Relative Importance of 7-Methylguanosine in Ribosome Binding and Translation of Vesicular Stomatitis Virus mRNA in Wheat Germ and Reticulocyte Cell-free Systems*

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Vesicular stomatitis virus mRNAs with these four types of 5'-termini, (a) mG'ppp'(m)Am, (b) ppp'(m)Am, (c) mG'ppp'(m)Am, and (d) G'ppp'A, were prepared and their translation and ribosome binding analyzed in wheat germ and reticulocyte cell-free protein synthesis systems. The relative efficiencies of translation of individual vesicular stomatitis virus (VSV) mRNAs having type 2 termini ranged from 23 to 29% of the control (type 1) RNA in the reticulocyte system and 6 to 7% of control RNA in the wheat germ system. A similar difference between the two systems was seen in ribosome-binding experiments in which type 2 RNA formed an 80 S initiation complex with high efficiency (70% of control type 1 RNA) in the reticulocyte system, but with low efficiency (17% of control RNA) in the wheat germ system. Similar differences in the importance of mG in translation in the two systems were seen when VSV mRNAs synthesized in vitro with type 3 and type 4 termini were analyzed. However, the analysis of type 4 RNA (which was synthesized in vitro in the presence of S-adenosylhomocysteine) was complicated by the presence of abnormally large poly(A) at its 3'-end. Another series of experiments showed that compounds such as 5'pm7G and mG'ppp'Np are potent and specific inhibitors of translation of all types of VSV mRNAs in eukaryotic cells (14-17). An apparently related finding is that compounds containing 7-methylguanosine 5'-phosphate (pm7G, ppm'G) are specific inhibitors of translation of several mRNAs in wheat germ extracts (18, 19); one study suggests that pm7G inhibits binding of only those mRNAs containing a 5'-terminal mG residue to an initiation factor (19).

Recent observations led us and others to question the requirement for 7-methylguanosine in translation. The 5'-terminal mG of poliovirus mRNA purified from polysomes of infected cells is pUp (20, 21). Also, VSV mRNA from which the 5'-terminal mG was removed chemically directed the synthesis of 25% of the normal amount of authentic VSV proteins in a lysate from rabbit reticulocytes (22). Further experiments showed that removal of the mG residue had little effect on the number of mRNA molecules which could participate in protein synthesis, although it did reduce the rate at which VSV mRNAs formed an 80 S initiation complex with reticulocyte ribosomes 3-fold (22). Similarly, Shih et al. showed that complete chemical removal of the 5'-mG from brome grass mosaic virus RNAs reduced the rate of protein synthesis in a wheat germ extract about 6-fold (23).

Because our previous results had suggested that the source of the translational components was an important factor in determining the relevance of mG to translation, we carried out a more thorough analysis of the differences between the reticulocyte and wheat germ translation systems. In this study we used VSV mRNAs with the 5'-ends mG'ppp'Ap and ppm'G in eukaryotic cell-free extracts from reticulocytes and wheat germ. We show here that wheat germ extracts are much less efficient in translating mRNAs lacking the terminal mG residue than are reticulocyte extracts. Fur-
ther, we show that compounds containing the 5'-monophosphate of m7G, such as 5'-m7Gppp5'Am, are potent inhibitors of translation of VSV mRNAs in wheat germ extracts, but have minimal effects on translation in the reticulocyte lysate. Thus, a 5'-terminal m7G has a more important role in recognition by translational components in the wheat germ system than in the reticulocyte lysate system.

MATERIALS AND METHODS

VSV mRNAs—Labeling and isolation of VSV mRNA from infected cells was described as was the procedure for removing the 5'-terminal m7G by sequential treatment with periodate and aniline (22). Synthesis of VSV mRNA by isolated virion transcriptase in the presence of AdoHcy or AdoMet was as detailed by Rose et al. (24).

Cell-free Protein Synthesis—Conditions for protein synthesis in cell-free extracts of rabbit reticulocytes (25) and wheat germ (26) have been described in detail. Conditions for studying binding of labeled VSV mRNA to ribosomes and incorporation into polysomes have also been described (22).

Reagents—P-L Biochemicals (Milwaukee) was the source of pm7G, m7GpppAm, m7GpppG, m7GpppGm, GpppG, and GpppGm. Concentrations of solutions were calculated from the optical density at 260 nm, assuming that the absorbance of the dinucleosides is the sum of the two component nucleotides. [3H]Methionine (100 to 300 mCi/μmol) was purchased from New England Nuclear. 32P-labeled m7GpppAm was isolated from an RNase P1 digest of 32P-labeled VSV mRNA by electrophoresis at pH 3.5 on DEAE-paper, as described previously (22).

Gel Electrophoresis and Radioautography—Pancreatic RNase (50 μg) was added to all reactions, followed by incubation at 37° for 5 min. Generally, 3 μl of the reaction were analyzed on a 13% polyacrylamide slab gel as detailed by Laemmli (27). The gels were fixed, dried, and subjected to radioautography with Kodak Royal Blue x-ray film. The films were scanned with a Joyce-Loebl microdensitometer. In these cases, the time of exposure of the film was such that the absorbance of the most intense VSV band (generally the N (28, dried, and subjected to radioautography with Kodak Royal Blue x-ray film for 47 h. Globin has migrated off the bottom of the gel. I, no additions; 2, 97 μM pm7G; 3, 250 μM pm7G; 4, 500 μM pm7G; 5, control VSV mRNA; 6, as 5, plus 97 μM pm7G; 7, as 5, plus 250 μM pm7G; 8, as 5, plus 500 μM pm7G; 9, periodate-oxidized, β-eliminated VSV mRNA; 10, as 9, plus 97 μM pm7G; 11, as 9, plus 250 μM pm7G; 12, as 9, plus 500 μM pm7G.

RESULTS

Translation of Modified VSV mRNAs—Oxidation of VSV mRNA with periodate, followed by β-elimination with aniline, yields the 5'-terminus ppmpAmpAMPp on all mRNA species (22). This structure has only partial base methylation of the terminal adenosine and partial base and ribose methylation of the second adenosine. For simplicity, we will indicate this structure with only the ribose methylation of the penultimate nucleotide. Previously we showed that this RNA is translated by unfractionated reticulocyte lysate 25 to 30% as efficiently as is normal VSV mRNA (5'-end m7Gppp5'AmAp...). and that these extracts did not add an m7G residue to the RNA (22). Translation of control and β-eliminated RNA is shown in Fig. 1, lanes 8 and 9. Table I shows the relative amounts of the three major VSV proteins produced in a similar experiment. In wheat germ extracts, by contrast, periodate-oxidized and β-eliminated RNA is translated only 8% as efficiently as is control RNA (Table I; Fig. 2).

Somewhat similar results were obtained with VSV mRNA, synthesized by virion transcriptase in the presence of AdoHcy (Table II). The 5'-end of this unmethylated RNA is GpppApAp, whereas that of RNA made in the presence of AdoMet is Gppp5'ApAp... (4, 12, 24). While methylated RNA has a normal-sized sequence of about 200 adenylate acid residues (poly(A)) at the 3'-end, that made in the presence of AdoHcy has a heterogeneous poly(A) sequence with an average size of about 700 nucleotides, but otherwise the two preparations of RNA are identical (24). In reticulocyte extracts, unmethylated RNA, which we will refer to as AdoHcy RNA, directs synthesis of 13% as much of the three predominant VSV proteins as does control methylated RNA (AdoMet RNA) (Table II). We do not know to what extents the lack of methylation and the very long poly(A) contribute to the reduction in translation. Wheat germ extracts, by contrast, translate this RNA only 1 to 2% as efficiently as they do normal VSV RNA (Table II; Fig. 5).

Attachment of Modified VSV mRNAs to Ribosomes—The above studies suggested that wheat germ ribosomes are much less efficient than reticulocyte ribosomes in their ability to translate VSV mRNAs lacking a 5'-terminal m7G residue. These conclusions were corroborated by two types of experiments in which the binding of labeled mRNAs to ribosomes was followed. As was shown previously, a large fraction of control VSV mRNA is incorporated into polysomes following incubation in a reticulocyte lysate under conditions of protein synthesis (22). These polysomes contained, on the average, 3.7 ribosomes per mRNA (Fig. 3; Table III). About half as much periodate-oxidized, β-eliminated RNA is incorporated into polysomes, and the attached mRNAs contain about 60% of the number of ribosomes, on the average, as does control RNA (Fig. 3; Table I). The variable decrease in the amount of β-eliminated RNA bound to ribosomes is due presumably to partial degradation of the RNA caused by the chemical treatment (22). In wheat germ extracts, by contrast, only 8% as much β-eliminated RNA as control RNA is incorporated into polysomes. Whereas the average size of polysomes formed by normal VSV mRNA in the two extracts is about the same, the size of polysomes formed by β-eliminated RNA is significantly

Fig. 1. Translation of normal and periodate-oxidized, β-eliminated VSV mRNA in reticulocyte extracts; effects of pm7G. Protein synthesis reactions (45 μl) contained a crude extract from rabbit reticulocytes, [35S]methionine (180 μCi/ml, 100,000 C/mmol), AdoHcy (1 × 10⁻⁶ M), other components described previously (26), and 1.5 μg of VSV mRNA or periodate-oxidized and β-eliminated VSV mRNA, as indicated. Incubation was at 30° for 60 min; 3 μl of the reaction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis; the fixed, dried gel was exposed to Kodak Royal Blue x-ray film for 47 h. Globin has migrated off the bottom of the gel. I, no additions; 2, 97 μM pm7G; 3, 250 μM pm7G; 4, 500 μM pm7G; 5, control VSV mRNA; 6, as 5, plus 97 μM pm7G; 7, as 5, plus 250 μM pm7G; 8, as 5, plus 500 μM pm7G; 9, periodate-oxidized, β-eliminated VSV mRNA; 10, as 9, plus 97 μM pm7G; 11, as 9, plus 250 μM pm7G; 12, as 9, plus 500 μM pm7G.

Fig. 3. Attachment of modified VSV mRNAs to ribosomes—The above studies suggested that wheat germ ribosomes are much less efficient than reticulocyte ribosomes in their ability to translate VSV mRNAs lacking a 5'-terminal m7G residue. These conclusions were corroborated by two types of experiments in which the binding of labeled mRNAs to ribosomes was followed. As was shown previously, a large fraction of control VSV mRNA is incorporated into polysomes following incubation in a reticulocyte lysate under conditions of protein synthesis (22). These polysomes contained, on the average, 3.7 ribosomes per mRNA (Fig. 3; Table III). About half as much periodate-oxidized, β-eliminated RNA is incorporated into polysomes, and the attached mRNAs contain about 60% of the number of ribosomes, on the average, as does control RNA (Fig. 3; Table II). The variable decrease in the amount of β-eliminated RNA bound to ribosomes is due presumably to partial degradation of the RNA caused by the chemical treatment (22). In wheat germ extracts, by contrast, only 8% as much β-eliminated RNA as control RNA is incorporated into polysomes. Whereas the average size of polysomes formed by normal VSV mRNA in the two extracts is about the same, the size of polysomes formed by β-eliminated RNA is significantly smaller. This is shown in Table III, which gives the average number of ribosomes per polysome for each type of mRNA.
Tracts; effect of pm'G. Protein synthesis reaction (50 pl) contained a different RNA concentrations.

Reticulocyte and wheat germ cell-free reactions were as in Figs. 1 and 2. Radioautograms of dried gels, on which the reaction products were analyzed, were scanned; tabulated here is the area, in arbitrary planimeter units, under the peaks corresponding to the VSV N, NS, and M proteins. The ratio "treated/control RNA" is that obtained from the average of the two sets of reactions with the use of two different RNA concentrations.

Table I: Translation of periodate-oxidized, β-eliminated VSV mRNA

| Extract                  | Relative amount of protein produced |
|--------------------------|-------------------------------------|
|                          | N       | NS     | M      |
| Reticulocyte VSV mRNA    | 38      | 34     | 63     |
| 7.7 μg/ml                | 57      | 59     | 113    |
| Periodate-treated, β-eliminated RNA | 10     | 8      | 14     |
| 7.7 μg/ml                | 18      | 15     | 27     |
| Treated/control RNA      | 0.29    | 0.24   | 0.23   |
| Wheat germ VSV mRNA      | 58      | 60     | 98     |
| 9.0 μg/ml                | 98      | 114    | 168    |
| Periodate-treated, β-eliminated RNA | 4.0   | 3.1    | 5.4    |
| 9.0 μg/ml                | 7.0     | 5.5    | 10.9   |
| Treated/control RNA      | 0.07    | 0.06   | 0.06   |

Fig. 2. Translation of normal, periodate-oxidized, β-eliminated VSV mRNA and Sindbis 26 S mRNA in wheat germ cell-free extracts; effect of pm'G. Protein synthesis reaction (50 pl) contained a preincubated, crude extract from wheat germ, (15'S)methionine (180 μCi/ml, 100,000 cpm/mmol), AdoHcy (7 x 10^{-4} M), other reagents described previously (25), and 1.5 pg of VSV mRNA. The ratio “treated/control RNA” is that obtained for translation of VSV mRNA by wheat germ extracts (18). All of these RNAs contain the 5'-sequence m7G5'ppp5'NpNpNp. This compound also blocked binding of these mRNAs to wheat germ ribosomes (18). In contrast, translation of satellite tobacco necrosis virus RNA, which has the 5'-terminal sequence pppApGp or pppGAp, is not affected by pm'G. It was concluded that pm'G specifically blocks binding of the 5'-m7G5'pppNpNp sequence on mRNA to a ribosome subunit or essential initiation factor. Figs. 5 and 6 and Table IV show that a similar result is obtained for translation of VSV mRNA by wheat germ extracts; pm'G, but not pG or Gp, is an inhibitor of translation of normal VSV mRNA. It is of interest that translation of AdoHcy RNA (5' end G5'ppp5'Ap) is also affected by pm'G, although less than is control RNA; 650 μm pm'G blocks translation of AdoHcy RNA over 98%, but that of AdoHcy RNA only 62% (Fig. 5; Table IV). Fig. 2 shows that translation of periodate-oxidized, β-eliminated RNA in wheat germ extracts is smaller in wheat germ extracts than in reticulocyte extracts (Fig. 3; Table III). The ability of labeled mRNAs to participate in cell-free protein synthesis can also be determined if the reactions contain 1 mg/ml of anisomycin, a specific inhibitor of polypeptide chain elongation. Under these conditions, a large fraction of control VSV mRNA is incorporated into 80 S initiation complexes (Fig. 4; Ref. 22). These complexes remain stable in a solution of 0.5 M NaCl and 0.03 M magnesium acetate, conditions which will dissociate 40 S to 60 S ribosome couples which do not contain mRNA and initiator tRNA. Fig. 4, using different mRNA preparations from the experiment in Fig. 3, shows that the β-eliminated is bound to reticulocyte ribosomes to almost the same extent as is control VSV. Wheat germ ribosomes, by contrast, incorporate only 8% of the β-eliminated RNA into monoribosomes. Taken together with results on cell-free protein synthesis (Figs. 1 and 2; Tables I and II), these results suggest that wheat germ ribosomes are far less efficient than reticulocyte ribosomes at binding to or initiating protein synthesis on VSV mRNA molecules lacking a terminal 5'-m7G residue. We showed previously that the rate of attachment of reticulocyte ribosomes to β-eliminated RNA molecules to form an 80 S initiation complex is about one-third that of control RNA (22). So little β-eliminated VSV mRNA binds to wheat germ ribosomes that we have not attempted to measure the rate of formation of the 80 S complex.

Table II: Translation of VSV mRNA synthesized in presence of AdoHcy

| Extract                  | Relative amount of protein produced |
|--------------------------|-------------------------------------|
|                          | N       | NS     | M      |
| Reticulocyte AdoHcy RNA  | 113     | 25     | 24     |
| 5 μg/ml                  | 167     | 45     | 44     |
| 10 μg/ml                 | 12      | 3.0    | 3.0    |
| 5 μg/ml                  | 25      | 6.0    | 6.2    |
| AdoHcy/AdoMet            | 0.12    | 0.13   | 0.13   |
| Wheat germ AdoHcy RNA    | 78      | 64     | 112    |
| 5.9 μg/ml                | 133     | 120    | 210    |
| 11.8 μg/ml               | 1.2     | 1.2    | 1.1    |
| AdoHcy/AdoMet            | 0.02    | 0.02   | 0.01   |

Fig. 3: Translation of periodate-oxidized, β-eliminated VSV mRNA and Sindbis 26 S mRNA in wheat germ cell-free extracts; effect of pm'G. Protein synthesis reaction (50 pl) contained a preincubated, crude extract from wheat germ, (15'S)methionine (180 μCi/ml, 100,000 cpm/mmol), AdoHcy (7 x 10^{-4} M), other reagents described previously (25), and 1.5 pg of VSV mRNA. The ratio “treated/control RNA” is that obtained for translation of VSV mRNA by wheat germ extracts (18). All of these RNAs contain the 5'-sequence m7G5'ppp5'NpNpNp. This compound also blocked binding of these mRNAs to wheat germ ribosomes (18). In contrast, translation of satellite tobacco necrosis virus RNA, which has the 5'-terminal sequence pppApGp or pppGAp, is not affected by pm'G. It was concluded that pm'G specifically blocks binding of the 5'-m7G5'pppNpNp sequence on mRNA to a ribosome subunit or essential initiation factor. Figs. 5 and 6 and Table IV show that a similar result is obtained for translation of VSV mRNA by wheat germ extracts; pm'G, but not pG or Gp, is an inhibitor of translation of normal VSV mRNA. It is of interest that translation of AdoHcy RNA (5' end G5'ppp5'Ap) is also affected by pm'G, although less than is control RNA; 650 μm pm'G blocks translation of AdoHcy RNA over 98%, but that of AdoHcy RNA only 62% (Fig. 5; Table IV). Fig. 2 shows that translation of periodate-oxidized, β-eliminated RNA in wheat germ extracts is smaller in wheat germ extracts than in reticulocyte extracts (Fig. 3; Table III).

The ability of labeled mRNAs to participate in cell-free protein synthesis can also be determined if the reactions contain 1 mg/ml of anisomycin, a specific inhibitor of polypeptide chain elongation. Under these conditions, a large fraction of control VSV mRNA is incorporated into 80 S initiation complexes (Fig. 4; Ref. 22). These complexes remain stable in a solution of 0.5 M NaCl and 0.03 M magnesium acetate, conditions which will dissociate 40 S to 60 S ribosome couples which do not contain mRNA and initiator tRNA. Fig. 4, using different mRNA preparations from the experiment in Fig. 3, shows that the β-eliminated is bound to reticulocyte ribosomes to almost the same extent as is control VSV. Wheat germ ribosomes, by contrast, incorporate only 8% of the β-eliminated mRNA into monoribosomes. Taken together with results on cell-free protein synthesis (Figs. 1 and 2; Tables I and II), these results suggest that wheat germ ribosomes are far less efficient than reticulocyte ribosomes at binding to or initiating protein synthesis on VSV mRNA molecules lacking a terminal 5'-m7G residue. We showed previously that the rate of attachment of reticulocyte ribosomes to β-eliminated RNA molecules to form an 80 S initiation complex is about one-third that of control RNA (22). So little β-eliminated VSV mRNA binds to wheat germ ribosomes that we have not attempted to measure the rate of formation of the 80 S complex.

Inhibitors Containing pm'G – Hickey et al. showed that pm'G, when used at high concentrations, was an inhibitor of translation of reovirus mRNA, tobacco mosaic virus RNA, and globin mRNA by wheat germ extracts (18). All of these RNAs contain the 5'-sequence m7G5'ppp5'NpNpNp. This compound also blocked binding of these mRNAs to wheat germ ribosomes (18). In contrast, translation of satellite tobacco necrosis virus RNA, which has the 5'-terminal sequence pppApGp or pppGAp, is not affected by pm'G. It was concluded that pm'G specifically blocks binding of the 5'-m7G5'pppNpNp sequence on mRNA to a ribosome subunit or essential initiation factor. Figs. 5 and 6 and Table IV show that a similar result is obtained for translation of VSV mRNA by wheat germ extracts; pm'G, but not pG or Gp, is an inhibitor of translation of normal VSV mRNA. It is of interest that translation of AdoHcy RNA (5' end G5'ppp5'Ap) is also affected by pm'G, although less than is control RNA; 650 μm pm'G blocks translation of AdoHcy RNA over 98%, but that of AdoHcy RNA only 62% (Fig. 5; Table IV). Fig. 2 shows that translation of periodate-oxidized, β-eliminated RNA in wheat germ extracts is smaller in wheat germ extracts than in reticulocyte extracts (Fig. 3; Table III).
**7-Methylguanosine and Translation of VSV mRNA**

FIG. 3. Incorporation of labeled VSV mRNA into polysomes. Reticulocyte cell-free reactions (120 µL, panels a and b) contained 10⁻⁴ M concentrations each of 20 amino acids, 7 × 10⁻⁴ M AdoHcy, and about 1.0 × 10⁵ cpm (0.15 µg) of ³²P-labeled control VSV mRNA (panel a) or periodate-treated and β-eliminated RNA (panel b). Incubation was at 30° for 4 min. Wheat germ cell-free reactions (145 µL, panels c and d) contained 10⁻⁴ M concentrations each of 20 amino acids, 7 × 10⁻⁴ M AdoHcy, and about 1.0 × 10⁵ cpm of the same preparation of control VSV mRNA (panel c) of periodate-oxidized, β-eliminated RNA (panel d). Incubation was at 25° for 10 min. Reaction tubes were chilled on ice, and 1.2 ml of Buffer A (0.5 M NaCl, 0.03 M Mg(acetate), 0.02 M N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid (Hepes), pH 7.5) was added. The samples were layered on a 36-ml 15 to 30% (w/v) linear sucrose gradient, made up in Buffer A, and centrifuged in a Beckman SW 27 rotor at 4° at 26,500 rpm for 4 h. Samples were collected through a flow cell in a Gilford recording spectrophotometer directly into scintillation vials. The arrows represent optical density peaks of the polysomes which in panel a is due predominantly to the endogenous globin mRNA. Each sample was bleached with alkaline hydrogen peroxide and counted with the use of Aquasol (New England Nuclear) in a Beckman scintillation spectrometer. In control reactions (which were not incubated) only 2 to 2.5% of the radioactivity was found in the monosome or polysome regions of the gradient (data not shown). Analysis of data from this experiment are in Table III.

...tracts is inhibited by pm⁷G to the same extent as is that of normal RNA; densitometer scans of these radioautograms showed that a concentration of pm⁷G (0.1 mM) which inhibits translation of control RNA 96% inhibited translation of treated RNA over 90%.

Figs. 5 and 6 also illustrate that several other analogues of 5'-ends of mRNAs, m¹G⁵pp⁵pG, m¹G⁵pp⁵p⁵Gm, m¹G⁵pp⁵p⁵p⁵Am, are also potent inhibitors of translation of VSV mRNA by wheat germ extracts. Equivalent inhibition of VSV protein synthesis by these compounds is achieved at about one-fifth the concentration required of pm⁷G (Fig. 6). Similar compounds lacking an m¹G residue, G⁵pp⁵pG and G⁵pp⁵p⁵Gm, are essentially inactive as inhibitors (Fig. 6).

By contrast, none of the aforementioned compounds have any discernible effect on translation of endogenous globin mRNA by reticulocyte lysates (data not shown). Neither do they have any significant effect on translation of exogenous VSV mRNA by these lysates (Figs. 6 and 7). In particular, a concentration of pm⁷G or m¹G⁵pp⁵p⁵Am which inhibits translation of VSV mRNA by a wheat germ lysate over 90% inhibits translation in a reticulocyte extract less than 10% (Fig. 7). The absence of significant inhibition by m¹GppAm in the reticulocyte lysate is not due to its degradation. By adding ±³²P-labeled m¹GppAm, we determined that half of the compound remains intact after 22 min of incubation in a reticulocyte lysate (Fig. 8), whereas no significant inhibition of translation of VSV mRNA is apparent after either 10 min (data not shown) or 20 min (Fig. 7) of incubation. The smaller peak of material seen in the unincubated sample (13 cm) is possibly m¹G⁵pp⁵p⁵Am containing an m¹G in the open ring form which is generated during elution of the compound from DEAE-paper with triethylamine bicarbonate.

**DISCUSSION**

The most significant aspect of this work is that the effect on translation of a 5' terminal m¹G residue in VSV mRNA is much less pronounced in the reticulocyte lysate system than in the wheat germ system. It is important to emphasize that in both extracts a large fraction of VSV mRNA will bind to ribosomes and participate in protein synthesis (Figs. 3 and 4). However, because the translation of animal virus mRNAs is being investigated, the results obtained from the reticulocyte lysate system would presumably be most relevant to an understanding of the importance of m¹G in translation of VSV mRNA in infected cells. Indeed, the rates of chain initiation and elongation on endogenous globin mRNA by reticulocyte...
TABLE III
Incorporation of labeled VSV mRNA into polysomes

The polysome gradients are depicted in Fig. 3. In calculating the data given in line 2, the amount of radioactivity in any region of a polysome gradient was multiplied by the number of ribosomes per polysome \((i)\), that is, by the number of ribosomes translating each mRNA in that peak. Hence, the total number of ribosomes translating the mRNAs is proportional to the sum of this product over all regions of the polysome gradient (line 2). Since the total radioactivity in polysomes (line 1) is proportional to the number of VSV mRNAs being translated, the average number of ribosomes per bound mRNA is given by line 3.

| Reticulocyte extract | Wheat germ extract |
|----------------------|--------------------|
| a. Control RNA       | b. Periodate-oxidized, \(\beta\)-eliminated RNA | c. Control RNA | d. Periodate-oxidized, \(\beta\)-eliminated RNA |
| Counts per min in mono- or polyribosomes | 23,502 | 15,997 | 11,796 | 1,265 |

Proportionate number of ribosomes bound to VSV mRNA

\[
R = \frac{1}{i} \sum C_i
\]

Ribosomes per bound mRNA

\[
r = \frac{R}{C}
\]

Total counts per min recovered from gradient \(C_i\)

\[
C_i = C/C_i
\]

lysat e ribosomes are within a factor of two of those found in the intact reticulocyte (30). Many crude cytoplasmic extracts from mammalian cells, such as those from ascites or L cells, and fractionated extracts from reticulocytes translate exogenous mRNAs much less efficiently (19, 31-34) and may not be appropriate for investigation of structure-function relationships in mRNA.

Several types of experiments revealed the differences between the reticulocyte and wheat germ systems. First was the demonstration that VSV mRNA from which the 5'-terminal m7G had been removed by periodate oxidation and \(\beta\) elimination bound to reticulocyte ribosomes to an extent which was 70% of control untreated mRNA (Fig. 4; Ref. 22). In contrast, this RNA bound only 17% of the extent of control RNA to wheat germ ribosomes (Fig. 4). Although all of our cell-free reactions contain the inhibitor of methylation, AdoHcy, we have not been able to rule out the possibility that the small amount of binding and protein synthesis in the presence of \(\beta\)-eliminated mRNA is not due to readdition of m7G to a few percent of the RNA molecules. However, no readdition of m7G occurs in the reticulocyte system (22). Parallel experiments on translation of this RNA showed that the \(\beta\)-eliminated RNA directed the synthesis of the authentic VSV proteins with an

![Fig. 4. Binding of \(^{32}P\)-labeled VSV mRNAs to ribosomes. Reactions contained 1 mg/ml of anisomycin, a specific inhibitor of polypeptide chain elongation (25) but were otherwise identical with those of Fig. 3. However, different preparations of RNA were used. Incubation gradient centrifugation and counting of the samples was as in Fig. 3. In reactions not incubated, 2.1 to 2.6% of the \(^{32}P\)-labeled RNA was found to sediment faster than 40 S (data not shown). The percentage of added \(^{32}P\)-labeled mRNA bound to ribosomes after the incubation was a, control RNA, reticulocyte extract, 71%; b, periodate-oxidized, \(\beta\)-eliminated RNA, reticulocyte extract, 48%; c, control RNA, wheat germ extract, 48%; d, periodate-oxidized, \(\beta\)-eliminated wheat germ extract, 8%.](http://www.jbc.org/Downloaded from)
Inhibition of wheat germ protein synthesis by analogs of 5' ends of messenger RNA

Reactions are described in the legend to Fig. 5, and a radioautogram of a gel analysis of the protein products is shown in Fig. 5. Tabulated here is the radioactivity incorporated into acid-precipitable polypeptides from a 5-μl sample of the reaction after 120 min of incubation. A background of 990 cpm, from reactions without added mRNA, has been subtracted from all values.

| Compound       | Concentration | Messenger RNA |
|----------------|---------------|---------------|
|                | μM            | AdoMet RNA    | AdoHcy RNA   |
| None           |               | 180,200       | 3,150        |
| pm7G           | 333           | 270           | 1,200        |
| pG             | 349           | 159,100       | 3,210        |
| m'GpppG        | 68            | 7,600         | 830          |
| GpppG          | 50            | 168,100       | 3,320        |
| m'GpppAm       | 60            | 3,700         | 630          |
| GpppGm         | 55            | 182,700       | 3,050        |

A second type of experiment which demonstrated the differences between the reticulocyte and wheat germ systems employed the RNA synthesized in vitro by the VSV virion transcriptase in its methylated (5' end m7Gppp5'Am) and unmethylated form (5' end Gppp5'Ap). The studies on these RNAs are complicated by the finding that the unmethylated RNA does not contain the correct RNA species because of the presence of large heterogeneous poly(A) on all mRNA species (24). Translation of these RNAs again revealed a discrepancy between the two systems because the AdoHcy RNA was translated with an efficiency of 12% of AdoMet RNA in the reticulocyte system but with only 1 to 2% efficiency in the wheat germ system. Because we do not know what the effect of the long poly(A) on translation might be, these experiments are less conclusive than those involving p elimination. If the poly(A) does not influence translation, then the greater discrepancy between the methylated and unmethylated RNAs in either system when compared to control and p-eliminated RNAs may indicate that the additional methylations which remain on 5'-Gppp5'Ap may be translated less efficiently than those with a pppA.

Fig. 5. Translation of AdoMet VSV mRNA and AdoHcy RNA in wheat germ extracts. Reactions (51 μl) contained wheat germ extract, [35S]methionine, AdoHcy, as described in the legend to Fig. 2. Reactions 1 through 7 contained 0.3 μg of VSV mRNA synthesized in vitro in the presence of AdoMet (AdoMet RNA); reactions 8 to 13 contained 0.3 μg of mRNA synthesized in vitro in the presence of AdoHcy (AdoHcy RNA). Incubation, gel electrophoresis, and radioautography conditions were also those of Fig. 2. Aliquots (5 μl) of the reaction after 120 min of incubation were used to determine the amount of acid-precipitable protein radioactivity; these results are tabulated in Table IV. Shown here are the radioautographs of the dried gel. No bands were evident in a sample (not shown) which contained no mRNA. Samples 1 and 8, no addition; 2 and 9, 333 FM pm7G; 3 and 10, 349 FM pG; 4 and 11, 68 FM m'GpppG; 5 and 12, 50 FM GpppG; 6 and 13, 60 FM m'GpppAm; 7, 55 FM GpppGm.

Fig. 6. Effect of pm7G and m'GpppAm on synthesis of VSV N protein. Reticulocyte or wheat germ reactions contained VSV mRNA purified from infected cells (see Figs. 1 and 2 for conditions) and the indicated amounts of pm7G (left panel) or m'GpppAm (right panel). Conditions of incubation, gel analysis, and radioautography are as in Figs. 1 and 2. An exposure time of the film was chosen so that the optical density of the VSV N protein band, as determined by scanning with a Joyce-Loebl microdensitometer, was within the linear range of response of the film (less than 1 absorbance unit). Plotted here are the relative areas of the N protein band in the scans of the gel analysis of the protein products is shown in Fig. 5. Efficiency varying from 23 to 29% of control RNA (depending on the protein examined) in the reticulocyte system but only 5 to 7% of control RNA in the wheat germ system.

Fig. 7. Effect of several compounds on translation of VSV mRNA by reticulocyte extracts. All reactions (36 μl, see Fig. 1) except no. 1 contained 0.2μg of normal VSV mRNA; other additions were as indicated. Incubation was at 30° for 20 min. Shown is a radioautograph of a gel in which 2 μl of the reaction products were analyzed; exposure of the film was for 8 days. 1, no additions; 2, VSV mRNA; 3, VSV mRNA plus 700 FM pm7G; 4, VSV mRNA plus 740 FM pG; 5, VSV mRNA plus 780 FM Gp; 6, VSV mRNA plus 130 FM m'GpppAm; 7, VSV mRNA plus 130 μM m'GpppAm; 8, VSV mRNA plus 120 μM GpppG; 9, VSV mRNA plus 120 μM m'GpppAm; 10, VSV mRNA plus 125 μM GpppGm.

TABLE IV

| Compound       | Concentration | Messenger RNA |
|----------------|---------------|---------------|
|                | μM            | AdoMet RNA    | AdoHcy RNA   |
| None           |               | 180,200       | 3,150        |
| pm7G           | 333           | 270           | 1,200        |
| pG             | 349           | 159,100       | 3,210        |
| m'GpppG        | 68            | 7,600         | 830          |
| GpppG          | 50            | 168,100       | 3,320        |
| m'GpppAm       | 60            | 3,700         | 630          |
| GpppGm         | 55            | 182,700       | 3,050        |
followed by in vitro translation studies. A study of this question will require β elimination and the removal of poly(A) from the RNAs synthesized in vitro. A third type of experiment illustrating a discrepancy between the wheat germ and reticulocyte systems in the response to m7G comes from studies of 5′ end analogues pm'G, m7G5'ppp5'Am, and m7GS'pppS'G(m) which inhibit translation in the reticulocyte system even when used at very high concentrations. Because the effects of pm'G in this fractionated reticulocyte system are far greater than those which we observe in the reticulocyte lysate, it is possible that an artifactual dependence on m7G-directed recognition of VSV mRNA is generated in the fractionated system, perhaps because other factors have become limiting. In addition to this possible artifact, we would like to suggest that in such studies a control RNA other than encephalomyocarditis virion RNA, whose binding to IF-M3 was not inhibited by pm'G, should be used because it is now known that poliovirus (a close relative of encephalomyocarditis) contains virion RNA in which the 5′ end is covalently linked to a protein.

Although a 5′-terminal mG is very important in translation of VSV mRNA in the wheat germ system, it may not be important for all mRNAs in this plant system since it is known that satellite tobacco necrosis virus (STNV, 5′-end ppAp or ppAp) and bacteriophage Qβ RNA can be translated by this system (18, 36). However, it has not been determined if mG is added on to a fraction of these RNAs, and little information is available on the efficiency of translation of these RNAs. A study which is relevant to this point is that of Shih et al. who have shown that the removal of mG from the coat protein mRNA of brome grass mosaic virus RNA (5′ end mG3'pppG) results in a 5- to 6-fold reduction in translation (23), an effect which seems significantly less than that which we find for VSV mRNAs in this system.

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