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 (l1, l2, l3), medium (m1, m2, m3), and small (s1, s2, s3, s4) 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\(^7\)G(5'ppp(5')Gmcp \ldots \) is the 5' nucleotide sequence of the methylated capped reovirus 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 (reovirus mRNA\(_{\text{U}}\)) by the core-associated reovirion transcriptase and capping enzymes in vitro (26). Experiments involving the use of reovirus mRNA\(_U\) and of methylated and capped reovirus mRNA's (reovirus mRNA\(_M\)) revealed that the presence of the methyl group at the 7 position of the 5' terminal guanylate residue of reovirus 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 reovirus mRNA\(_U\) in extracts of uninfected control EAT cells (S30\(_0\)) with that in extracts of infected, interferon-treated EAT cells (S30\(_{\text{INT}}\)) (25). We found that the methylation of added reovirus mRNA\(_U\) by the host enzymes in S30\(_{\text{INT}}\) 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 reovirus mRNA\(_U\) in the reaction mixture (21, 25).


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.; [\(\text{\textsuperscript{3}H}\)]SAM (methyl labeled; specific activity, 12.6 Ci/mmol) and [8-\(\text{\textsuperscript{32}P}\)]GTP (specific activity, 9.35 Ci/mmol) from New England Nuclear; \(\beta,\gamma\)-[G\(\text{\textsuperscript{32}P}\)]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 Amer sham/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\(^8\) 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 (\({\textsuperscript{\ast}}\text{G-0230901-52}\)).

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, poly(U)-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 28S 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\(_{\text{INT}}\)) and from untreated cells (S30\(_0\)) 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 [S30poly(I)-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 mRNAα) included 100 μM SAM, and those mixtures (for preparing unmethylated reovirus mRNA (reo mRNAγ)) included 100 μM SAH. Both preparations consisted of species with heterogeneous 5' terminal structures occurring in approximately the following proportions: reo mRNAα (m7GpppGm7 .. . 75%, ppG .... 25%; GpppG ...., 1 to 2%) and reo mRNAγ (ppG ...., 70 to 75%; GpppG ...., 25 to 30%) (4). For preparing "CAP" ([32P]-labeled reo mRNA, β,γ-[32P]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 mRNAα methylation. This was based on determining the amount of [3H]CH₃ residues transferred from [3H]CH₃-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 mRNAα 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 SW28 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 mRNAα prepared with β,γ-[32P]GTP and in reo mRNAγ methylated in S30 with [3H]SAM. This was done according to the method of Furuchi et al. (10). Reo mRNAα 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 absorbance 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 absorbance 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 mRNAγ (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 mRNAα methylation is not due to faster cleavage of reo mRNA in S30INT than in S30C. The 5' terminal sequence of the molecules serving as substrates for methylation in our reo mRNAα preparation (cap-labeled, alkaline phosphatase-treated reo mRNAα) 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 mRNAα reached 50 to 60% of its final level after 1 min and stopped after 5 min in both S30c and S30INT. The final level of methylation was about

![Graph](https://via.placeholder.com/150)

FIG. 1. Characterization of cap-labeled reo mRNAα. Reo mRNAα was synthesized by viral cores in the presence of β,γ-[32P]GTP and SAM. Treatment with alkaline phosphatase was used to remove the two phosphate residues from RNA molecules with 5' terminal p*pG ... moieties. (p* indicates a [32P]-labeled phosphate moiety). As expected, approximately 70% of the 32P 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 mRNAα preparation.
Fig. 2. Comparison of the kinetics of methylation and degradation of CAP-labeled roc mRNA\textsubscript{v} in S30\textsubscript{c} and S30\textsubscript{INT}. In all experiments CAP \textsuperscript{32}P-labeled alkaline phosphatase-treated roc mRNA\textsubscript{s} (designated as CAP-labeled roc mRNA) were used. (For the characterization of CAP-labeled roc mRNA\textsubscript{s}, see the legend to Fig. 1.) (A) Kinetics of methylation of CAP-labeled roc mRNA\textsubscript{u} in S30\textsubscript{c} and S30\textsubscript{INT}. CAP-labeled roc mRNA\textsubscript{u} (100 \textmu g/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 roc mRNA\textsubscript{u}. (B) Kinetics of degradation of CAP-labeled roc mRNA\textsubscript{u} and roc mRNA\textsubscript{v} to acid-soluble material in S30\textsubscript{c} and S30\textsubscript{INT}. A 100- \textmu g portion of CAP-labeled roc mRNA\textsubscript{u} or roc mRNA\textsubscript{v} per ml was incubated under methylation conditions, except that \[^{3}H\text{SAM}\] was absent and 800 \mu M 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\textsubscript{c}; the discontinuous lines indicate incubation in S30\textsubscript{INT}. (C) Kinetics of degradation of CAP-labeled roc mRNA\textsubscript{u} in S30\textsubscript{c} and S30\textsubscript{INT} analysis on sucrose gradients. A 100- \textmu g portion of CAP-labeled roc mRNA\textsubscript{u} per ml was incubated under methylation conditions for 1 min, except that unlabeled SAM was substituted for \[^{3}H\text{SAM}\]. 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\textsubscript{c}; discontinuous lines indicate incubation in S30\textsubscript{INT}. The peaks of sedimentation of large, medium, and small size classes of roc mRNA\textsubscript{s} are represented as 1, 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\textsubscript{INT} than in S30\textsubscript{c}. Under the same conditions the rates of hydrolysis of cap-labeled, alkaline phosphatase-treated roc mRNA\textsubscript{u} and roc mRNA\textsubscript{u} into acid-soluble products in S30\textsubscript{INT} were indistinguishable from those in S30\textsubscript{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\_\text{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\_\text{INT} of reo mRNA\_C 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\_C incubated under methylation conditions in S30\_\text{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 \(^{32}\text{P}\)-labeled reo mRNA\_C and reo mRNA\_A in S30\_\text{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\_C methylation in S30\_\text{INT} is not due to an irreversible inactivation of reo mRNA\_C.** The experiments outlined in Fig. 3 were performed to test if the impairment of reo mRNA\_C methylation in S30\_\text{INT} could be due to an irreversible inactivation of reo mRNA\_C as a substrate for methylation. For this purpose portions of reo mRNA\_C were methylated in S30\_C and S30\_\text{INT} until methylation stopped, and the extent of methylation in each extract was determined. Thereafter total RNA (including reo mRNA\_A) was isolated from the reaction mixtures and portions of it were added to S30\_C and S30\_\text{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\_C methylation, total RNA was also isolated from S30\_C and S30\_\text{INT} which had been incubated without added reo mRNA\_C and further methylated in S30\_C and S30\_\text{INT}.) The data in Fig. 3 indicate that: (i) as expected, the methylation of reo mRNA\_C was much less in S30\_\text{INT} (13,552 cpm) than in S30\_C (24,976 cpm) and (ii) reo mRNA that had been incubated in S30\_\text{INT} until its methylation stopped could be reisolated and further methylated in S30\_C. Actually such reo mRNA reisolated from reaction mixtures containing S30\_\text{INT} was a much better substrate for further methylation in S30\_C (18,952 cpm) than reo mRNA reisolated from reaction mixtures containing S30\_\text{INT} (8,645 cpm). Interestingly, the total methylation of reo mRNA\_A first in S30\_\text{INT} and thereafter in S30\_C (32,504 cpm) was not very different from that of reo mRNA\_C 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\_\text{INT} cannot be the consequence of the irreversible inactivation of reo mRNA\_C as a substrate for methylation.

**Freshly added reo mRNA\_C can be methylated in an S30\_\text{INT}-containing reaction mixture in which the methylation of previously added reo mRNA\_C has stopped.** To eliminate the possibility that the impairment of reo mRNA methylation in S30\_\text{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\_C was methylated in one set of two reaction mixtures including S30\_C and a second set of two reaction mixtures including S30\_\text{INT}. As expected, there was less methylation of reo mRNA\_C in the reaction mixtures with S30\_\text{INT} than in those with S30\_C. After the methylation of reo mRNA\_C had stopped in all reaction mixtures, further reo mRNA\_C 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\_\text{INT} than in the one with S30\_C. The finding of the second burst of methylation in S30\_\text{INT} supplemented with further reo mRNA\_C seems to indicate that the impairment of methylation is not due to the lack of the methylating capacity of S30\_\text{INT} but might rather be a consequence of the unavailability of reo mRNA\_C for methylation.

**Impaired methylation of reo mRNA\_A in S30\_\text{INT} is probably not a consequence of impaired capping.** Only about 25 to 30% of the molecules in the reo mRNA\_A preparation have GppG . . . 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\_\text{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\_A preparation with alkaline phosphatase to convert the ppG termini to G termini without affecting the GppG termini. The molecules having G at the 5' termini
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<sub><i>U</i></sub> in S30<sub><i>C</i></sub> and S30<sub><i>INT</i></sub>. The data revealed that the methylation of treated reo mRNA<sub><i>U</i></sub> (which is presumably no substrate for capping) in S30<sub><i>INT</i></sub> is inhibited at least to the same degree as that of untreated mRNA<sub><i>U</i></sub>. This seems to indicate that methylation proper is impaired in S30<sub><i>INT</i></sub>. The finding that treated and untreated reo mRNA<sub><i>U</i></sub> are methylated to a similar extent in S30<sub><i>C</i></sub> indicates that little, if any, capping occurs under the conditions of the experiment.

**Impairment of reo mRNA<sub><i>U</i></sub> methylation in S30<sub><i>INT</i></sub> is not affected by the addition of reovi...**
FIG. 4. freshly added reo mRNAU can be methylated in an S30NT-containing reaction mixture in which the methylation of previously added reo mRNAU has stopped. two sets of 20 μl of reaction mixtures were incubated: the first set of two tubes with S30c (∧ and ■); the second set of two tubes with S30NT (○ and □). Each tube was supplemented with 3 μg of reo mRNAU at zero time. 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 mRNAU in 2 μl of H2O was added to one tube from each set (■ and □) and 2 μl of H2O 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 methylation values of reo mRNAU. 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 mRNAU. the amounts subtracted (in cpm, × 10^4/ml) are as follows. for S30c: S30c: (5 min), 4.10 (10 min), and 6.6 (15 min); for S30NT: 2.2 (5 min), 4.4 (10 min), and 6.6 (15 min).

We have noted earlier that various exogenous RNAs, including reo mRNAU, are degraded faster in S30NT than in S30, 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 S30NT. In the experiment described in Table 2 a reo mRNAU 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 mRNAU preparation in S30c and in S30NT. the results in Table 2 indicate that the addition of double-stranded RNA had no effect on either the extent of methylation in S30c or its impairment in S30NT under our experimental conditions.

Effect of the addition of various single-stranded RNAs on the impairment of reo mRNAU methylation in S30NT. we have reported earlier that the impairment of reo mRNAU methylation in S30NT decreases upon increasing the concentration of reo mRNAU 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 mRNAU. seventy-five percent of the molecules in the reo mRNAU preparation have m7GpppGmp . . . 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.

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Impairment of methylation in S30s prepared from cells treated with homologous or heterologous double-stranded RNA. We have noted earlier that various exogenous RNAs, including reo mRNAU, are degraded faster in S30NT than in S30, 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).

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Impairment of methylation in S30s prepared from cells treated with homologous or heterologous double-stranded RNA. We have noted earlier that various exogenous RNAs, including reo mRNAU, are degraded faster in S30NT than in S30, 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 S30NT. In the experiment described in Table 2 a reo mRNAU 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 mRNAU preparation in S30c and in S30NT. The results in Table 2 indicate that the addition of double-stranded RNA had no effect on either the extent of methylation in S30c or its impairment in S30NT under our experimental conditions.

Effect of the addition of various single-stranded RNAs on the impairment of reo mRNAU methylation in S30NT. We have reported earlier that the impairment of reo mRNAU methylation in S30NT decreases upon increasing the concentration of reo mRNAU 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 mRNAU. Seventy-five percent of the molecules in the reo mRNAU preparation have m7GpppGmp . . . 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 mRNAU methylation in S30NT (Table 3) when tested at a concentration at which reo mRNAU or reo mRNAU does partially overcome the inhibition.
**Table 2. Inhibition of the methylation in S30<sub>INT</sub> of reo mRNA<sub>u</sub> (presumably free of double-stranded RNA) and effect of added double-stranded reovirus RNA on the inhibition**

<table>
<thead>
<tr>
<th>Double-stranded reovirus RNA added (µg/ml)</th>
<th>Methylation RNA</th>
<th>([^{3}H]CH_{3}) residues incorporated (cpm, \times 10^{3}/ml)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endogenous</td>
<td>S30&lt;sub&gt;C&lt;/sub&gt;</td>
<td>S30&lt;sub&gt;INT&lt;/sub&gt;</td>
</tr>
<tr>
<td>-</td>
<td>+ reo mRNA&lt;sub&gt;u&lt;/sub&gt; - endogenous</td>
<td>570</td>
<td>464</td>
</tr>
<tr>
<td>-</td>
<td>Endogenous</td>
<td>915</td>
<td>277</td>
</tr>
<tr>
<td>5</td>
<td>+ reo mRNA&lt;sub&gt;u&lt;/sub&gt; - endogenous</td>
<td>597</td>
<td>486</td>
</tr>
<tr>
<td>5</td>
<td>- Endogenous</td>
<td>874</td>
<td>234</td>
</tr>
</tbody>
</table>

* The reo mRNA<sub>u</sub> 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 adenyate-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.

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**Fig. 5. Release of the inhibition of reo mRNA<sub>u</sub> methylation in S30<sub>INT</sub> by added reo mRNA<sub>u</sub>.** The reo mRNA<sub>u</sub> concentration was 50 µg/ml, and the incubations were for 10 min. For each point shown in the figure the contributions due to the methylation of the endogenous RNA and of any added reo mRNA<sub>u</sub> have been subtracted. Methylation of endogenous RNA accounted for 5 × 10<sup>4</sup> cpm/ml in S30<sub>C</sub> and for 4 × 10<sup>4</sup> cpm/ml in S30<sub>INT</sub>. Net methylation due to the addition of 33 µg of reo mRNA<sub>u</sub> per ml was 2.6 × 10<sup>4</sup> cpm/ml in S30<sub>C</sub> and 2.0 × 10<sup>4</sup> cpm/ml in S30<sub>INT</sub>. Symbols: •, net methylation of reo mRNA<sub>u</sub> in S30<sub>C</sub>; ○, net methylation of reo mRNA<sub>u</sub> in S30<sub>INT</sub>.

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<sub>INT</sub>. To test whether the inhibitor is a macromolecule or not, the small molecules were removed from S30<sub>C</sub> and S30<sub>INT</sub> by gel filtration through Sephadex G-25. The data in Table 5 show that the impairment is manifested in Sephadex-treated S30<sub>INT</sub>. 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<sub>INT</sub> 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<sub>C</sub> and S30<sub>INT</sub> at 37°C for various lengths of time (preincubation) before testing their capacity to methylate reo mRNA<sub>u</sub>. 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 <sub>U</sub> methylation in S30<sub>int</sub><sup>a</sup>

<table>
<thead>
<tr>
<th>Additions</th>
<th>Methylation of RNA</th>
<th>(&lt;sup&gt;3&lt;/sup&gt;H)CH&lt;sub&gt;3&lt;/sub&gt; residues incorporated (cpm, × 10&lt;sup&gt;-4&lt;/sup&gt;/ml) in:</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>S30&lt;sub&gt;C&lt;/sub&gt;</td>
<td>S30&lt;sub&gt;int&lt;/sub&gt;</td>
</tr>
<tr>
<td>None</td>
<td>Endogenous</td>
<td>222</td>
<td>193</td>
</tr>
<tr>
<td>Poly(U) (100 µg/ml)</td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>293</td>
<td>116</td>
</tr>
<tr>
<td></td>
<td>Endogenous</td>
<td>231</td>
<td>180</td>
</tr>
<tr>
<td>EAT, ribosomal RNA (100 µg/ml)</td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>361</td>
<td>98</td>
</tr>
<tr>
<td>EMC RNA (66 µg/ml)</td>
<td>Endogenous</td>
<td>254</td>
<td>255</td>
</tr>
<tr>
<td></td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>355</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>218</td>
<td>235</td>
</tr>
<tr>
<td></td>
<td></td>
<td>285</td>
<td>68</td>
</tr>
</tbody>
</table>

<sup>a</sup> The reo mRNA<sub>U</sub> concentration was 33 µg/ml. The reaction mixtures were incubated for 10 min.

Table 4. Inhibition of reo mRNA<sub>U</sub> 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<sup>a</sup>

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Cell treatment</th>
<th>(&lt;sup&gt;3&lt;/sup&gt;H)CH&lt;sub&gt;3&lt;/sub&gt; residues incorporated (cpm, × 10&lt;sup&gt;-4&lt;/sup&gt;/ml) in:</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endogenous</td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>198</td>
<td>811</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (20 U/ml)</td>
<td>105</td>
<td>405</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (60 U/ml)</td>
<td>146</td>
<td>234</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>195</td>
<td>165</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (60 U/ml; 1.5 h)</td>
<td>246</td>
<td>221</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (60 U/ml; 18 h)</td>
<td>241</td>
<td>82</td>
</tr>
<tr>
<td>3</td>
<td>None</td>
<td>534</td>
<td>554</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (50 U/ml)</td>
<td>469</td>
<td>338</td>
</tr>
<tr>
<td></td>
<td>Mengo virus infection</td>
<td>362</td>
<td>734</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (50 U/ml) and Mengo</td>
<td>482</td>
<td>414</td>
</tr>
<tr>
<td></td>
<td>virus infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>None</td>
<td>476</td>
<td>406</td>
</tr>
<tr>
<td></td>
<td>Mouse interferon (50 U/ml)</td>
<td>323</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>Human interferon (200 U/ml)</td>
<td>328</td>
<td>394</td>
</tr>
<tr>
<td>5</td>
<td>DEAE-dextran</td>
<td>281</td>
<td>549</td>
</tr>
<tr>
<td></td>
<td>DEAE-dextran + poly(I)·poly(C)</td>
<td>217</td>
<td>281</td>
</tr>
</tbody>
</table>

<sup>a</sup> The reo mRNA<sub>U</sub> 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<sub>U</sub> methylation in S30<sub>int</sub><sup>a</sup>

<table>
<thead>
<tr>
<th>Sephadex G-25</th>
<th>Methylation of RNA</th>
<th>(&lt;sup&gt;3&lt;/sup&gt;H)CH&lt;sub&gt;3&lt;/sub&gt; residues incorporated (cpm, × 10&lt;sup&gt;-4&lt;/sup&gt;/ml) in:</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>treatment of S30s</td>
<td></td>
<td>S30&lt;sub&gt;C&lt;/sub&gt;</td>
<td>S30&lt;sub&gt;int&lt;/sub&gt;</td>
</tr>
<tr>
<td>No</td>
<td>Endogenous</td>
<td>329</td>
<td>293</td>
</tr>
<tr>
<td>No</td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>299</td>
<td>79</td>
</tr>
<tr>
<td>Yes</td>
<td>Endogenous</td>
<td>136</td>
<td>112</td>
</tr>
<tr>
<td>Yes</td>
<td>+ reo mRNA&lt;sub&gt;U&lt;/sub&gt; - endogenous</td>
<td>441</td>
<td>228</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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<sub>U</sub> concentration was 50 µg/ml, and the reaction mixtures were incubated for 10 min.

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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$_{in}$ 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$_{in}$. 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).

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$_{in}$ by centrifugation through sucrose gradients (Fig. 7). Reo mRNA's from all the three size classes were methylated in S30$_c$. The
There is no clear-cut difference between S30\textsubscript{C} and S30\textsubscript{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\textsubscript{INT} than in S30\textsubscript{C}. The majority of these products carried -4 net charge and probably consists of the partially methylated cap structure: m\textsuperscript{7}GpppGp. The product with about -4.5 charge is probably m\textsuperscript{7}GpppGmpCp (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\textsuperscript{7}GpppGmpxp (a product designated as cap 2 which might be formed by further methylation of reo mRNA\textsubscript{v}) or it might be a partially methylated cap 1 structure with a ring opened m\textsuperscript{7}G moiety. These results indicate that there is a general impairment in cap structure methylation in S30\textsubscript{INT}.

In a further experiment RNA samples isolated from incubated reaction mixtures containing [\textsuperscript{3}H]SAM, reo mRNA\textsubscript{v}, and either S30\textsubscript{C} or S30\textsubscript{INT} were digested to nucleosides by treatment with P\textsubscript{i} 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\textsubscript{INT} was 74\%. (The methylation of endogenous RNA, i.e., not reo mRNA\textsubscript{v}, was established in S30 and incorporated into 7 methyl-guanosine. The corresponding value in S30\textsubscript{INT} was 74\%. (The methylation of endogenous RNA, i.e., not reo mRNA\textsubscript{v}, 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\textsubscript{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\textsubscript{INT}. The treatment of reo viruses 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\textsubscript{C} with that of the addition of S30\textsubscript{INT} on the core transcription and core methylation processes (Fig. 9). The rate of core transcription, as measured by [\textsuperscript{3}H]GMP incorporation, was similar in reaction mixtures containing S30\textsubscript{C} or S30\textsubscript{INT} (Fig. 9A) or S30\textsubscript{poly(I)-poly(C)} (Fig. 9B). The S30\textsubscript{poly(I)-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 [\textsuperscript{3}H]methyl residues from [\textsuperscript{3}H]SAM was lower in reaction mixtures with S30\textsubscript{INT} (Fig. 9C) or S30 poly(I)-poly(C) (Fig. 9D) than in those with S30\textsubscript{C}.

The impairment of core methylation in S30\textsubscript{INT} is apparently not a consequence of a faster degradation of the products in S30\textsubscript{INT} than in S30\textsubscript{C}. This was established (Fig. 9C) by (i) allowing methylation to take place for 10 min in one reaction mixture with S30\textsubscript{C} and another one with S30\textsubscript{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
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\textsubscript{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\textsubscript{INT}. The impairment of core methylation in S30\textsubscript{INT} decreases upon the addition to the reaction mixture of reo mRNA\textsubscript{M} in increasing concentrations (data not shown). Furthermore, the preincubation of S30\textsubscript{INT} results in the inactivation of the inhibitor of core methylation (Fig. 10). As indicated earlier, the inhibition of reo mRNA\textsubscript{M} methylation by cellular enzymes is similarly overcome by either the addition of reo mRNA\textsubscript{M} or the preincubation of the S30\textsubscript{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\textsubscript{M} methylation is lower in S30\textsubscript{INT} than in S30\textsubscript{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\textsubscript{INT} that has been incubated for 30 min before the addition of reo mRNA\textsubscript{U}. It impairs the methylation of the cap structures of reo mRNA\textsubscript{M} from all three size classes.

The impairment of reo mRNA\textsubscript{M} methylation in S30\textsubscript{INT} is not a consequence of: (i) the degradation of reo mRNA\textsubscript{M} prior to methylation, (ii) the cleavage of reo mRNA after it has been methylated, (iii) the irreversible inactivation of products of methylation are equally stable in the presence of S30\textsubscript{C} and S30\textsubscript{INT}. Further studies revealed that under our conditions of core methylation, the extent of methylation of endogenous RNA is negligible in both S30\textsubscript{C} and S30\textsubscript{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 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.
reo mRNA as a substrate for methylation, or (iv) the impairment of the activity of the capping enzymes.

Most of the impairment persists in S30\textsubscript{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\textsubscript{INT} of either unlabeled SAM (which would lower the specific activity of the \textsuperscript{3}H]SAM used in the methylation assays) or of SAH (an inhibitor of methylation).

A faster production of unlabeled SAM in S30\textsubscript{INT} during incubation, which would again lower the specific activity of \textsuperscript{3}H]SAM, is also unlikely. At least, (i) the methylation of endogenous RNA in S30\textsubscript{INT} (Sephadex treated or not) is impaired, if at all, only to a much lesser extent than that of reo mRNA, and (ii) partially methylated reo mRNA can be reisolated from S30\textsubscript{INT} and further methylated in S30\textsubscript{C} to a similar extent as reo mRNA, which was methylated twice in S30\textsubscript{C}.

An increased rate of production of SAH in S30\textsubscript{INT} probably does not contribute to the impairment. The rate of conversion of SAM to SAH is the same in S30\textsubscript{C} and S30\textsubscript{INT} (data not shown). Moreover, added SAH (0.8 or 80 \textmu M) diminishes the extent of methylation in S30\textsubscript{C} and S30\textsubscript{INT} equally and, thus, the percent inhibition of impairment in S30\textsubscript{INT} compared to S30\textsubscript{C} is unaffected.

Recently, we reported that double-stranded RNA and ATP enhance the endonuclease activity in S30\textsubscript{INT} but not, or only to a lesser extent, in S30\textsubscript{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\textsubscript{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, 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\textsubscript{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\textsubscript{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 in
the reaction mixture or by preincubating the S30\textsubscript{INT} prior to methylation. Thus it is conceivable that the action of both types of enzymes is impaired by the same inhibitor.

In S30\textsubscript{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\textsubscript{INT} might, in principle, be a consequence of the degradation of reo mRNA\textsubscript{C}. 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\textsubscript{INT} with no SAM added is indistinguishable from that synthesized in S30\textsubscript{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\textsubscript{INT}.** Reo mRNA\textsubscript{C} that have been incubated in S30\textsubscript{INT} until their methylation ceased can be reisolated by phenol extraction and further methylated in S30\textsubscript{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\textsubscript{C} in S30\textsubscript{INT} partially overcomes the impairment. The simplest hypothesis accounting for these features is that the inhibitor binds to the reo mRNA\textsubscript{C}, making its 5' terminal cap region inaccessible for methylation. The addition of the reo mRNA\textsubscript{C} 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\textsubscript{C} 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\textsubscript{C} methylation in S30\textsubscript{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\textsubscript{U}, which had been incubated in S30, until its methylation ceased, can be reisolated and further methylated in S30\textsubscript{C}. This indicates that an inhibitor of methylation might also be present in S30\textsubscript{C}. It is obvious, however, that methylation is less impaired in S30\textsubscript{C} than in S30\textsubscript{INT}.

Since our interferon preparation is not pure, we cannot prove that the agent increasing the activity of the methylation inhibitor in S30\textsubscript{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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