The Absence of a m\(^7\)G Cap on \(\beta\)-Globin mRNA and Alfalfa Mosaic Virus RNA 4 Increases the Amounts of Initiation Factor 4F Required for Translation*

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\(\beta\)-Globin mRNA and alfalfa mosaic virus (AMV) RNA 4, two naturally capped mRNAs, and satellite tobacco necrosis virus (STNV) RNA, a naturally uncapped mRNA, were prepared by in vitro transcription with and without a 5' m\(^7\)G cap structure (m\(^7\)G(5')ppp(5')N). The translation of the capped and uncapped forms of these mRNAs was measured in a crude S30 system and a partially purified system from wheat germ. In the S30 system the uncapped forms of \(\beta\)-globin mRNA and AMV RNA 4 are much less active (\(\leq 10\%\)) than their capped forms, whereas the uncapped and capped forms of STNV RNA are equally active. The low activity of uncapped \(\beta\)-globin mRNA and AMV RNA 4 in the S30 system is due, in part, to inactivation of the uncapped mRNAs in this system. Additional studies, carried out in the partially purified system in which very little inactivation of the mRNAs occurs, show that the uncapped and capped forms of \(\beta\)-globin mRNA or AMV RNA 4 differ markedly with respect to the amount of eukaryotic initiation factor (eIF)-4F required for translation. For \(\beta\)-globin mRNA the absence of the 5' cap structure increases the concentration of eIF-4F required for half-maximal translation about 6-fold (from 10 to 60 nM) and for AMV RNA 4 it increases the concentration of eIF-4F about 12-fold (from 5 to 60 nM). The concentrations of eIF-3, eIF-4A, and eIF-4B required for half-maximal translation of the uncapped forms of \(\beta\)-globin mRNA and AMV RNA 4 are either the same or only slightly higher (1.5- to 2-fold) than the concentrations required for the capped forms. With STNV RNA the concentration of eIF-4F required for half-maximal translation of either uncapped or capped STNV RNA is 3 nM, and the concentrations of eIF-3, eIF-4A, and eIF-4B required for the two forms are also the same. The translation of the capped and uncapped forms of \(\beta\)-globin mRNA and AMV RNA 4 is inhibited strongly by low concentrations of m\(^7\)GTP in the partially purified system containing low concentrations of eIF-4F. Under the same conditions, the translation of capped or uncapped STNV RNA is inhibited only slightly by m\(^7\)GTP. These findings suggest the possibility that the mechanism by which eIF-4F interacts and initiates translation of naturally capped mRNAs may not be identical to the mechanism by which eIF-4F interacts and initiates translation of naturally capped mRNAs.

Previous work has shown that the absence of a 5' cap structure (m\(^7\)G(5')ppp(5')N (11)) on a naturally capped mRNA such as reovirus RNA, VSV RNA, or globin mRNA greatly decreases its ability to be translated (2), whereas capping of a naturally uncapped mRNA such as STNV RNA does not enhance its ability to be translated (3). There have been several reports (4-6) indicating that the translation of AMV RNA 4 is less sensitive than other naturally capped mRNAs to inhibition by m\(^7\)G cap analogs (4, 5) or by antibody to the 24-kDa cap binding protein (4). Also, AMV RNA 4 is translated in extracts from poliovirus-infected cells (7) and in eIF-4E-deficient extracts from a temperature-sensitive yeast mutant (8).

On the basis of these observations, it has been concluded the translation of AMV RNA 4 is less dependent upon the 5' cap structure than other capped mRNAs.

In this investigation we have prepared capped and uncapped AMV RNA 4, as well as capped and uncapped \(\beta\)-globin mRNA and STNV RNA, by in vitro transcription and have measured the abilities of these mRNAs to be translated in a crude S30 system and a partially purified system from wheat germ. We find that translation of AMV RNA 4 is dependent upon the cap structure. In the wheat germ S30 system, the uncapped forms of AMV RNA 4 and \(\beta\)-globin mRNA are inactivated more rapidly than the capped forms of these mRNAs. Further work carried out in the partially purified system in which very little inactivation of the capped or uncapped forms of these mRNAs occurs showed that the absence of a 5' cap on AMV RNA 4 or \(\beta\)-globin mRNA increases the amount of eIF-4F required for translation 6- to 12-fold. In addition, we find that m\(^7\)GTP strongly inhibits the translation of both the capped and uncapped forms of AMV RNA 4 and \(\beta\)-globin mRNA in the partially purified system containing low concentrations of eIF-4F. In contrast, the uncapped form of STNV RNA is as stable as the capped form in the S30 system. In addition, the amounts of eIF-4F required for translation of the capped and uncapped forms in the partially purified system are the same, and m\(^7\)GTP has very little effect on the translation of STNV RNA, either capped or uncapped.

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1 The abbreviations used are: VSV, vesicular stomatitis virus; eIF, eukaryotic initiation factor; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; STNV, satellite tobacco necrosis virus; AMV, alfalfa mosaic virus.

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EXPERIMENTAL PROCEDURES

Materials—The S30 supernatant (prepared in 120 mM KCl), high-salt washed ribosomes, 40-70% ammonium sulfate fraction from the postribosomal supernatant, and highly purified preparations of eIF-2, eIF-3, and eIF-4A were obtained from wheat germ as described previously (9), eIF-4F and eIF-4B were purified by the procedure of Browning et al. (10), eIF-4C was purified by the following procedure. A strain of Escherichia coli carrying the TT RNA polymerase gene on a plasmid (kindly supplied by Dr. J. J. Dunn, Brooklyn National Laboratories).

Construction of cDNA Clones for Rabbit b-Globin mRNA, AMV RNA 4, and STNV RNA—The plasmid containing the cDNA for STNV RNA (pSTNViso) was constructed as described previously (13). This construct contains ten extra bases at the 5' end of the transcribed mRNA (see Fig. 1). Rabbit globin mRNA was isolated as described previously (14); the CDNA was prepared and the subcloning was carried out as described previously (13). The oligonucleotides for first strand synthesis (5'TTTTTTTTTTTTTTTTTTTTTCGAACCGC') and for second strand synthesis contain a HindIII restriction site (underlined), the TT RNA polymerase promoter (underlined), and a phosphocellulose column equilibrated in buffer B (20 mM Hepes, KOH, pH 7.6, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol) containing 100 mM KCl. The column was developed with the same buffer, l-ml fractions were collected, and the fractions containing eIF-4C eluted from the column between 550 and 650 mM KCl. Fractions containing eIF-4C as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (generally six fractions) were pooled, concentrated in an Amicon concentrator equipped with a YM-10 membrane, and then dialyzed in Spectropore tubing (M, cut 12,000-14,000) against buffer B containing 100 mM KCl.

T-RNA polymerase was purified as described previously (12) from an Escherichia coli strain carrying the TT RNA polymerase gene on a plasmid. The DNA sequence across the cloning junctions was confirmed by DNA sequencing. This construct adds two extra nucleotides at the 5' end of the transcribed mRNA (Fig. 1). A plasmid, pSP65A4, containing the AMV RNA 4 sequence (15) was digested with EcoRI and Smal, subcloned into the EcoRI and Smal sites of pUC18, and designated pAMV4. This construct contains eight extra nucleotides at the 5' end of the transcribed mRNA (Fig. 1).

Preparation of Capped and Uncapped b-Globin mRNA, AMV RNA 4, and STNV RNA—Viro Transcription—Capped mRNA were prepared in a 0.5 ml reaction mixture containing: 40 mM Tris/HCl, pH 8.3, 10 mM DTT, 8 mM MgCl2, 1 mM cap analog (mG'5'ppp5'G), 0.4 mM GTP, 1 mM UTP, 1 mM CTP, 1 mM [α-32P]ATP (25-50 cpn/pmol), 4 mM spermidine, 1000 units of RNasin (Pharmacia LKB Biotechnology Inc. or Promega), 11 μg of T-RNA polymerase, and 30-50 μg of either pβ-HB DNA linearized with HindIII, pAMV4 linearized with Smal, or pSTNViso linearized with BamHI. To prepare uncapped mRNAs, 1 mM GMP was substituted for the cap analog in the reaction mixture. After incubation for 60 min at 37 °C, 10 units of RQ DNase (Promega) were added, and the incubation was continued for 15 min. The reaction mixture was extracted once with phenol/chloroform and once with chloroform, applied to a 20-ml G-100 (DNA grade, Pharmacia) column equilibrated with 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, 150 mM KCl. The column was developed with the same buffer, 1-ml fractions were collected, and the fractions containing mRNA (generally fractions 9-11) were pooled. The pooled fractions were precipitated with ethanol at -20 °C for 2 h, collected by centrifugation, washed with 80% ethanol, dissolved in 0.2 ml of sterile water, and stored at -70 °C. The amount of mRNA obtained was determined from the absorbance at 260 nm, assuming 25 A_{260} units is equivalent to 1 mg/ml. Approximately 200-400 μg of the capped or uncapped mRNAs (containing 3000-6000 cpn/pmol) were obtained by this procedure.

Electrophoretic analysis on 8 M urea, 4% polyacrylamide gels showed that the mRNAs migrated to single bands corresponding to rabbit globin mRNA, viral STNV RNA, and viral AMV RNA 4. To determine the percent of mRNA that was capped, mRNA was synthesized with [α-32P]GTP instead of [α-32P]ATP and was completely digested with a mixture of RNase T1, T2, and A at 50 °C for 1 h. The digestion products were separated by electrophoresis on DEAE paper and the percent capping was determined (16). The mRNAs were found to be greater than 70% capped.

Translation Assays—The S30 system described previously (17) was modified to contain 90 mM KOAc, 40 mM KCl, 2 mM Mg(OAc)2, 10 μl of S30 supernatant, 5 μg of eIF-3, and 25 units of RNasin (Pharmacia or Promega). The partially purified assay system described previously (17) was modified to contain 90 mM KOAc, 40 mM KCl, 2.5 mM Mg(OAc)2, 180 μg of 40-70% ammonium sulfate fraction, 6 μg of eIF-4C, 5 μg of eIF-3, 3.5 μg of eIF-4F, 8 μg of eIF-4A, and 1.2 μg of eIF-4B. Incubation was for 30 min at 27 °C.

Binding of mRNA to 40 S Ribosomal Subunits—Binding of mRNA to 40 S ribosomal subunits was measured by sucrose density gradient (10-30%) analysis described previously (13). The reaction mixture was modified to contain 90 mM KOAc, 40 mM KCl, 3 μg of eIF-2, 13 μg of eIF-3, 12 μg of eIF-4A, 2 μg of eIF-4B, and 4 μg of eIF-4F.

RESULTS

Capped and uncapped β-globin mRNA, AMV RNA 4, and STNV RNA were prepared by in vitro transcription of the plasmid constructs described under "Experimental Procedures." The nucleotide sequences of the 5' ends of the RNA transcripts are shown in Fig. 1. The abilities of the mRNAs to direct polypeptide synthesis in a crude S30 system from wheat germ are shown in Fig. 2. In this system polypeptide synthesis directed by capped β-globin mRNA increased linearly with increasing amounts of capped β-globin mRNA and increased linearly with time up to about 45 min. Under the same conditions the amount of polypeptide synthesis obtained with uncapped β-globin mRNA was less than 10% of that obtained with capped β-globin mRNA. Similar results were obtained with AMV RNA 4. The amount of polypeptide synthesized in the presence of uncapped AMV RNA 4 was only 10-15% of that obtained in the presence of capped AMV RNA 4. In contrast to the results obtained with β-globin mRNA and AMV RNA 4, the amount of polypeptide synthesis obtained with uncapped STNV RNA was the same as that obtained with capped STNV RNA, and the rates of incorporation were the same.

When the abilities of the capped and uncapped mRNAs to direct polypeptide synthesis were measured in a partially purified system from wheat germ, the results shown in Fig. 3 were obtained. The fractionated system contained purified eIF-3, eIF-4A, eIF-4B, eIF-4F, eIF-4C, and a small amount of 40-70% ammonium sulfate fraction sufficient to provide aminoacyl-tRNA synthetases, eIF-2, eIF-5, and elongation factors. In this system uncapped β-globin mRNA was about 40% as active as capped β-globin mRNA. Uncapped AMV RNA 4 was 40-50% as active as capped AMV RNA 4 and uncapped and capped STNV RNA were equally active. Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the products synthesized in the partially purified system (labeled with [35S]Met instead of [14C]Leu) showed that the polypeptides synthesized in the presence of uncapped mRNAs were the same size as those synthesized in the presence of the capped mRNAs (data not shown).

The results described above suggested that uncapped β-globin mRNA and uncapped AMV RNA 4 were being inactivated in the S30 system. When the ATP-labeled mRNAs were incubated for 10 min in the S30 system lacking amino acids and then precipitated with cold trichloroacetic acid, 80-90% of the ATP was recovered in the precipitate (data not shown). When the mRNAs were incubated in the S30 system,
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**FIG. 1.** Nucleotide sequence of the 5' ends of \(\beta\)-globin mRNA, AMV RNA 4, and STNV RNA transcripts. Nucleotides added to the 5' end during the cloning procedures are in parentheses. The numbers at the right in parentheses are references.

\[\beta\text{-globin} \quad m^7\text{Gppp(GG)ACACUUGCUUUUGACAC~CUGUGUUUACUUGCAAUCCCC~AACAGACAGA~GUG... (16)\]  
AMV 4 \[m^7\text{Gppp(GGG~UUC)GUUUUUAUUUUU~UUUUCUUUC~AUACUUCCAUC~AGU... (19)\]  
STNV \[p(GGGAAAGCUU)AGUAAAGACAGGAAACUUUACUGACUAAC~mGCA... (20, 21)\]

**FIG. 2.** Translation of capped and uncapped \(\beta\)-globin mRNA, AMV RNA 4, and STNV RNA in an S30 system from wheat germ. In the panels on the left, the S30 system described under "Experimental Procedures" was supplemented with capped (O- - - O) or uncapped (O- - - O) mRNAs, in the amounts indicated. In the panels on the right, the S30 system was supplemented with 10 pmol of \(\beta\)-globin mRNA, 5 pmol AMV RNA 4, or 5 pmol of STNV RNA and was incubated for the times indicated.

**FIG. 3.** Translation of capped and uncapped \(\beta\)-globin mRNA, AMV RNA 4, and STNV RNA in a partially purified system from wheat germ. In the panels on the left, the partially purified system described under "Experimental Procedures" was supplemented with capped (O- - - O) or uncapped (O- - - O) mRNA, in the amounts indicated. In the panels on the right, the partially purified system was supplemented with 10 pmol of \(\beta\)-globin mRNA, 5 pmol of AMV RNA 4, or 5 pmol of STNV RNA and was incubated for the times indicated.

recovered by phenol extraction, and analyzed by electrophoresis on a 4% polyacrylamide, 6 M urea gel, the mRNAs appeared to be intact, indicating that gross degradation had not occurred. However, when the mRNAs treated in this manner were tested for their ability to support polypeptide synthesis in the partially purified system, the results shown in Table I were obtained. Treated capped \(\beta\)-globin mRNA was about 90% as active as untreated capped \(\beta\)-globin mRNA, whereas treated uncapped \(\beta\)-globin mRNA was only 20% as active as untreated uncapped \(\beta\)-globin mRNA. Similarly, treated capped AMV RNA 4 was 90% as active as the untreated capped AMV RNA 4, and treated uncapped AMV RNA 4 was only 27% as active as the untreated uncapped AMV RNA 4. Treated and untreated uncapped STNV RNA were equally active. These data showed that inactivation of the uncapped forms of \(\beta\)-globin mRNA and AMV RNA 4 occurred when the mRNAs were incubated in the S30 system. It is not known whether inactivation was due to removal of a small number of bases which would not be detected by the procedures described above or to some other modification of the mRNAs.

A comparison was made of the abilities of the capped and uncapped mRNAs to bind to 40 S ribosomal subunits in a system containing \[^{35}S\]Met-tRNA and highly purified initiation factors, eIF-2, eIF-3, eIF-4A, eIF-4B, and eIF-4F. The results given in Table II show that uncapped \(\beta\)-globin mRNA was 50–60% as active as capped \(\beta\)-globin mRNA. Uncapped AMV RNA 4 was about 70% as active as capped AMV 4 RNA, and capped and uncapped STNV RNA were equally active. These data indicate that the difference in the ability of capped and uncapped \(\beta\)-globin mRNA or AMV RNA 4 to support polypeptide synthesis in the partially purified translation system is due, primarily, to a difference in ability to bind to 40 S ribosomal subunits.

The amounts of eIF-3, eIF-4A, eIF-4B, and eIF-4F required for translation of the capped and uncapped mRNAs were determined in the partially purified polymerization system, and the results obtained are shown in Figs. 4–6. The concentrations of the factors required for half-maximal translation are given in Table III. These values were obtained from double-reciprocal plots of the data in Figs. 4–6. The double-reciprocal plots of the responses to eIF-4F are shown in Fig. 7. In the case of STNV RNA the concentrations of eIF-3, eIF-4A, eIF-4B, and eIF-4F required for half-maximal translation were the same for the uncapped and capped forms of this RNA. Also, there were no significant differences in the concentrations of eIF-3 required for half-maximal translation of
TABLE I
Effect of incubation in the S30 system on the activity of capped and uncapped mRNAs

| mRNA     | Activity | Untreated | Incubated | Percent |
|----------|----------|-----------|-----------|---------|
| β-Globin    |          |           |           |         |
| Capped  | 16       | 14        | 88        |         |
| Uncapped | 10       | 2         | 20        |         |
| AMV 4     |          |           |           |         |
| Capped  | 18       | 17        | 94        |         |
| Uncapped| 11       | 3         | 27        |         |
| STNV      |          |           |           |         |
| Capped  | ND       | ND        | 96        |         |
| Uncapped| 28       | 27        |           |         |

*Each of the mRNAs (~100 pmol) were incubated for 10 min at 27 °C in 0.5 ml of the S30 incubation mixture lacking amino acids. The reaction mixture was extracted with phenol and the RNA was precipitated with ethanol, washed, dried, and suspended in 50 μl of sterile water. The concentration of mRNA was calculated from the amount of 32P recovered. The mRNAs were then assayed for the ability to support polypeptide synthesis in the partially purified system. The values given are picomoles of leucine incorporated into polypeptide per pmol of mRNA.

TABLE II
Binding of capped and uncapped mRNAs to 40 S ribosomal subunits

| mRNA     | Amount added | Amount bound | Percent of capped |
|----------|--------------|--------------|------------------|
|          | pmol         | Capped pmol  | Uncapped pmol    |         |
| β-Globin    | 6            | 1.2          | 0.6              | 50      |
|            | 12           | 1.6          | 0.9              | 56      |
| AMV 4     | 6            | 0.92         | 0.66             | 72      |
|            | 12           | 1.3          | 0.86             | 66      |
| STNV      | 0            | 1.2          | 1.3              | 108     |
|            | 12           | 2.0          | 1.9              | 95      |

TABLE III
The concentrations of eIF-3, eIF-4A, eIF-4B, and eIF-4F required for the translation of capped and uncapped mRNAs

| mRNA     | eIF-3 | eIF-4A | eIF-4B | eIF-4F |
|----------|-------|--------|--------|--------|
| β-Globin    |       |        |        |        |
| Capped  | 10    | 1000   | 90     | 10     |
| Uncapped | 12    | 1400   | 160    | 60     |
| AMV 4     |       |        |        |        |
| Capped  | 27    | 750    | 26     | 5      |
| Uncapped| 25    | 1700   | 22     | 60     |
| STNV      |       |        |        |        |
| Capped  | 34    | 450    | 24     | 3      |
| Uncapped| 33    | 450    | 26     | 3      |

*The concentrations required for half-maximal translation were obtained from double-reciprocal plots of the data in Figs. 4, 5, and 6, given the following M₀: eIF-3, 700,000; eIF-4A, 50,000; eIF-4B, 59,000; eIF-4F, 330,000 (9).
STNV (capped or uncapped) are not very different, for half-maximal translation of capped AMV RNA and concentrations of eIF-4F (data not shown). As shown above translation of the uncapped forms was overcome by higher AMV RNA was inhibited by m7GTP. Inhibition of the translation of the uncapped forms of β-globin mRNA and the results are shown in Fig. 8. The effects of m7GTP on the translation of the capped forms of these mRNAs carried at different concentrations of eIF-4F are shown in the panels on the left. At a low concentration of eIF-4F (5 nM), the concentration of m7GTP required to inhibit translation 50% was 5 μM for β-globin mRNA and 10 μM for AMV RNA 4. At a 4-fold higher concentration of eIF-4F (20 nM), the concentration of m7GTP required to obtain 50% inhibition increased to approximately 20 μM for capped β-globin mRNA and to approximately 100 μM for capped AMV RNA 4. Increasing the concentration of eIF-4F not only increased the concentration of m7GTP required for 50% inhibition, it also decreased the maximal inhibition obtained at saturating amounts of m7GTP. With AMV RNA 4 increasing the concentration of eIF-4F from 5 to 20 nM decreased the maximum inhibition from 90 to 50%. When the concentration of eIF-4F was increased to 40 nM, the maximal inhibition obtained was only about 40%. The data in the panels on the right show that the translation of the uncapped forms of β-globin mRNA and AMV RNA 4 was inhibited by m7GTP. Inhibition of the translation of the uncapped forms was overcome by higher concentrations of eIF-4F (data not shown). As shown above (Fig. 7 and Table III) the concentrations of eIF-4F required for half-maximal translation of capped AMV RNA 4 and STNV (capped or uncapped) are not very different, 5 versus 3 nM, respectively. However, as shown in Fig. 8, at 5 nM eIF-4F close to the Kₐ for both of these mRNAs, there was a marked difference in the inhibitory effect of m7GTP. The translation of STNV RNA was inhibited less than 20% at 50 μM m7GTP, whereas translation of AMV RNA 4 was inhibited more than 80%.

**Discussion**

In this investigation we find that uncapped β-globin mRNA and uncapped AMV RNA 4 are much less active (≤10%) than their capped counterparts in directing polypeptide synthesis in a crude S30 system from wheat germ, whereas capped and uncapped STNV RNA are equally active. The low activity of uncapped β-globin mRNA and AMV RNA 4 in the S30 system is due, at least in part, to rapid inactivation of these mRNAs. The uncapped forms of β-globin mRNA and AMV RNA 4 are also less active than their capped forms when translation is carried out in a partially purified system from wheat germ in which very little inactivation of the uncapped mRNAs occurs. In contrast to the results obtained with the naturally capped mRNAs, the naturally uncapped mRNA, STNV RNA, is not rapidly inactivated in the crude S30 system, and the capped and uncapped forms of this mRNA are equally active in the crude and purified systems.

Additional studies carried out in a partially purified system show that the difference in the activities of the capped and uncapped forms of β-globin mRNA and AMV RNA 4 is due primarily to the amounts of eIF-4F required. For β-globin mRNA the absence of the 5′ cap structure increases the concentration of eIF-4F required for half-maximal translation about 6-fold (from 10 to 60 nM), and for AMV RNA 4 it increases the concentration of eIF-4F required about 12-fold (from 5 to 60 nM). At infinitely high concentrations of eIF-4F the maximal translational velocities of the uncapped forms approach those of the capped forms. The concentrations of eIF-3, eIF-4A, and eIF-4B required for half maximal translation of uncapped forms of β-globin mRNA and AMV RNA 4 are either the same or only slightly higher (1.5- to 2-fold) than the concentrations required for the capped forms. With STNV RNA the concentrations of eIF-4F, as well as eIF-3, eIF-4A, and eIF-4B, required for translation are the same for the capped and uncapped forms. These data show that the 5′ cap structures of these mRNAs...
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