Robard, D., & Chrambach, A. (1974) in *Electrophoresis and Isoelectric Focusing in Polyacrylamide Gel* (Allen, R. C., & Maurer, H. R., Eds.) de Gruyter, New York.
Ross, C. A., & Harris, W. J. (1978) *Biochem. J. 171*, 241.
Sarngadharan, M. G., Robert-Guroff, M., & Gallo, R. C. (1978) *Biochim. Biophys. Acta 516*, 419.
Sedwick, W. D., Wang, T. S.-F., & Korn, D. (1975) *J. Biol. Chem. 250*, 7045.
Setlow, P. (1974) *Methods Enzymol. 29*, 3.
Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346.
Topal, M. D., & Fresco, J. R. (1976) *Nature (London) 263*, 285.
Wang, T. S.-F., Sedwick, D., & Korn, D. (1974) *J. Biol. Chem 249*, 841.
Weissbach, A., Baltimore, D., Bollum, F., Gallo, R., & Korn, D. (1975) *Science 190*, 401.
Wintersberger, E. (1974) *Eur. J. Biochem. 50*, 41.
Yarranton, G. Y., & Banks, G. R. (1977) *Eur. J. Biochem. 77*, 521.
Yoshida, S., Kondo, T., & Ando, T. (1974) *Biochim. Biophys. Acta 353*, 463.

# Accumulation of Methyl-Deficient Rat Liver Messenger Ribonucleic Acid on Ethionine Administration†

Biswendu B. Goswami and Opendra K. Sharma*

ABSTRACT: Highly purified poly(adenylic acid)-containing RNA isolated from livers of rats fed 0.25% DL-ethionine in the diet for 7 days accepted methyl groups from *S*-adenosyl[*methyl*-$^3$H]methionine, when incubated in vitro with mRNA methyltransferases from vaccinia virus or Ehrlich ascites cells, whereas RNA from control rats had no such activity. Nuclease digestion followed by chromatographic analyses of mRNA methylated in vitro revealed that the methyl groups were incorporated at the 5′ end into cap 1 structures ($m^7$GpppNmp...) by the viral enzyme, whereas both cap 0 ($m^7$GpppNp...) and cap 1 ($m^7$Gpppm$^6$Am...) structures were formed by the Ehrlich ascites cell enzymes. The methyl-deficient mRNA isolated from the liver of ethionine-fed rats differed in its translational properties from mRNA isolated from control animals in an in vitro protein synthesizing system from wheat germ.

Several eukaryotic viral and cellular mRNAs as well as heterogeneous nuclear RNA (hnRNA) contain at their 5′ terminus a blocked methylated structure (often referred to as "cap") of the type $m^7$GpppN′mpN″mp....[1] In this structure, the 7-methylguanosine and the penultimate nucleoside are joined by the 5′-hydroxyl groups through a triphosphate bridge. Nucleosides N′ and N″ are often methylated in the 2′-O position and an $N^6$,2′-*O*-dimethyladenosine may be found in position N′ (Shatkin, 1976). Certain mammalian, low molecular weight nuclear RNAs, whose functions are not known, have 5′ termini of a similar configuration (Busch, 1976). Additional methylation of several mRNAs occurs internally between its 5′ cap and the 3′-poly(A) end, yielding 6-methyladenosine (Shatkin, 1976) and 5-methylcytosine (Dubin & Stollar, 1975).

The 5′-terminal 7-methylguanosine in mRNAs is apparently required for their efficient translation in vitro (Both et al., 1975; Muthukrishnan et al., 1975, 1978), and is also implicated in protection against degradation by exonucleases (Furuichi et al., 1977). The physiological significance of ribose methylation and internal methylation remains obscure at present.

Considerable information about the enzymatic mechanisms involved in the posttranscriptional modifications of mRNA has been obtained mainly through the use of coupled viral transcription and methylation systems (Wei & Moss, 1974; Furuichi et al., 1976; Furuichi & Shatkin, 1977), or of isolated nuclei, capable of carrying out some of these modifications (Winicov & Perry, 1976). Methyl-deficient RNA synthesized in vitro by viral cores has been used to study reactions catalyzed by enzymes solubilized from purified virus by detergent treatment (Ensinger et al., 1975; Martin et al., 1975) or by purifying some of the modifying enzymes (Ensinger & Moss, 1976; Barbosa & Moss, 1978; Keith et al., 1978). Partially modified mRNAs isolated from purified virus particles have also been used as substrates (Moss, 1977). A homologous substrate for studying these modification reactions in vitro has been isolated from a methionine auxotroph of *Neurospora crassa* under conditions of methionine starvation (Germershausen et al., 1978). However, attempts to produce homologous methyl-deficient mRNA by methionine starvation from mammalian cells have not been successful (Kaehler et al., 1977).

Administration of the hepatocarcinogen ethionine to rats is known to result in the accumulation in liver of undermethylated tRNAs, which are capable of accepting methyl groups from *S*-adenosyl[*methyl*-$^3$H]methionine in vitro by homologous enzymes (Rajalakshmi, 1973; Kerr, 1975; Friedman, 1977; Wainfan et al., 1977).

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[1] Abbreviations used: $m^7$G, 7-methylguanosine; N, ribonucleoside; Nm, 2′-O-methylated ribonucleoside; $m^6$A, $N^6$-methyladenosine; $m^6$Am, $N^6$,2′-*O*-dimethyladenosine; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; hnRNA, heterogeneous ribonucleic acid; NETS buffer, 10 mM Tris-HCl, 1 mM EDTA, 0.4 M NaCl, and 0.5% $NaDodSO_4$; ETS buffer, 10 mM Tris-HCl, 1 mM EDTA, and 0.5% $NaDodSO_4$; $Cl_3AcOH$, trichloroacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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We report here that administration of ethionine to rats resulted in the production of undermethylated poly(A)-containing liver RNA. This RNA accepted methyl groups from *S*-adenosyl[*methyl*-$^3$H]methionine in vitro when exposed to methyltransferases from vaccinia virus or Ehrlich ascites cells.

## Experimental Procedures

*S*-Adenosyl[*methyl*-$^3$H]methionine (specific activity, 10.2 Ci/mmol) was purchased from New England Nuclear. Nucleoside triphosphates and ribonucleases $T_1$ and $T_2$ were from Sigma Chemical Company. Snake venom phosphodiesterase and bacterial alkaline phosphatase were obtained from Worthington Chemical Company. *Penicillium* ($P_1$) nuclease was from Boehringer Mannheim. GpppAm, GpppGm, GpppA, and GpppG were procured from P-L Biochemical Company.

*Animals.* Male Holtzman rats (150–170 g body weight) were injected intraperitoneally with L-ethionine (0.25 mg/g body weight) and adenine (0.125 mg/g body weight) daily for 3 days. In other experiments, rats were fed a low protein diet (basal diet) supplemented with 0.25% DL-ethionine (Farber, 1956). Control rats were fed a basal diet. Both of these diets were bought in pellet form from Bio-Serv, Inc., Frenchtown, N.J.

*Cells and Virus.* Procedures for the growth of mouse L929 cells and purification of vaccinia virus from infected cells have been described previously (Goswami et al., 1979).

*Preparation of Methyl-Deficient Vaccinia mRNA.* Methyl-deficient vaccinia virus mRNA was synthesized in vitro in the absence of methyl donor *S*-adenosylmethionine. The reaction mixture contained in a total volume of 50 to 100 mL: 4–5 $A_{260}$ units/mL of purified vaccinia virus, 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM DTT, 0.05% Nonidet P-40, and 2.5 mM each of ATP, GTP, CTP, and UTP. After 90 min of incubation at 37 °C, viral cores were removed by centrifugation, and RNA from the supernatant was purified (Goswami et al., 1979).

*Isolation of Vaccinia Viral Methyltransferases.* Vaccinia virus cores were prepared from purified virus by treatment with 50 mM Tris-HCl (pH 8.5), 50 mM DTT, and 0.5% Nonidet P-40 and disrupted with 0.1% sodium deoxycholate in 0.3 M Tris-HCl (pH 8.4), 50 mM DTT, and 0.25 M NaCl. Insoluble proteins were removed by centrifugation at 136000*g* for 60 min, and the supernatant was passed through a DEAE-cellulose column (Paoletti et al., 1974; Goswami et al., 1979). The enzyme preparation contained about 1.1 mg of protein per mL.

*mRNA Methyltransferases from Ehrlich Ascites Cells.* Ehrlich ascites cells were propagated in BDF male mice. Seven days after infection, cells were collected in 0.15 M NaCl (pH 7.5), and were washed 5–6 times with 0.035 M Tris-HCl (pH 7.5) and 0.146 M NaCl. A 30000*g* supernatant (S-30) was prepared from 5 mL of packed cells by homogenizing in 2.5 volumes of hypotonic buffer in a tight-fitting Dounce homogenizer and centrifuging at 30000*g* for 15 min (Sharma et al., 1975). The S-30 was centrifuged at 160000*g* for 90 min; the upper four-fifths of the supernatant fraction was saved for the preparation of an ammonium sulfate fraction, and the rest was discarded. The ribosome-free supernatant fraction was adjusted to 0.3 M KCl by adding a 4 M KCl solution, was passed through DEAE-cellulose in 0.3 M KCl, and was fractionated by adding solid ammonium sulfate. The 30–80% saturated ammonium sulfate precipitate was dissolved in 1–1.5 mL of 0.01 M potassium phosphate (pH 7.0), 1 mM dithiothreitol, and 10% (w/v) sucrose and was dialyzed extensively for 5–6 h against the above buffer. The reddish colored solution was clarified by centrifugation at 10000*g* for 10 min, stored frozen in aliquots at −80 °C, and used as a source of mRNA methyltransferase.

*Isolation of Rat Liver RNA.* RNA from livers of normal rats and from rats exposed to ethionine was isolated by homogenizing the tissue from three rats in 5 volumes of 50 mM Tris-HCl (pH 8.3), 5 mM EDTA, 75 mM NaCl, 0.5% sodium dodecyl sulfate, and an equal volume of buffer-saturated phenol in a blender for 1 min at 4 °C. The aqueous phase was reextracted with phenol 3–4 times, and the RNA was precipitated from the aqueous phase by the addition of 0.1 volume of 20% potassium acetate (pH 5.0) and 2.5 volumes of ethanol.

*Oligo(dT)–Cellulose Chromatography.* Poly(A)-containing RNA from bulk liver RNA was isolated by affinity chromatography on oligo(dT)-cellulose (Aviv & leder, 1972). The RNA was dissolved in NETS buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 0.4 M NaCl, and 0.5% $NaDodSO_4$] and applied to a column of oligo(dT)-cellulose equilibrated with NETS. After extensive washing with NETS buffer to remove non-poly(A)-containing RNA, the bound RNA was eluted with ETS buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.5% $NaDodSO_4$]. The RNA was precipitated with ethanol and passed through an oligo(dT)-cellulose column a second time as described above. After ethanol precipitation of the bound RNA, it was dissolved in ETS buffer, heated to 60 °C for 5 min, and quickly cooled in an ice bath. After adjusting the salt concentration to 0.4 M NaCl, it was passed through an oligo(dT)-cellulose column for the third time. The bound RNA was eluted with ETS and precipitated with ethanol. This procedure was routinely followed to ensure complete removal of any contaminating ribosomal and transfer RNAs from the poly(A)-containing RNA (Desrosiers et al., 1975).

*mRNA Methyltransferase Assay.* The standard mRNA methyltransferase reaction mixture contained in a total volume of 100 μL: 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 1.9 μM *S*-adenosyl[*methyl*-$^3$H]methionine (10.2 Ci/mmol, 10000 cpm/pmol), 10 μg of vaccinia viral methyltransferases or 160 to 200 μg of Ehrlich ascites cell methyltransferases, and indicated amounts of various RNAs. After 30 min of incubation at 37 °C, cold $Cl_3AcOH$ precipitable radioactivity was measured (Goswami et al., 1979). For analysis of products of methyltransferase reaction, the reaction mixtures were scaled up 5- to 20-fold. After incubation, the reaction mixtures were adjusted to 0.05 M Tris-HCl (pH 8.5) and 0.5% $NaDodSO_4$ and extracted twice with water-saturated phenol in cold, and the RNA was precipitated by ethanol. The RNA was reprecipitated several times with ethanol, and was dissolved in water for various ribonuclease digestions.

*Enzymatic Digestions and Chromatographic Analysis of RNase Digests.* Enzymatically $^3$H-methyl-labeled RNA or desalted DEAE-cellulose column fractions were digested with different ribonucleases according to published methods (Ensinger & Moss, 1976; Lockard & RajBhandary, 1976; Winicov & Perry, 1976; Boone & Moss, 1977; Faust & Millward, 1977). RNase $T_1$, $T_2$, and A digests of $^3$H-methyl-labeled RNAs were separated on a DEAE-cellulose column (1 × 15 cm) in the presence of 7 M urea to separate molecules according to their net charge (Tener, 1967). The digest, together with markers generated by limited RNase A digestion of *Escherichia coli* tRNA, were applied to the column in 10 mM Tris-HCl (pH 7.6) and 7 M urea. A 400-mL linear gradient of 0 to 0.3 M NaCl in 10 mM Tris-HCl (pH 7.6) and 7 M urea was used to elute the column. Pooled radioactive peaks were desalted by absorption on small DEAE-cellulose columns

(bicarbonate form) and elution with 2 M ammonium bicarbonate, which was removed by successive evaporation from water.

Whatman No. 1 paper was used for all paper chromatography. Two solvent systems were used: (A) isobutyric acid–0.5 N $NH_4OH$ (10:6, v/v) and (B) 2-propanol–concentrated $NH_4OH$–water (7:1:2, v/v). The position of internal markers was detected under ultraviolet light, and papers were cut into 1-cm strips and counted for radioactivity in toluene-based scintillation fluid.

*Assay of Protein Synthesis in Wheat Germ Protein Synthesizing System.* The incubation mixture contained in 50 μL: 15 μL of wheat germ extract (Marcu & Dudock, 1974), 20 μM Hepes (pH 7.4), 2 mM DTT, 1 mM ATP, 20 μM GTP, 0.25 mM spermidine, 0.3 mM *S*-adenosylhomocysteine, 8 mM creatine phosphate, 40 μg/mL creatine phosphokinase, 1.5 mM magnesium acetate, 100 mM KCl, 5 μCi of [$^{35}$S]methionine (sp act. 893 Ci/mmol, New England Nuclear), 0.05 mM each of 19 nonradioactive amino acids, and 5 μg of mRNA (Sharma et al., 1976). The tubes were incubated at room temperature for 90 min. To determine amino acid incorporation into polypeptides, 0.2 mL of 2 N KOH was added to an aliquot of the reaction mixture, and the incubation was continued for an additional 20 min. The tubes were chilled in ice, 1 mL of 5% icecold $Cl_3AcOH$ was added, and the samples were filtered through Whatman GF/C filters, washed 4–5 times with 5% $Cl_3AcOH$, and once with ethanol. The filters were dried and counted for radioactivity. For 5–15% gradient polyacrylamide slab gel electrophoresis containing 0.1% $NaDodSO_4$ (Studier, 1973), aliquots of the reaction mixture were precipitated with 5% $Cl_3AcOH$.

## Results

Poly(A)-containing liver RNA from rats exposed to ethionine accepted methyl groups from *S*-adenosyl[*methyl*-$^3$H]-methionine when incubated in vitro with vaccinia viral mRNA methyltransferases. RNA from control rats had only marginal substrate activity (Table I, part A). Methyl acceptor activity of RNA from rats exposed to ethionine was enhanced by rigorous purification by passing through oligo(dT)-cellulose under conditions which minimized contamination by ribosomal RNAs (Desrosiers et al., 1975).

Any possible contamination of poly(A)-containing RNA with tRNA was ruled out, since tRNA is not retained by oligo(dT)-cellulose under the conditions of purification of poly(A)-containing RNA. Moreover, tRNA, when tested under assay conditions for mRNA methylation with vaccinia methyltransferases, did not significantly accept methyl groups (data not shown). Wei & Moss (1974) have observed that the tRNAs from *E. coli* and yeast are not substrates for vaccinia mRNA methyltransferases. Poly(A)-containing RNA from rats administered ethionine for 1, 4, 8, and 24 weeks also accepted methyl groups. However, RNA from rats fed ethionine for 7 days accepted the maximum number of methyl groups, although the acceptor activity was one-third that of in vitro synthesized, methyl-deficient vaccinia mRNA. Incorporation of methyl groups into poly(A)-containing RNA from rats exposed to ethionine by an enzyme fraction from Ehrlich ascites cells was also observed (Table I part B).

To determine whether the methyl groups were incorporated at the 5′ end of the poly(A)-containing RNA, $^3$H-methyl-labeled RNA was digested with nuclease $P_1$ and alkaline phosphatase and analyzed by paper chromatography. In vitro synthesized methyl-deficient vaccinia RNA, methylated by vaccinia mRNA methyltransferase, was also analyzed. Nuclease $P_1$ cleaves phosphodiester bonds regardless of the presence of 2′-*O*-methyl groups; it does not cleave the triphosphate linkage of the cap structure at the 5′ end of mRNAs. As shown in Figure 1A, treatment with nuclease $P_1$ and alkaline phosphatase of methyl-deficient vaccinia RNA, methylated in vitro by the vaccinia methyltransferases, in agreement with published reports (Muthukrishnan et al., 1978) produced in almost equal amounts two peaks of radioactivity whose migration was similar to authentic cap markers $m^7$GpppGm and $m^7$GpppAm (designated as cap 1 structure). Poly(A)-containing RNA isolated from ethionine-treated rats and methylated in vitro by enzymes from vaccinia virus also yielded two peaks of radioactivity corresponding to $m^7$GpppGm and $m^7$GpppAm markers (Figure 1B). But the amount of radioactivity in the $m^7$GpppAm caps was almost three times that present in $m^7$GpppGm caps. (This could simply reflect the ratio of Am to Gm caps normally present in rat liver poly(A)-containing RNA.) Complete digestion with snake venom phosphodiesterase and alkaline phosphatase of vaccinia RNA or poly(A)-containing RNA from the liver of ethionine-treated rats methylated in vitro by vaccinia methyltransferases yielded the expected nucleosides $m^7$G, Gm, and Am (Figure 2). From vaccinia RNA labeled in vitro, the radioactivity recovered in nucleoside $m^7$G was equal to that present in Gm and Am (Figure 2A). From ethionine-treated rat liver poly(A)-containing RNA, the amount of radioactivity recovered in Am was three times that of either $m^7$G or Gm (Figure 2B). This is in agree with data from Figure 1B, showing three times more radioactivity in $m^7$GpppAm caps than in $m^7$GpppGm cap structures.

Since vaccinia 2′-O-methyltransferase methylated only the penultimate guanosine and adenosine moieties, we decided to use a different enzyme source to determine whether any other undermodified base was present at the 5′ end of poly(A)-containing RNA, isolated from ethionine-treated rats. A partially purified enzyme fraction from Ehrlich ascites cells was used as a source of methyltransferase (Table I, part B).

Table I: Incorporation of Methyl Groups from *S*-Adenosyl[*methyl*-$^3$H]methionine into Various RNAs by mRNA Methyltransferases

| additions | [$^3$H]methyl incorp (cpm) |
|---|---|
| A. Vaccinia mRNA Methyltransferases | |
| no RNA | 500 |
| unmethylated vaccinia RNA (6.8 μg) | 55 000 |
| methylated vaccinia RNA (8.8 μg) | 10 000 |
| normal rat liver poly(A)-containing mRNA (22 μg) | 1 700 |
| basal diet fed rat liver poly(A)-containing mRNA (20 μg) | 2 000 |
| 3d ethionine injected rat liver poly(A)-containing mRNA (20 μg) | 27 000 |
| 7d ethionine fed rat liver poly(A)-containing mRNA (20 μg) | 54 000 |
| B. Ehrlich Ascites Cell mRNA Methyltransferases | |
| no RNA | 11 000 |
| Ehrlich ascites cellular poly(A)-containing mRNA (20 μg) | 13 400 |
| mouse liver poly(A)-containing mRNA (20 μg) | 13 000 |
| normal liver poly(A)-containing mRNA (20 μg) | 16 400 |
| basal diet fed rat liver poly(A)-containing mRNA (20 μg) | 16 000 |
| 7d ethionine fed rat liver poly(A)-containing mRNA (20 μg) | 37 000 |
| unmethylated vaccinia mRNA (13.6 μg) | 58 000 |
| methylated vaccinia mRNA (17.6 μg) | 28 400 |

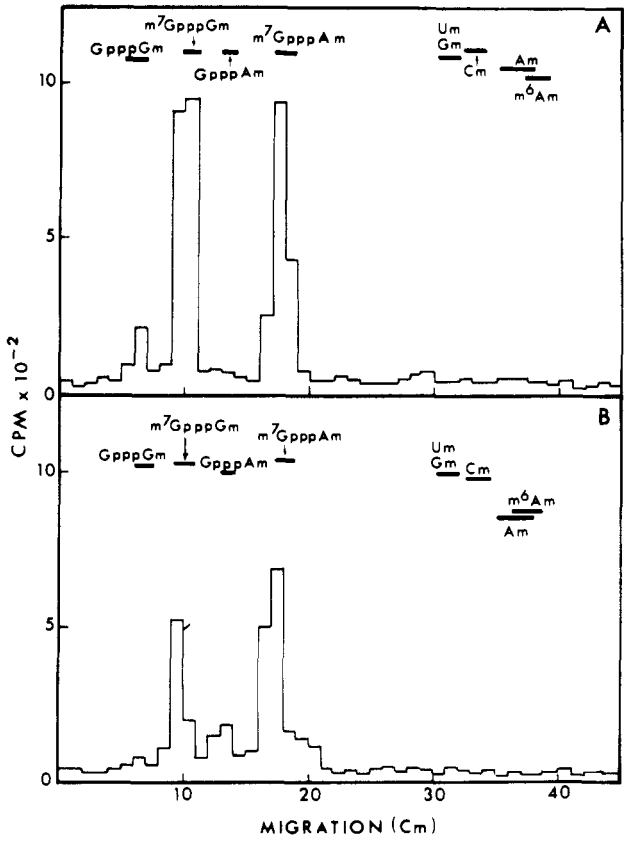


FIGURE 1: Analysis of nuclease $P_1$ digestion products of vaccinia RNA and rat liver poly(A)-containing RNA methylated by vaccinia methyltransferases in vitro. 50 $\mu$g of RNA was labeled in 500-$\mu$L reaction mixtures with 50 $\mu$g of vaccinia methyltransferases. Methyl-labeled RNA was isolated from the reaction mixture and digested with *Penicillium* nuclease $P_1$ and bacterial alkaline phosphatase and analyzed by descending paper chromatography in solvent A: (A) vaccinia methyl-deficient mRNA; (B) undermethylated rat liver poly(A)-containing mRNA.

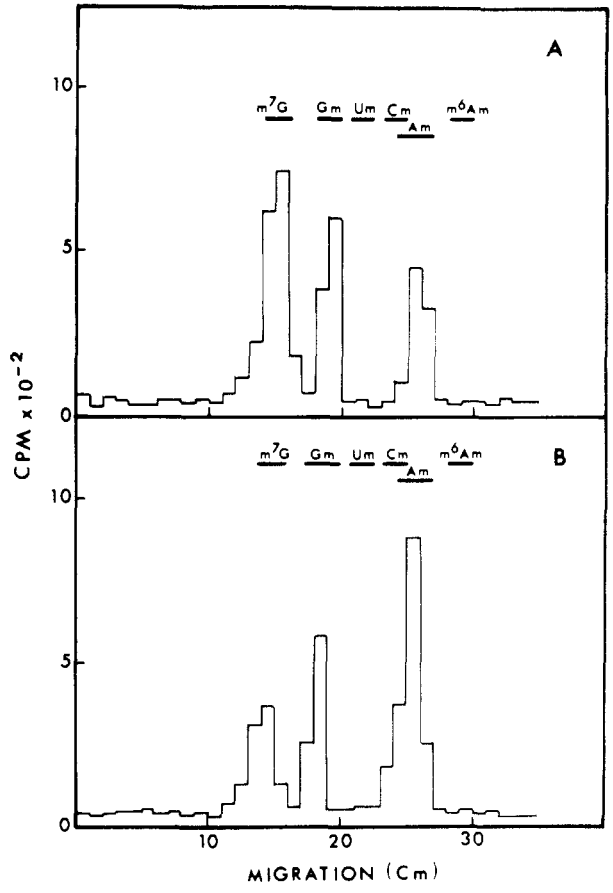


FIGURE 2: Methylated nucleoside analysis of vaccinia mRNA and rat liver poly(A)-containing RNA labeled by vaccinia methyltransferases in vitro as described in Figure 1. In vitro methyl-labeled RNAs were digested to nucleosides by snake venom phosphodiesterase and bacterial alkaline phosphatase and analyzed by descending paper chromatography in solvent B; (A) vaccinia methyl-deficient mRNA; (B) rat liver undermethylated poly(A)-containing mRNA.

Since this system has a rather high background incorporation of radioactivity in the absence of any added RNA, we determined whether it in any way would affect our subsequent analyses. Therefore, we incubated Ehrlich ascites enzyme at a concentration similar to that used for methylating RNA. The assay mixture was scaled up to 20 times, and incubation was carried out without adding an RNA substrate. The reaction mixture was extracted with phenol; carrier tRNA was added to the aqueous phase and RNA precipitated with ethanol. The $^3H$-labeled radioactive material precipitated with ethanol from 2 mL of reaction mixture was incubated with RNAse $T_2$, $T_1$, and A, and chromatographed on a DEAE-cellulose–urea column (Figure 3). Most of the radioactive material eluted in two major peaks, corresponding to approximately 1– and 2– charged materials, and a small amount (3%) eluted at the elution position of cap structure with a net charge of 4– to 5–. Since the level of contamination by the endogenous activity of material eluting with a net charge of 4– to 5– was negligible, we proceeded to analyze the types of cap structure produced by the Ehrlich ascites methyltransferases with both in vitro synthesized methyl-deficient vaccinia RNA and undermethylated RNA from rat liver.

In addition to a peak of radioactivity eluting with a net charge of 2–, there was an additional major peak of radioactivity eluted corresponding approximately to the 4– net charge from in vitro methylated vaccinia RNA (Figure 4A), and two major peaks of radioactivity with net charges of 4– and 5– from undermethylated rat liver RNA (Figure 4B). The absence of any radioactivity in the dinucleotide (net charge 3–) region further indicated that there was no contamination of ribosomal RNA in our mRNA preparations. Radioactive material from each of these peaks was desalted, hydrolyzed with nuclease $P_1$ and alkaline phosphatase, and analyzed by paper chromatography. The radioactive material which eluted with a net charge of 4– on DEAE-cellulose–urea column chromatography (Figure 4) from vaccinia RNA showed two peaks of radioactivity migrating with markers GpppGm and GpppAm in almost equal amounts (Figure 5A). Another portion of the 4– charged oligonucleotide was digested to nucleosides with snake venom phosphodiesterase and alkaline phosphatase. Its analysis by paper chromatography revealed that the major peak of radioactivity migrated with authentic $m^7G$ (Figure 6A). Thus, the 4– charged oligonucleotide was a mixture of $m^7GpppG$ and $m^7GpppA$. Similar analyses of the 4– charged oligonucleotide from undermethylated rat liver RNA also produced two peaks of radioactivity migrating with the cap markers, GpppGm and GpppAm (Figure 5B). But the ratio of GpppGm and GpppAm was approximately 1:3. This is in close agreement with data obtained with the vaccinia methyltransferase and undermethylated rat liver poly(A)-containing RNA. Complete digestion of the 4– charged oligonucleotide to nucleosides with snake venom diesterase and bacterial alkaline phosphatase, followed by paper chromatography, yielded only one major peak of radioactivity corresponding to $m^7G$ (Figure 6B). Therefore, the $P_1$ resistant oligonucleotide fragments are of the type $m^7GpppN$ (cap 0,

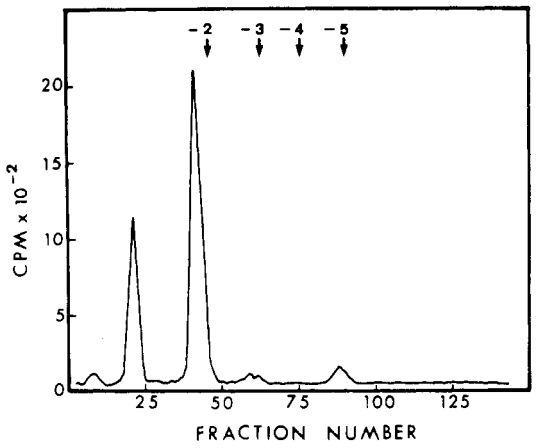


FIGURE 3: Characterization of the nature of endogenous activity of methyltransferases from Ehrlich ascites cells. The assay mixture for methyltransferase was scaled up 20 times: 4 mg of protein was incubated in vitro as described under Experimental Procedures, without any added mRNA substrate. After extraction with phenol of this reaction mixture, carrier *E. coli* tRNA was added to the aqueous phase and RNA precipitated with ethanol. The precipitation was repeated two times to remove any residual *S*-[$^3$H]adenosylmethionine. The precipitate was dissolved in water and digested with ribonucleases $T_2$, $T_1$, and A, and analyzed by DEAE-cellulose–urea column chromatography. 2.5-mL fractions were collected and 0.2-mL aliquots were counted for radioactivity in 10 mL of Biofluor. The numbers on top of arrows indicate the net charge of nucleotides eluting at those positions.

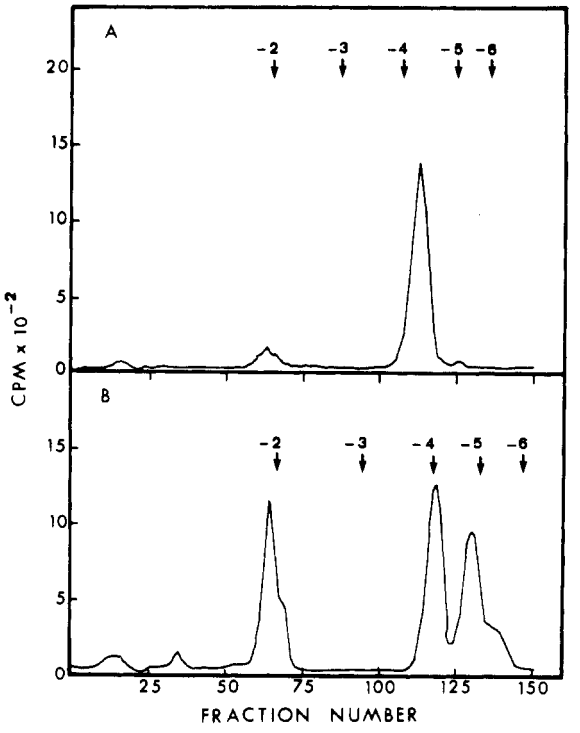


FIGURE 4: Analysis of methylated products synthesized in vitro by Ehrlich ascites methyltransferase in response to added RNA. Methyl-deficient vaccinia RNA or undermethylated rat liver poly(A)-containing RNA was labeled in vitro: (A) 35 $\mu$g of methyl-deficient vaccinia RNA labeled with 320 $\mu$g of enzyme in a 350-$\mu$L reaction mixture; (B) 120 $\mu$g of undermethylated RNA labeled with 2.4 mg of enzyme in a 1.2-mL reaction mixture. Methyl-labeled RNA was isolated from the reaction mixture by phenol extraction and precipitation with ethanol. Methyl-labeled RNA was hydrolyzed with ribonucleases $T_2$, $T_1$, and A, and analyzed by DEAE-cellulose chromatography in 7 M urea.

N = G or A). The radioactive material eluting with a net charge of 5– on nuclease $P_1$ and alkaline phosphatase digestion produced a minor peak migrating with GpppAm and a major

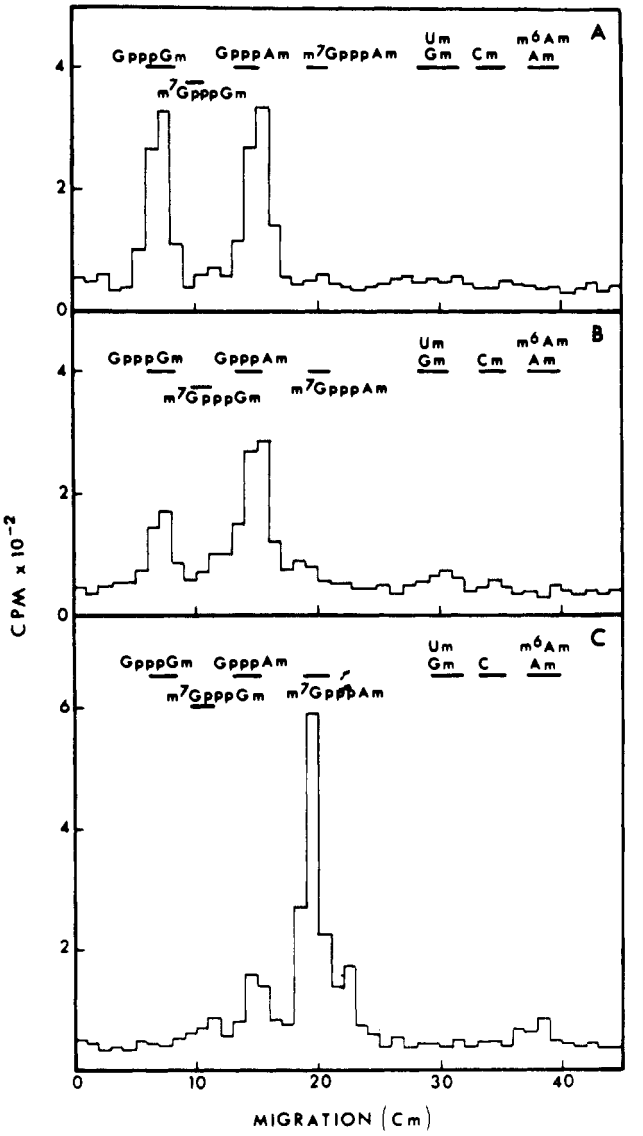


FIGURE 5: Analysis of the cap structures produced by Ehrlich ascites cell methyltransferases in response to added RNA. The oligonucleotide eluting with a net charge of approximately 4– or 5– in Figure 4 was desalted, treated with nuclease $P_1$ and bacterial alkaline phosphatase, and analyzed by descending paper chromatography in solvent A; (A) oligonucleotide with a net charge of 4– in Figure 4A; (B) oligonucleotide with a net charge of approximately 4– in Figure 4B; (C) oligonucleotide with a net charge of approximately 5– in Figure 4B.

peak of radioactivity migrating with the marker $m^7$GpppAm. The minor peak of radioactivity is most likely a contaminant of 4– charged oligonucleotide. Complete digestion of 5–charged oligonucelotide (Figure 5C) with snake venom phosphodieterase and alkaline phosphatase produced $m^7$G and a peak of radioactivity migrating with the marker $m^6$Am (Figure 6C). Therefore, the 5– charged oligonucleotide is $m^7$Gpppm$^6$Am (cap 1).

The radioactivity eluting with a net charge of 2– (Figure 4B) was identified as $m^6$A by hydrolysis with alkaline phosphatase, followed by paper chromatography in solvent B. Less radioactivity was recovered from control incubation (without added poly(A)-containing RNA) in this area.

Since poly(A)-containing liver RNA from rats exposed to ethionine was found to be methyl deficient, it was of interest to examine the ability of such RNA to participate in protein synthesis. Translation of mRNA was carried out in wheat germ extracts in the presence of 0.3 mM *S*-adenosylhomocysteine to inhibit any remethylation of mRNA during protein

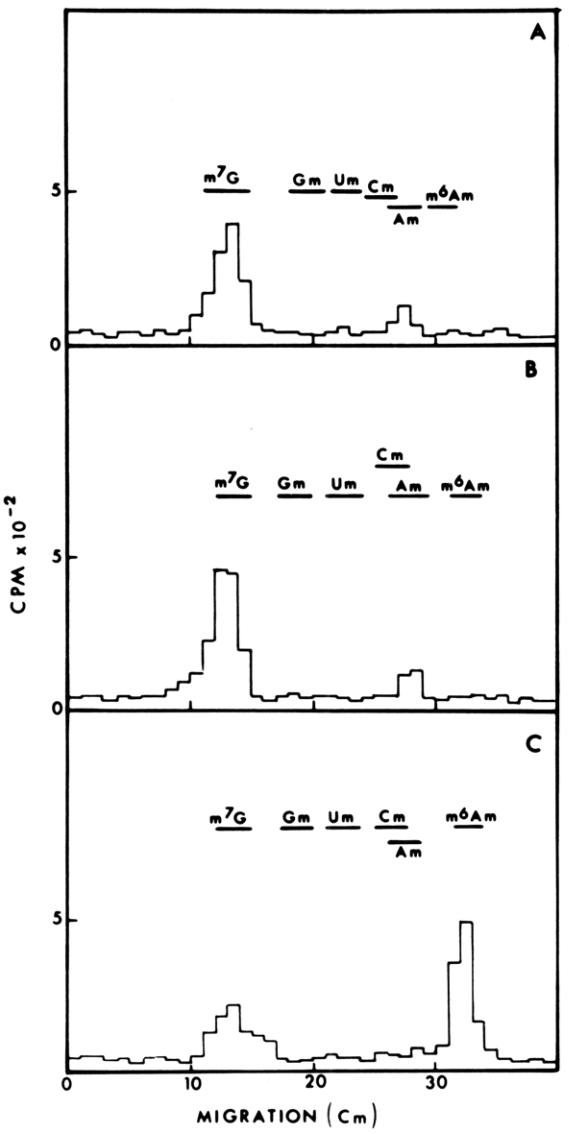


FIGURE 6: Analysis of methylated nucleosides in cap structures synthesized by Ehrlich ascites cell methyltransferases in response to added RNA. Oligonucleotides eluting with a net charge of 4– in Figure 4A and 4– and 5– in Figure 4B were desalted and digested with snake venom phosphodiesterase and bacterial alkaline phosphatase, and the resultant nucleosides were analyzed by descending paper chromatography in solvent B: (A) oligonucleotide eluted with a net 4– charge in Figure 4A; (B) oligonucleotides with a 4– net charge in Figure 4B; (C) oligonucleotides with a 5– net charge in Figure 4B.

synthesis. The electrophoretic pattern of proteins synthesized in the presence of poly(A)-containing RNA from rats administered ethionine was different from those maintained on a basal diet (Figure 7).

## Discussion

Methylation of mRNA in vivo has been shown to be inhibited by cycloleucine (Bachellerie et al., 1978; Dimock & Stoltzfus, 1979), *S*-tubericidinylhomocysteine (Pugh et al., 1977; Kaehler et al., 1979), and *S*-isobutyladenosine (Jacquemont & Huppert, 1977). However, methyl-deficient mammalian mRNA, a substrate for mRNA methyltransferases, has not been isolated heretofore. Administration of ethionine to rats resulted in the accumulation in the liver of methyl-deficient poly(A)-containing RNA. This RNA accepted methyl groups in vitro from *S*-adenosyl[$^3$H]-methionine and enzymes from vaccinia virus or Ehrlich ascites cells. That the methyl acceptor activity of this RNA was not due to the presence of any contaminating tRNA or ribosomal

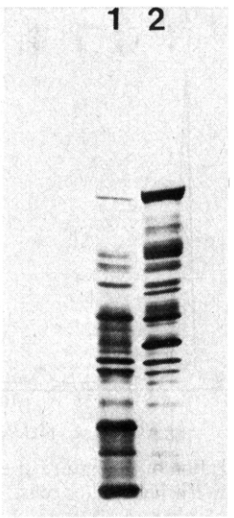


FIGURE 7: Autoradiograph of [$^{35}$S]methionine-labeled products of cell-free protein synthesis in wheat germ extracts: (slot 1) poly-(A)-containing liver RNA from rats fed basal diet; (slot 2) poly-(A)-containing liver RNA from rats fed a basal diet supplemented with 0.25% ethionine for 7 days. Both slots were loaded with 300 000 cpm.

RNA species was ascertained by repeated purification by affinity chromatography on oligo(dT)-cellulose under conditions which remove any adventitious RNA. Furthermore, analysis of the products of in vitro methylation of poly(A)-containing RNA revealed that methyl groups were located in the 5′-cap structures, a characteristic of posttranscriptional modification of poly(A)-containing mRNAs and heterogeneous nuclear RNA (Shatkin, 1976). Poly(A)-containing RNA isolated from rats on exposure to ethionine and methylated in vitro by vaccinia methyltransferase showed that ethionine inhibited methylation of the guanine at the 5′ terminus, and methylation of ribose of the penultimate adenosine and guanosine. All four 2-O′-methylated nucleosides have been detected in cap structures of cellular mRNAs (Desrosiers et al., 1974); apparently vaccinia mRNA methyltransferase lack activities for the methylation of ribose in penultimate uridine and cytidine.

Inhibition of methylation of 5′-terminal guanine by ethionine was also discerned with mRNA methyltransferase from Ehrlich ascites cells. However, we did not detect in vitro methylation of penultimate adenosine and guanosine to Am and Gm as evidenced by the isolation of partially methylated cap structures (cap 0). This is not due to the absence of unmethylated penultimate A and G in the cap structure from rat liver RNA, as synthesis of penultimate Am and Gm in the cap structure was observed when vaccinia methyltransferases were used to methylate rat liver RNA. Sen et al. (1977) have also detected synthesis of partially methylated cap structures (cap 0) when reovirus mRNA was methylated in vitro by extracts from Ehrlich ascites cells. However, we have observed synthesis of $N^6$,2′-*O*-dimethyladenosine ($m^6Am$), a nucleoside in the cap structure of undermethylated liver RNA by Ehrlich ascites enzymes. Such an enzymatic activity was absent from vaccinia enzymes. Since we have not detected the synthesis of penultimate Am alone in the cap structure from rat liver RNA with Ehrlich ascites enzymes, it appears that Am, already present in this RNA, was further methylated in the $N^6$ position by Ehrlich ascites cell enzymes to produce $m^6Am$. Enzyme activity methylating the internal adenosine residues to 6-methyladenosine is different from the enzyme activity methylating penultimate 2′-O-methyladenosine in cap structure (Keith et al., 1978).

Methylation of internal adenine residues to $N^6$-methyladenosine in rat liver poly(A)-containing RNA by Ehrlich ascites cell methyltransferases was also observed. However, with enzymes from vaccinia virus, no such activity was detected, as vaccinia viral cores lack such an enzymatic activity (Wei & Moss, 1974; Ensinger et al., 1975). The differences in the products of methylation of vaccinia virus and Ehrlich ascites cell methyltransferases clearly indicate the need for a variety of RNA substrates for studying in vitro the enzymes involved in posttranscriptional methylation of mRNA.

Prolonged feeding of rats with ethionine results in a high frequency of hepatic carcinoma (Farber, 1963). The mechanism of such carcinogenesis is not known. Unlike most carcinogens, ethionine causes at least a 100-fold greater extent of alkylation of tRNA than DNA (Farber et al., 1967; Swann et al., 1971). We have observed that ethylation of tRNA is not random; an isoacceptor species of lysyl-tRNA is the major target for the ethylation (Kuchino et al., 1978). Ethionine ethylates reovirus mRNA synthesized in vitro (Furuichi et al., 1979), it remains to be explored whether exposure of rats to ethionine results in the alkylation of mRNAs. Exposure of rats to ethionine induces a rapid decrease in hepatic ATP concentration, accumulation of *S*-adenosylethionine (Shull et al., 1966), and inhibition of RNA (Shull et al., 1966) and protein synthesis (Farber & Corban, 1958). *S*-Adenosylethionine is an inhibitor of tRNA methyltransferases (Moore & Smith, 1969; Pegg, 1971). Consequently, hepatic tRNA (Rajalakshmi, 1973; Kerr, 1975; Friedman, 1977; Wainfan et al., 1977) and DNA (Cox & Irving, 1977) isolated from rats administered ethionine and adenine are hypomethylated. Ethionine also affects tRNA methyltransferases which are aberrant in tumor tissues (Kerr, 1978). tRNA methyltransferase activity decreases during the first 2 days of ethionine administration, and then increases steadily (Wainfan et al., 1977). Even though these methylation enzymes are highly elevated, the tRNAs isolated after 3 days or 4 weeks of ethionine administration are equally deficient in methylated nucleosides (Friedman, 1977). It has been observed earlier in our laboratory that administration of ethionine to immature chicks produces a steroid hormone imbalance which results in a 10-fold increase in serum progesterone levels (Sharma & Borek, 1977). Whether the findings reported here have any relevance to carcinogenesis by ethionine remains obscure at present.

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## References

Aviv, H., & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1408.

Bachellerie, J., Amalric, F., & Caboche, M. (1978) *Nucleic Acids Res. 5*, 2927.

Barbosa, E., & Moss, B. (1978) *J. Biol. Chem. 253*, 7692.

Boone, R. F., & Moss, B. (1977) *Virology 79*, 67.

Both, G. W., Banerjee, A. K., & Shatkin, A. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1189.

Busch, H. (1976) *Perspect. Biol. Med. 19*, 549.

Cox, R., & Irving, C. C. (1977) *Cancer Res. 37*, 222.

Desrosiers, R. C., Friderici, K., & Rottman, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3971.

Desrosiers, R. C., Friderici, K., & Rottman, F. (1975) *Biochemistry 14*, 4367.

Dimock, K., & Stoltzfus, C. M. (1979) *J. Biol. Chem. 254*, 5591.

Dubin, D. T., & Stollar, V. (1975) *Biochem. Biophys. Res. Commun. 66*, 1373.

Ensinger, M. J., & Moss, B. (1976) *J. Biol. Chem. 251*, 5283.

Ensinger, M. J., Martin, S. A., Paoletti, E., & Moss, B. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 2525.

Farber, E. (1956) *Cancer Res. 16*, 142.

Farber, E. (1963) *Adv. Cancer Res. 7*, 383.

Farber, E., & Corban, M. S. (1958) *J. Biol. Chem. 233*, 625.

Farber, E., McConomy, J., Franzen, B., Marroquin, F., Stewart, G. A., & Magee, P. N. (1967) *Cancer Res. 27*, 1761.

Faust, M., & Millward, S. (1977) *Anal. Biochem. 79*, 16.

Friedman, S. (1977) *Nucleic Acids Res. 4*, 1853.

Furuichi, Y., & Shatkin, A. J. (1977) *Virology 77*, 566.

Furuichi, Y., Muthukrishnan, S., Tomasz, J., & Shatkin, A. J. (1976) *J. Biol. Chem. 251*, 5043.

Furuichi, Y., LaFiandra, A., & Shatkin, A. J. (1977) *Nature (London) 266*, 235.

Furuichi, Y., Morgan, M. A., & Shatkin, A. J. (1979) *J. Biol. Chem. 254*, 6732.

Germershausen, J., Goodman, D., & Somberg, E. W. (1978) *Biochem. Biophys. Res. Commun. 82*, 871.

Goswami, B. B., Borek, E., Sharma, O. K., Fujitaki, J., & Smith, R. A. (1979) *Biochem. Biophys. Res. Commun. 89*, 830.

Jacquemont, B., & Huppert, J. (1977) *J. Virol. 22*, 160.

Kaehler, M., Coward, J., & Rottman, F. (1977) *Biochemistry 16*, 5770.

Kaehler, M., Coward, J., & Rottman, F. (1979) *Nucleic Acids Res. 6*, 1161.

Keith, J. M., Ensinger, M. J., & Moss, B. (1978) *J. Biol. Chem. 253*, 5033.

Kerr, S. J. (1975) *Cancer Res. 35*, 2969.

Kerr, S. J. (1978) *Methods Cancer Res. 15*, 163.

Kuchino, Y., Sharma, O. K., & Borek, E. (1978) *Biochemistry 17*, 144.

Lockard, R. E., & RajBhandary, U. L. (1976) *Cell 9*, 747.

Marcu, K., & Dudock, B. (1974) *Nucleic Acids Res. 1*, 1385.

Martin, S. A., Paoletti, E., & Moss, B. (1975) *J. Biol. Chem. 250*, 9322.

Moore, B. G., & Smith, R. C. (1969) *Can. J. Biochem. 47*, 561.

Moss, B. (1977) *Biochem. Biophys. Res. Commun. 74*, 374.

Muthukrishnan, S., Both, G. W., Furuichi, Y., & Shatkin, A. J. (1975) *Nature (London) 255*, 33.

Muthukrishnan, S., Moss, B., Cooper, J. A., & Maxwell, E. S. (1978) *J. Biol. Chem. 253*, 1710.

Paoletti, E. Rosemond-Hornbeak, H., & Moss, B. (1974) *J. Biol. Chem. 249*, 3273.

Pegg, A. E. (1971) *FEBS Lett. 16*, 13.

Pugh, C. S. G., Borchardt, R. T., & Stone, H. O. (1977) *Biochemistry 16*, 3928.

Rajalakshmi, S. (1973) *Proc. Am. Assoc. Cancer Res. 14*, 39 Abstr.

Sen, G. C., Shaila, S., Lebleu, B., Brown, G. E., Desrosiers, R. C., & Lengyel, P. (1977) *J. Virol. 21*, 69.

Sharma, O. K., & Borek, E. (1977) *Nature (London) 265*, 748.

Sharma, O. K., Roberts, W. K., Beezley, D. N., & Borek, E. (1975) *Biochim. Biophys. Acta 390*, 327.

Sharma, O. K., Hruby, D. E., & Beezley, D. N. (1976) *Biochem. Biophys. Res. Commun. 72*, 1392.

Shatkin, A. J. (1976) *Cell 9*, 645.
Shull, K. H., McConomy, J., Vogt, M., Castillo, A., & Farber, E. (1966) *J. Biol. Chem. 241*, 5060.
Studier, F. W. (1973) *J. Mol. Biol. 79*, 237.
Swann, P. F., Pegg, A. E., Hawks, A., Farber, E., & Magee, P. N. (1971) *Biochem. J. 123*, 175.
Tener, G. M. (1967) *Methods Enzymol. 12A*, 398.
Wainfan, E., Tscherne, J. S., Maschio, F. A., & Balis, M. E. (1977) *Cancer Res. 37*, 865.
Wei, C. M., & Moss, B. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3014.
Winicov, I., & Perry, R. P. (1976) *Biochemistry 15*, 5039.

# Aminoacyl Transfer Ribonucleic Acid Binding Site of the Bacterial Elongation Factor Tu†

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ABSTRACT: Hydrolysis protection experiments were used for a quantitative determination of the binding of several aminoacyl-tRNAs to the *Escherichia coli* elongation factor Tu. The observed differences could not be rationalized in terms of structural properties of the tRNAs. The experimental results support, however, a model according to which the differences in the affinity of naturally occurring aminoacyl-tRNAs are determined mainly by the nature of the amino acid esterified to the tRNA. Aminoacyl-tRNAs with polar amino acid side chains are bound less strongly than those with apolar ones. This model is substantiated by results obtained with misacylated and modified aminoacyl-tRNAs. Furthermore, it could be shown that the aminoacyl group of the aminoacyl-tRNA must be in the L configuration; EF-Tu in this way prevents blocking of the ribosomal A site or even incorporation of D-amino acids into protein. The data have been used for a schematic description of the structure of a part of the aminoacyl-tRNA binding site of the bacterial elongation factor Tu.

Elongation factor (EF-Tu)[1] supplies the bacterial ribosome with aminoacyl-tRNAs (aa-tRNAs) [for recent reviews, cf. Lucas-Lenard & Beres (1974), Miller & Weissbach (1977), and Ofengand (1977)]. For this purpose a ternary complex consisting of one molecule each of EF-Tu·GTP and aa-tRNA is formed. The structural requirements of both the nucleotide and the tRNA for ternary complex formation have been the subject of several investigations.

The involvement of the acceptor and TΨCG stems of the tRNA in the aa-tRNA·EF-Tu·GTP complex formation has been demonstrated by Jekowsky et al. (1977) in nuclease digestion experiments. The importance of contributions from the end of the acceptor stem to ternary complex formation has been deduced from experiments with several aa-tRNAs lacking the 5′-terminal phosphate. These aa-tRNAs are drastically impaired in their ability to form stable ternary complexes (Schulman et al., 1974). Factor-dependent binding to the ribosome is diminished by a replacement of the terminal adenosine by formycin (Baksht et al., 1975) and by lengthening the CCA terminus by introducing another CMP residue (Thang et al., 1972). Furthermore, several aminoacylated single-stranded fragments of aa-tRNAs have been demonstrated not to bind to EF-Tu·GTP; i.e., within the concentration ranges investigated strong complex formation with $K_{assoc} > 10^5$ $M^{-1}$ could be excluded (Kawakami et al., 1975; Krausskopf et al., 1972). This finding, however, is not necessarily in disagreement with the reported binding of smaller fragments, e.g., CpA-Phe. In these experiments high concentrations of the aminoacyl dinucleoside phosphate had been employed; binding constants of the order of $10^5$ $M^{-1}$ had been deduced (Ringer & Chladek, 1975; Jonak et al., 1978). With respect to the aminoacyl moiety, it had been shown that *N*-acyl-aminoacyl-tRNAs are not bound by EF-Tu·GTP (Ravel et al., 1967; Weissbach et al., 1978). Hydroxyacyl-tRNAs, however, are bound (Fahnenstock et al., 1972). Similarly, modifications in the 3′-terminal nucleotide affect ternary complex formation: Phe-tRNA(Phe, yeast)(ox–red) does not form a complex with EF-Tu·GTP (Ofengand & Chen 1972); Tyr-tRNA(Tyr, yeast) lacking the vicinal OH group either in the 2′ or 3′ position is bound weakly to EF-Tu·GTP, and Phe-tRNA(Phe, yeast) with a 2′- or 3′-$NH_2$ group carrying the aminoacyl residue does not interact with EF-Tu·GTP (Sprinzl et al., 1977).

There are considerable differences in the association constants for the binding of some naturally occurring tRNAs to EF-Tu·GTP (Pingoud et al., 1977). We have, therefore, extended this investigation by analyzing in precise physicochemical terms the binding of several other aa-tRNAs, unmodified and modified ones; this allows us now to put forward

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[1] Abbreviations used: EF-Tu, elongation factor Tu from *E. coli*; aa-tRNA, aminoacyl transfer ribonucleic acid; Tyr-tRNA (Tyr, *E. coli*), tyrosine-specific tyrosyl-tRNA from *E. coli* (other aminoacyl-tRNAs are abbreviated analogously); p-F-Phe-tRNA (Phe, yeast), tRNA(Phe, yeast) aminoacylated with *p*-fluorophenylalanine; Tyr-tRNA(Tyr, yeast)(3′-dA), Tyr-tRNA(Tyr, yeast) lacking the 3′-OH group at the terminal adenosine [analogously for Tyr-tRNA(Tyr, yeast)(2′-dA)]; Phe-tRNA(Phe, yeast)(CCF), Phe-tRNA(Phe, yeast) with the terminal adenosine replaced by formycin; Phe-tRNA(Phe, yeast)(ox–red) is Phe-tRNA(Phe, yeast) with the ribose ring of the terminal adenosine cleaved by periodate oxidation with a subsequent $NaBH_4$ reduction; Tris, tris(hydroxymethyl)aminomethane.

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