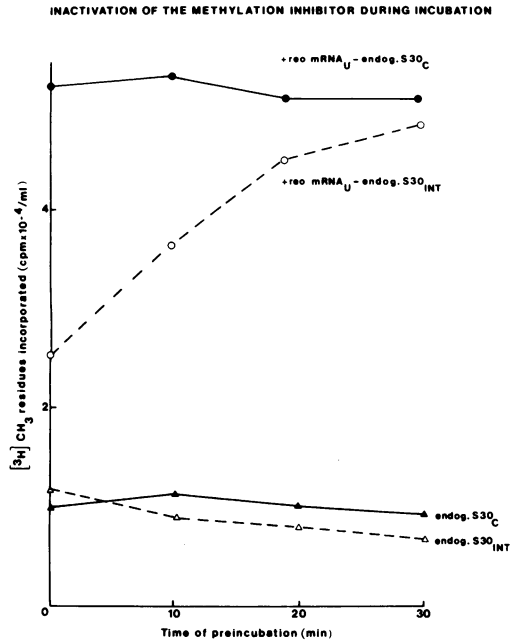

FIG. 6. *Inactivation of the methylation inhibitor in S30$_{INT}$ during incubation. S30$_C$ and S30$_{INT}$, which had been passed through a Sephadex G25 column in TKM buffer (see Table 5) to remove small molecules, were incubated at 37°C for the indicated lengths of time (time of preincubation) and then used in regular methylation assays. The reo mRNA$_U$ concentration was 50 μg/ml, and the reaction mixtures were incubated for 10 min. Symbols: ▲, methylation of endogenous RNA in S30$_C$; △, methylation of endogenous RNA in S30$_{INT}$; ●, net methylation of reo mRNA$_U$ in S30$_C$; ○, net methylation of reo mRNA$_U$ in S30$_{INT}$.*

tion in S30$_C$ was unaffected by the preincubation, whereas the extent of methylation in S30$_{INT}$ increased with an increasing length of preincubation. Thus there was no significant impairment of methylation in S30$_{INT}$ that had been preincubated for 30 min. Similar results were obtained by preincubating S30 extracts that had not been treated with Sephadex. These findings indicate that the inhibitor is labile. Thus it is conceivable that part or all of the decrease in the impairment of methylation in Sephadex-treated S30$_{INT}$ may be a consequence of the partial inactivation of the inhibitor during gel filtration.

Characterization of the products of methylation. The reo mRNA$_U$ preparation contains three size classes of reo mRNA's: small (*s*), medium (*m*), and large (*l*). We analyzed the products of reo mRNA$_U$ methylation in S30$_C$ and S30$_{INT}$ by centrifugation through sucrose gradients (Fig. 7). Reo mRNA's from all the three size classes were methylated in S30$_C$. The amount of methyl residues incorporated was the highest in the *s* size class, lower in *m*, and lowest in *l*. The distribution of methyl residues in the three size classes might be accounted for by the fact that the reo mRNA$_U$ preparation used was the richest in molecules of the *s* size class and poorest in molecules of the *l* size class. Moreover, each reo mRNA molecule (irrespective of its size) is methylated only in its 5′ terminal region (26). The curves in Fig. 7 reveal that in S30$_{INT}$ the methylation of reo mRNA$_U$'s from all three size classes was impaired. Further studies will be needed to establish if the methylation of the 10 reo mRNA's is impaired equally or not.

The products were also analyzed for determining the sites of methylation. For this purpose portions of reo mRNA$_U$ were methylated in S30$_C$ and S30$_{INT}$. Then total RNA was isolated from the reaction mixtures and digested with T2 RNase. This enzyme hydrolyzes phosphodiester bonds between unmethylated nucleosides and produces molecules with 3′ phosphate termini (10). The digestion products were analyzed on a DEAE-cellulose column in the presence of urea (Fig. 8). This column fractionates molecules according to their net charge (10).

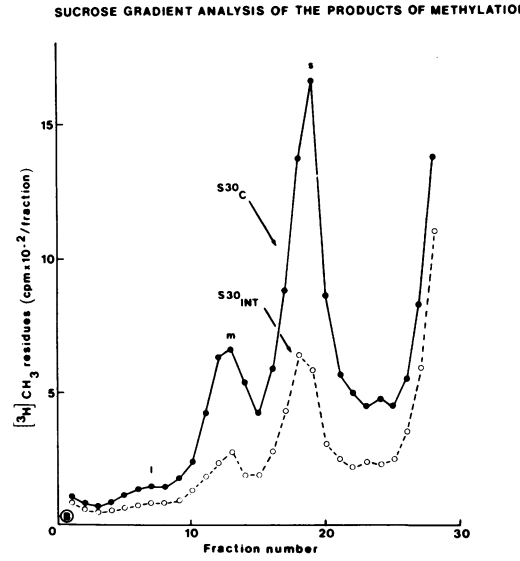

FIG. 7. *Sucrose gradient analysis of the products of methylation of reo mRNA$_U$ in S30$_C$ and S30$_{INT}$. Reo mRNA$_U$ (50 μg/ml) was incubated with S30$_C$ or S30$_{INT}$ and [^{3}H]SAM under methylation conditions. The methylated reo mRNA$_U$ was analyzed on sucrose gradients as described in Materials and Methods. Symbols: ●, reo mRNA$_U$ methylated in S30$_C$; ○, reo mRNA$_U$ methylated in S30$_{INT}$. The peaks of large, medium, and small size classes of reo mRNA's in the gradient are designated as l, m, and s, respectively.*

There is no clear-cut difference between $S30_C$ and $S30_{INT}$ in the amounts of methylated products carrying between 0 and −3 net charge. These products probably originate from the methylation of endogenous RNA. The amount of methylated products carrying between −4 and −6 net charge is much less in $S30_{INT}$ than in $S30_C$. The majority of these products carried −4 net charge and probably consists of the partially methylated cap structure: $m^7GpppGp$. The product with about −4.5 charge is probably $m^7GpppGmpCp$ (7a, 10, 26). This type of cap structure is designated as cap 1. It is present in lesser amounts than the partially methylated cap structure. The small amount of products with about −5 net charge might be either $m^7GpppGmCmpXp$ (a product designated as cap 2 which might be formed by further methylation of reo $mRNA_U$ by cellular enzymes) or might be a partially methylated cap 1 structure with a ring opened m^7G moiety. These results indicate that there is a general impairment in cap structure methylation in $S30_{INT}$.

In a further experiment RNA samples isolated from incubated reaction mixtures containing [^{3}H]SAM, reo $mRNA_U$, and either $S30_C$ or $S30_{INT}$ were digested to nucleosides by treatment with P_1 RNase, nucleotide pyrophosphatase, and bacterial alkaline phosphatase. The resulting digests were supplemented with unlabeled nucleoside markers and analyzed by liquid chromatography on an Aminex-A-5 column (not shown) according to published procedures (7, 7a). The total nucleoside methylation in $S30_{INT}$ was 74%. (The methylation of endogenous RNA, i.e., not reo $mRNA_U$, was estab- nucleosides in $S30_C$ was incorporated into 7 methyl-guanosine. The corresponding value in $S30_{INT}$ was 74%. (The methylation of endogenous RNA, i.e., not reo $mRNA_U$, was established in separate control experiments and has been subtracted from the above values.) Over 90% of the decrease in total nucleoside methylation in $S30_{INT}$ could be accounted for by a decrease in 7 methyl-guanosine formation.

Methylation of de novo synthesized reo mRNA by the virion-associated enzymes is inhibited by $S30_{INT}$. The treatment of reo virions with chymotrypsin results in the cleavage and removal of some of the outer coat proteins (19). The treatment converts the virions into reovirus cores and results in the activation of the virion-associated RNA transcriptase, the capping enzyme, and the RNA methylase(s) (26). We compared the effect of the addition of $S30_C$ with that of the addition of $S30_{INT}$ on the core transciption and core methylation proc-

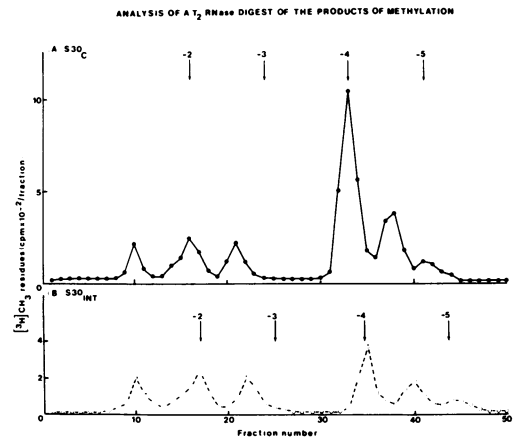

FIG. 8. *Analysis of T2 RNase digests of the products of reo $mRNA_U$ methylation in $S30_C$ and $S30_{INT}$. Reo $mRNA_U$ (50 μg/ml) was methylated in either $S30_C$ (in A) or $S30_{INT}$ (in B) in the presence of [^{3}H]SAM. Total RNA was isolated from the reaction mixtures by phenol extraction. The RNA in the resulting aqueous phase was precipitated with ethanol in the presence of added carrier RNA. The precipitation with ethanol was repeated twice to ensure complete removal of residual [^{3}H]SAM. The isolated RNA was digested with T2 RNase and analyzed on a urea-DEAE-cellulose column as described in Materials and Methods. (A) Analysis of the products from reo $mRNA_U$ methylation in $S30_C$. (B) Analysis of the products from reo $mRNA_U$ methylation in $S30_{INT}$. The numbers on top of the arrows indicate the net charge of nucleotides eluting at those positions.*

esses (Fig. 9). The rate of core transcription, as measured by [^{3}H]GMP incorporation, was similar in reaction mixtures containing $S30_C$ or $S30_{INT}$ (Fig. 9A) or $S30_{poly(I)\cdot poly(C)}$ (Fig. 9B). [The $S30_{poly(I)\cdot poly(C)}$ was prepared from EAT cells that had been treated not with the interferon preparation but with the interferon inducer poly(I)·poly(C) (6).] However, the rate of core methylation as measured by the incorporation of [^{3}H]methyl residues from [^{3}H]SAM was lower in reaction mixtures with $S30_{INT}$ (Fig. 9C) or S30 poly(I)·poly(C) (Fig. 9D) than in those with $S30_C$.

The impairment of core methylation in $S30_{INT}$ is apparently not a consequence of a faster degradation of the products in $S30_{INT}$ than in $S30_C$. This was established (Fig. 9C) by (i) allowing methylation to take place for 10 min in one reaction mixture with $S30_C$ and another one with $S30_{INT}$, (ii) stopping further methylation (by the addition of SAH), and (iii) continuing the incubations and assaying for methylated RNA. The results obtained indicate that the

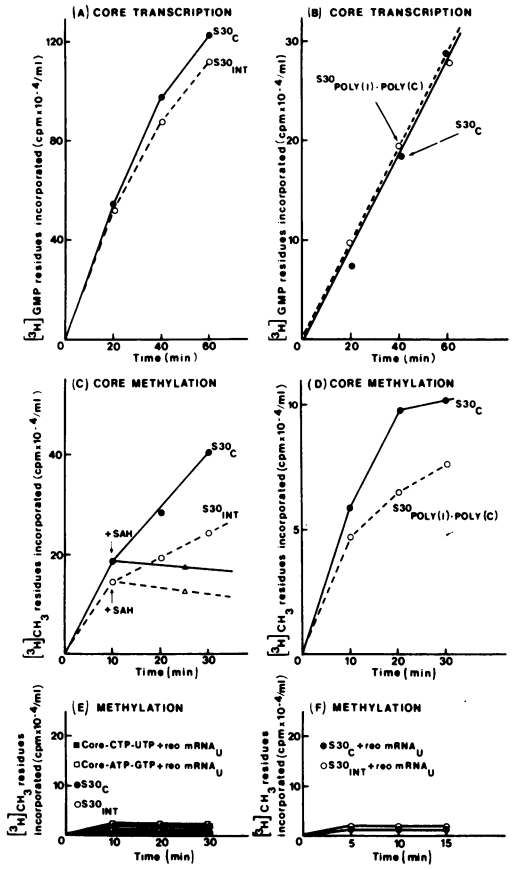

FIG. 9. *Effect of $S30_C$, $S30_{INT}$, and $S30_{poly(I)\cdot poly(C)}$ on the transcription and methylation of reo mRNA's as catalyzed by reovirus cores. Core transcription in (A) $S30_C$ and $S30_{INT}$ and in (B) $S30_C$ and $S30_{poly(I)\cdot poly(C)}$. Core methylation (i.e., methylation of de novo synthesized reo mRNA's by the cores) in (C) $S30_C$ and $S30_{INT}$ and in (D) $S30_C$ and $S30_{poly(I)\cdot poly(C)}$. Methylation of added reo $mRNA_U$ under core methylation conditions (E) by cores (F) by $S30_C$ and $S30_{INT}$. The reaction mixtures (50 μl) contained reovirus cores (prepared by treatment with chymotrypsin of 400 μg of a reovirus preparaion per ml in [A] and [C] and of 260 μg of another reovirus preparaion per ml in [B] and [D] [see Materials and Methods]) as well as one or more of the following components as indicated: 7.5 μl of $S30_C$, $S30_{INT}$, or $S30_{poly(I)\cdot poly(C)}$, 24 μg of reo $mRNA_U$ per ml, 3.2 μM [^{3}H]GTP (specific activity, 7.9 Ci/mol; for assaying core transcription), and 8 μM [^{3}H]SAM (specific activity, 11.6 Ci/mol; for assaying methylation). SAH (400 μM) was added (in C) after a 10-min incubation (as indicated by the arrows) to stop the further methylation. All reaction mixtures also contained, unless otherwise specified (i.e., in E), all four unlabeled ribonucleoside triphosphates, 2 mM each for core methylation and 2 mM ATP, CTP, and UTP but only 0.5 mM GTP for core transcription. The other components of the reaction mixtures were as specified by Both et al. (5). The reaction mixtures in (E) and (F) did not contain core unless so specified. All incubations were at 37°C for the times indicated.*

products of methylation are equally stable in the presence of $S30_C$ and $S30_{INT}$. Further studies revealed that under our conditions of core methylation, the extent of methylation of endogenous RNA is negligible in both $S30_C$ and $S30_{INT}$ (Fig. 9E) and so is the extent of methylation of added reo mRNA by both the core-associated methylase(s) (Fig. 9E) and the cellular methylase(s) (Fig. 9F).

The apparent lack of activity of the cellular methylases in these experiments might be due to the fact that the composition of the reaction mixtures (5) was different from that under which the methylation by cellular RNA methylases was usually assayed (25). The lack of reo $mRNA_U$ methylation by the core methylase may be due either to the inaccessibility of the RNA to the enzyme or the inability of the enzyme to methylate long RNA chains.

Apparent similarity between the inhibition of the core methylase(s) and the cellular methylase in $S30_{INT}$. The impairment of core methylation in $S30_{INT}$ decreases upon the addition to the reaction mixture of reo $mRNA_M$ in increasing concentrations (data not shown). Furthermore, the preincubation of $S30_{INT}$ results in the inactivation of the inhibitor of core methylation (Fig. 10). As indicated earlier, the inhibition of reo $mRNA_U$ methylation by cellular enzymes is similarly overcome by either the addition of reo $mRNA_M$ or the preincubation of the $S30_{INT}$.

DISCUSSION

The data presented in this and previous communications (21, 25; S. Shaila, B. Lebleu, G. C. Sen, G. E. Brown, and P. Lengyel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S209, p. 239; B. Lebleu, G. C. Sen, S. Shaila, G. E. Brown, and P. Lengyel, Abstr. Annu. Meet. Am. Soc. Microbiol. 1976, S210, p. 239) reveal that the extent of reo $mRNA_U$ methylation is lower in $S30_{INT}$ than in $S30_C$. The inhibitor (or inhibitors) causing the impairment of methylation is a macromolecule. It is apparently unstable, since its effect is not manifested in $S30_{INT}$ that has been incubated for 30 min before the addition of reo $mRNA_U$. It impairs the methylation of the cap structures of reo $mRNA_U$ from all three size classes.

The impairment of reo $mRNA_U$ methylation in $S30_{INT}$ is not a consequence of: (i) the degradation of reo $mRNA_U$ prior to methylation, (ii) the cleavage of reo mRNA after it has been methylated, (iii) the irreversible inactivation of

reo $mRNA_U$ as a substrate for methylation, or (iv) the impairment of the activity of the capping enzymes.

Most of the impairment persists in $S30_{INT}$ from which the small molecules have been removed by gel filtration through Sephadex G-25. (The small decrease in the impairment upon this treatment is most probably due to the lability of the macromolecular inhibitor.) This persistence indicates that the impairment cannot be a consequence of a higher concentration in $S30_{INT}$ of either unlabeled SAM (which would lower the specific activity of the [^{3}H]SAM used in the methylation assays) or of SAH (an inhibitor of methylation).

A faster production of unlabeled SAM in $S30_{INT}$ during incubation, which would again lower the specific activity of [^{3}H]SAM, is also unlikely. At least, (i) the methylation of endogenous RNA in $S30_{INT}$ (Sephadex treated or not) is impaired, if at all, only to a much lesser extent than that of reo $mRNA_U$, and (ii) partially methylated reo $mRNA_U$ can be reisolated from $S30_{INT}$ and further methylated in $S30_C$ to a similar extent as reo $mRNA_U$, which was methylated twice in $S30_C$.

An increased rate of production of SAH in $S30_{INT}$ probably does not contribute to the impairment. The rate of conversion of SAM to SAH is the same in $S30_C$ and $S30_{INT}$ (data not shown). Moreover, added SAH (0.8 or 80 μM) diminishes the extent of methylation in $S30_C$ and $S30_{INT}$ equally and, thus, the percent inhibition of impairment in $S30_{INT}$ compared to $S30_C$ is unaffected.

Recently, we reported that double-stranded RNA and ATP enhance the endonuclease activity in $S30_{INT}$ but not, or only to a lesser extent, in $S30_C$ (6; G. E. Brown, B. Lebleu, G. C. Sen, M. Kawakita, S. Shaila, and P. Lengyel, Fed. Proc. 35:1414, 1976). The impairment of methylation in $S30_{INT}$ is not affected by the addition of double-stranded RNA at a concentration at which it boosts endonuclease activity. However, this negative result does not rule out the possibility of an involvement of double-stranded RNA in the impairment: the reo $mRNA_U$ used as a substrate for methylation, though purified, may still be contaminated with small amounts of double-stranded RNA, and this in turn may obscure the effect of the addition of further double-stranded RNA.

Mechanism and significance of the impairment of methylation by the virion-associated enzymes. The methylation of the 5′ terminal and the second nucleotide of reo mRNA are catalyzed in vivo presumably by the virion-associated enzymes (26). Thus the likelihood of the physiological relevance of the observed impairment of reo mRNA methylation in $S30_{INT}$ is increased by the finding that the methylation of do novo synthesized reo mRNA by the virion-associated methylases (core methylases) is also impaired by $S30_{INT}$.

The inhibition of the reo mRNA methylating activity of both the cellular and the core methylases can be overcome in the same way: by increasing the concentration of reo $mRNA_M$ in

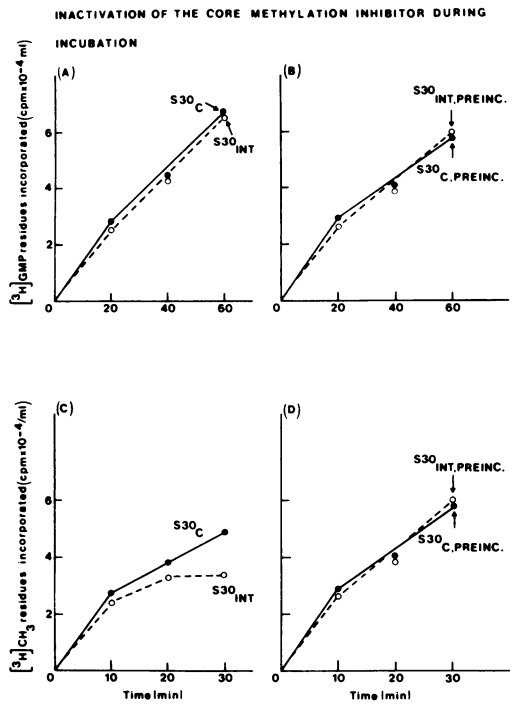

FIG. 10. *Inactivation of the core methylation inhibitor during incubation. Effect on the transcription by reovirus cores of (A) $S30_C$ and $S30_{INT}$ and (B) preincubated $S30_C$ ($S30_{C\ preincubated}$) and preincubated $S30_{INT}$ ($S30_{INT\ preincubated}$). Effect on the methylation of de novo synthesized reo mRNA by reovirus cores of (C) $S30_C$ and $S30_{INT}$ and (D) $S30_{C\ preincubated}$ and $S30_{INT\ preincubated}$. $S30_{C\ preincubated}$ and $S30_{INT\ preincubated}$ are $S30_C$ and $S30_{INT}$ which had been incubated at 37°C for 30 min before addition to the reaction mixtures for assaying core transcription or core methylation. Each reaction mixture contained reovirus cores (prepared by treatment with chymotrypsin of 260 μg of a reovirus preparation per ml [see Materials and Methods]) and 7.5 μl of $S30_C$ or $S30_{INT}$ or $S30_{C\ preincubated}$ or $S30_{INT\ preincubated}$, as well as 3.2 μM [^{3}H]GTP (specific activity, 7.9 Ci/mol) in (A) and (B) or 8 μM [^{3}H]SAM (specific activity, 11.6 Ci/mol) in (C) and (D). All reaction mixtures also contained all four unlabeled ribonucleoside triphosphates, 2 mM each for core methylation, and 2 mM ATP, CTP, UTP but only 0.5 mM GTP for core transcription. The other components of the reaction mixtures were as specified by Both et al. (5). The reaction mixtures were incubated at 37°C for the times indicated.*

the reaction mixture or by preincubating the $S30_{INT}$ prior to methylation. Thus it is conceivable that the action of both types of enzymes is impaired by the same inhibitor.

In $S30_{INT}$ the extent of the impairment of core methylases is consistently less than that of the cellular methylases. This might be due to one or more of the following reasons. (i) The access of the inhibitor to the site of RNA synthesis within the cores may be limited. (ii) The ionic conditions used for core methylation might be unfavorable for the action of the inhibitor. (iii) Since the inhibitor of methylation is unstable, it might be partially inactive during the longer incubation periods of the core methylation experiments.

The impairment of core methylation in $S30_{INT}$ might, in principle, be a consequence of the degradation of reo $mRNA_U$. This is unlikely, however, since the impairment is well manifested after a 15-min incubation, whereas the size distribution of reo mRNA synthesized by reovirus cores in a 15-min incubation in $S30_{INT}$ with no SAM added is indistinguishable from that synthesized in $S30_C$ (data not shown). The possibility that the impairment of core methylation is a consequence of an impairment of capping has not been ruled out.

Inhibitor of methylation in $S30_{INT}$. Reo mRNA's that have been incubated in $S30_{INT}$ until their methylation ceased can be reisolated by phenol extraction and further methylated in $S30_C$. This observation, together with some of the other data presented, indicates that phenol extraction either inactivates the inhibitor or removes it from the mRNA. An increase in the concentration of reo $mRNA_U$ in $S30_{INT}$ partially overcomes the impairment. The simplest hypothesis accounting for these features is that the inhibitor binds to the reo $mRNA_U$, making its 5′ terminal cap region inaccessible for methylation. The addition of the reo $mRNA_M$ preparation seems to overcome the impairment too. This might seem to indicate that the inhibitor may also bind to methylated, capped reo mRNA. This is, however, uncertain, since the reo $mRNA_M$ preparation contains unmethylated molecules, and it is possible that only these are responsible for overcoming the inhibition. However, if the inhibitor would bind to methylated reo mRNA, then it might also block the binding of ribosomes to mRNA, i.e., initiation complex formation. Indeed, it is conceivable that the impairment of methylation is only one of the manifestations of the inhibitor and may not even be the most important one.

Poly(U), ribosomal RNA, and EMC RNA do not affect the impairment of reo $mRNA_U$ methylation in $S30_{INT}$. These RNAs are neither capped nor are they thought to be substrates for the capping enzymes. Thus, the inhibitor does not recognize at least some RNAs with such characteristics.

It remains to be established if the inhibitor: (i) recognizes uncapped RNAs that are substrates for the capping enzymes; (ii) acts equally on all capped viral mRNA's; or (iii) acts on cellular mRNA's.

Reo $mRNA_U$, which had been incubated in $S30_C$ until its methylation ceased, can be reisolated and further methylated in $S30_C$. This indicates that an inhibitor of methylation might also be present in $S30_C$. It is obvious, however, that methylation is less impaired in $S30_C$ than in $S30_{INT}$.

Since our interferon preparation is not pure, we cannot prove that the agent increasing the activity of the methylation inhibitor in $S30_{INT}$ is interferon and not another component of the preparation. It is in accord with this possibility, however, that the requirements for obtaining the impairment of methylation in the cell extract seem to be identical in our tests to those needed for establishing the antiviral state.

The impairment of methylation in extracts of interferon-treated cells is apparently not restricted to the Ehrlich ascites cell-mouse interferon system. We observed a similar impairment in an extract from HeLa cells which had been treated with a human interferon preparation (S. Shaila and B. Lebleu, manuscript in preparation).

The replication of picornaviruses is also inhibited in interferon-treated cells (9), though the mRNA's of these viruses were reported to be neither capped nor methylated (8, 17, 23; P. Fellner, D. Frisby, J. Goodchild, A. Porter, and N. H. Carey, Abstr. Int. Congr. Virol. Madrid, 3:161). Thus, it is unlikely that an impairment of methylation should be the only mechanism by which virus replication is blocked in interferon-treated cells.

The obvious should be noted: studies with intact, interferon-treated, virus-infected cells will be needed to establish if the agent(s) which impairs reo mRNA methylation in vitro is involved in mediating the antiviral action of interferons.

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