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Regulation of Brome Mosaic Virus Gene Expression by Restriction of Initiation of Protein Synthesis

JADWIGA CHROBOCZEK,* LUDMIŁA PUCHKOVA,† AND WŁODZIMIERZ ZAGÓRSKI

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-532 Warsaw, Poland

The translation of total and individual brome mosaic virus (BMV) RNAs was examined in a wheat germ cell-free system in the presence of various inhibitors. Inhibitors of the initiation of polypeptide synthesis, e.g., potassium ions, 7-methylguanosine 5'-monophosphate, and aurintricarboxylic acid, were shown not only to inhibit overall BMV protein synthesis but also to change the ratio of BMV polypeptides synthesized. Under conditions restrictive for initiation, the translation of nonstructural BMV genes was suppressed, but coat protein synthesis proceeded at a high rate. A similar discrimination among BMV messengers was exerted by a regulatory protein kinase isolated from wheat germ. These results suggest that the regulation of the expression of BMV genes is based on a difference in the mechanism of formation of initiation complexes for individual BMV messages.

Investigation of the in vitro translation of total mRNA's derived from plant viral multipartite genomes allows us to study the simultaneous translation of a population of messages. It creates the situation similar to that encountered in normal cells, where many messengers are interplaying at the same time. One of the best-characterized plant viruses with a divided genome is brome mosaic virus (BMV) (9). The total RNA extracted from BMV virions is composed of four monocistronic messengers, designated RNA 1, RNA 2, RNA 3, and RNA 4, in the order of decreasing molecular weight (6). All of these mRNA's contain a cap structure on their 5' terminus (6, 16). These RNAs are translated in vitro into a collection of four polypeptides: 1a, 2a, 3a, and 4a (23). Polypeptide 4a is a coat protein, and the other polypeptides are nonstructural viral polypeptides (6).

It is clear that the ratio of synthesis of nonstructural proteins should be variable during viral development (3, 6, 22). At an early phase, nonstructural proteins thought to be involved in RNA replication accumulate, whereas at a late phase, synthesis of coat protein required for capsid organization dominates.

It is accepted that the regulation of the formation of individual BMV proteins occurs on the translational level (6). However, the particular mechanism responsible for this regulation is unknown. In this paper we demonstrate that upon restriction of the initiation of polypeptide synthesis, the formation of coat protein prevails over nonstructural BMV proteins. From our results, it seems that the mechanism of regulation of BMV protein synthesis is similar to that described by Lodish for globin chain formation (10).

† Permanent address: Institute of Experimental Medicine, 197022 Leningrad, USSR.

MATERIALS AND METHODS

7-Methylguanosine 5'-monophosphate ($m^7G^{5'}p$), sodium salt, was from P-L Biochemicals, Inc., Milwaukee, Wis.; aurintricarboxylic acid (ATA), ammonium salt, was from Sigma Chemical Co., St. Louis, Mo.; cycloheximide, B grade, was from Calbiochem, La Jolla, Calif.; [^{14}C]leucine (specific activity, 211 mCi/mmol) was from UVVVR, Prague, Czechoslovakia.

The preparation of a cell-free system from wheat germ and tRNA from wheat germ, the isolation of total BMV RNA, and radioactivity measurements were performed by methods described previously (22). Individual BMV RNAs were separated on a sucrose gradient by the method of Stubbs and Kaesberg (18). Preparations of RNA 4 and RNA 3 were shown to be electrophoretically pure. RNA 1 and RNA 2 do not separate on a sucrose gradient, and they were used as a mixture, referred to as RNA 1+2. This mixture was shown to be electrophoretically free from other RNAs and contained equimolar amounts of RNA 1 and RNA 2. Preparations of total BMV RNA contained individual components in a molar ratio of 1:1:4:4.

The translation of BMV total and individual RNAs was performed in the wheat germ unfractionated system. Details are given in the legend to Fig. 1. Translation products were analyzed by electrophoresis on 12% polyacrylamide slab gels containing sodium dodecyl sulfate by the method of Laemmli (8). This was followed by fluorography by the method described by Bonner and Laskey (4). Polish blue base X-ray film produced by Foton, Warsaw, Poland, was used. When necessary, the quantitation of radioactive products was performed by densitometry.

RESULTS

One of the best-known inhibitors of initiation in eucaryotic cell-free protein-synthesizing systems is $m^7G^{5'}p$. It is thought to be a competitive inhibitor of the 5'-terminal cap structure present in many eucaryotic mRNA's (16, 19). The inhibitory effect of the cap analog was shown to depend on the potassium ion concentration in the wheat germ translation system primed with vaccinia mRNA (21). Thus, before we investigated the effect of $m^7G^{5'}p$ on BMV RNA translation, we studied the effect of potassium ions on protein synthesis in the presence of total and individual BMV RNAs (Fig. 2). Maximum translation of total BMV RNA was achieved at about a 90 mM concentration of potassium in the system, and the same occurred for RNA 3 and RNA 1+2. On the other hand, maximum incorporation in the presence of RNA 4 was shifted to a higher potassium ion concentration (about 110 mM).

It is believed that the most sensitive step arrested by a higher-than-optimal concentration of K^+ ions is the initiation of polypeptide synthesis (15, 20). This notion is also supported by observations that polyuridylic acid-directed

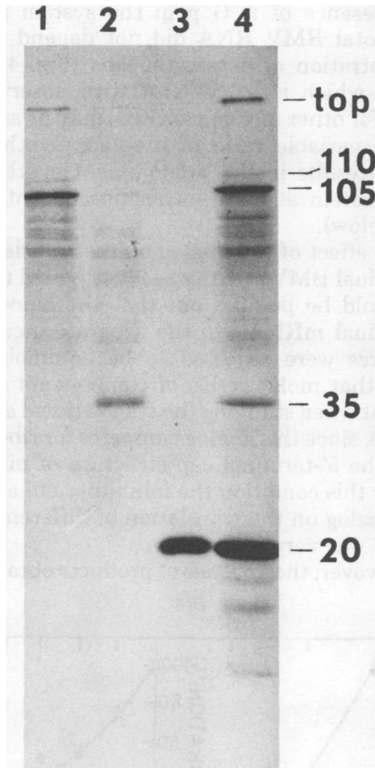

FIG. 1. *Analysis of products of the translation of BMV RNAs in the cell-free wheat germ system. The incorporation mixture (25 or 50 µl) contained 3 mM magnesium acetate, 95 mM potassium acetate, 4.8 mM Tris-acetate (pH 7.6), 60 µM spermine tetrachloride, 0.48 mM dithiothreitol, 1 mM ATP, 0.375 mM GTP, 20 mM creatine phosphate, 125 µg of creatine kinase per 25 µl, 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-KOH buffer (pH 7.6), 125 µM unlabeled amino acids, 0.1 µCi of [^{14}C]leucine (specific activity, 211 mCi/mmol), 12 µl of extract per 25 µl (30 mg/ml), and the indicated amounts of BMV RNA. With buffer and salt concentrations, the contribution of the extract is taken into account. Translation was performed with the following: 1.5 µg of RNA 1+2 per 25 µl (lane 1); 1 µg of RNA 3 per 25 µl (lane 2); 0.75 µg of RNA 4 per 25 µl (lane 3); and 2 µg of total BMV RNA per 25 µl (lane 4). Samples of 2 to 10 µl containing 3,300 cpm for lane 1, 2,600 cpm for lane 2, 3,700 cpm for lane 3, and 11,000 cpm for lane 4 were submitted to electrophoresis. A molecular weight estimation was performed by comparing positions of products with the following molecular weight markers: cytochrome c, chymotrypsinogen A, ovalbumin, bovine serum albumin, and Escherichia coli polymerase subunits (M_r, 12,400, 25,000, 45,000, 67,000, 39,000, 155,000, and 165,000, respectively). Labels on the right indicate $M_r \times 10^{-3}$.*

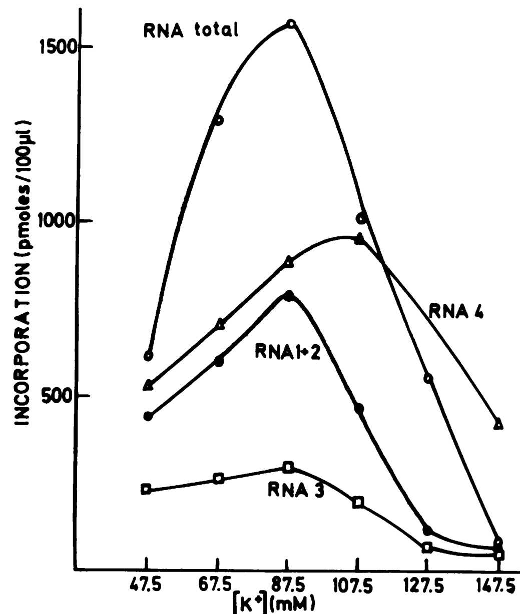

FIG. 2. *Effect of potassium ion concentrations on the translation of BMV RNAs in the wheat germ cell-free system. The translation was performed in 25 µl as described in the legend to Fig. 1. The concentration of potassium ions was adjusted to obtain values indicated on the abscissa. The concentrations of RNAs were as follows: total RNA, 2 µg; RNA 1+2, 1.2 µg; RNA 3, 1 µg; RNA 4, 1 µg.*

translation, which can be taken as a measure of elongation processes, is optimal at a 170 mM potassium ion concentration (13) and that when the translation of a natural message is initiated at a low potassium ion concentration and continued at 170 mM K^+, it proceeds at a high rate, but no chain reinitiation occurs (17). Therefore, a supraoptimal potassium ion concentration may be regarded as inhibitory for initiation, and according to the model proposed by Lodish (10), it may affect the ratio of viral polypeptides formed in the cell-free system primed with total BMV RNA.

This supposition was confirmed by an analysis of products formed at different potassium ion concentrations (Fig. 3). The synthesis of coat protein increased with potassium ion concentrations of up to 107 mM, whereas the synthesis of longer polypeptides was already inhibited at this potassium ion concentration. At 127 mM potassium, there was virtually no product other than coat protein. It is noteworthy that a decreased synthesis of full-length viral proteins was not associated with an increased appearance of unfinished polypeptides. It confirms the supposition that initiation, but not polypeptide elongation, is affected at supraoptimal potassium ion concentrations.

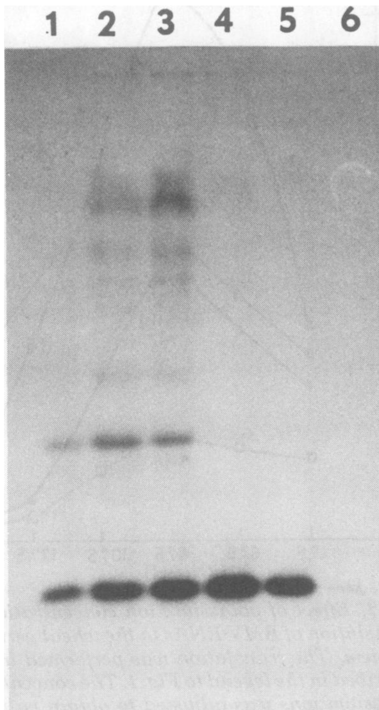

FIG. 3. *Effect of potassium ion concentration on the products of the translation of total BMV RNA. The translation was performed as described in the legend to Fig. 1, with 2 μg of total RNA per 25 μl. Lane 1, 47.5 mM K^+; lane 2, 67.5 mM K^+; lane 3, 87.5 mM K^+; lane 4, 107.5 mM K^+; lane 5, 127.5 mM K^+; lane 6, 147.5 mM K^+.*

Overall inhibition of protein synthesis due to the presence of $m^7G^{5'}p$ in the system primed with total BMV RNA did not depend on the concentration of potassium ions (Fig. 4). This result, which is in contrast with observations made for other messengers (21), may be ascribed to the variable ratio of products synthesized, which results in the predominant synthesis of coat protein at high potassium concentrations (see below).

The effect of cap analog on the translation of individual BMV RNAs was investigated (Fig. 5). It should be pointed out that the amounts of individual mRNA's in the separate incubation mixtures were adjusted to be equimolar. We think that molar ratios of components are important when studying the translational effect of $m^7G^{5'}p$, since this analog competes for ribosomes with the 5'-terminal cap structure of mRNA's. Under this condition the inhibitory effect of the cap analog on the translation of different BMV RNAs was very similar.

However, the analysis of products obtained by

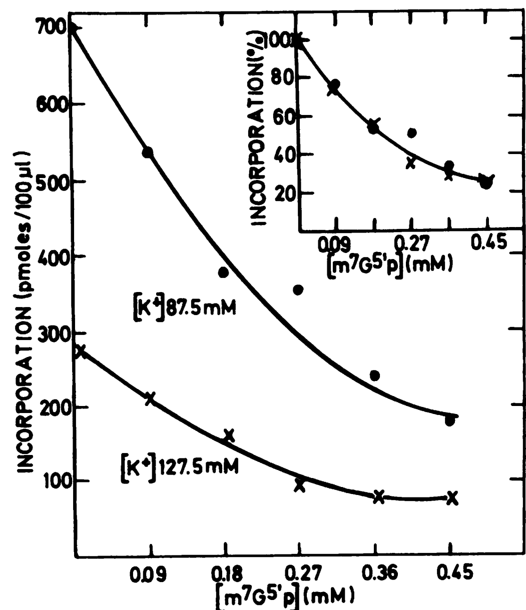

FIG. 4. *Inhibition of protein synthesis primed with total BMV RNAs by $m^7G^{5'}p$. The translation was performed as described in the legend to Fig. 1, at 87.5 and 127.5 mM potassium ion concentrations. Mixtures were supplemented with 2 μg of total BMV RNA in each 25 μl and with the indicated amount of $m^7G^{5'}p$.*

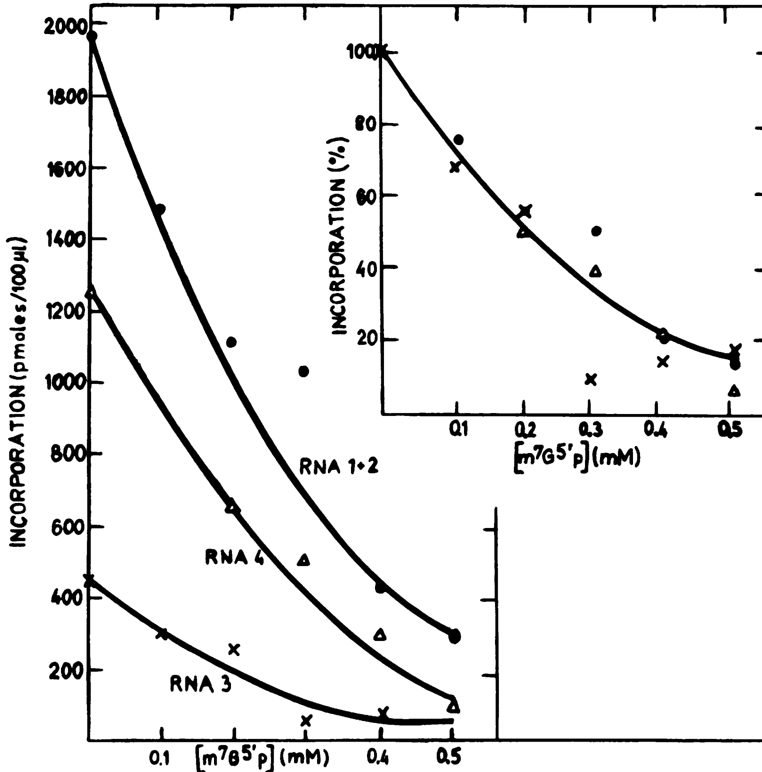

FIG. 5. *Effect of $m^7G^{5'}p$ on the translation of individual BMV RNAs. The incubation was performed as described in the legend to Fig. 1, with a 90 mM potassium ion concentration. Incubation mixtures (25 µl) were supplemented, respectively, with equimolar amounts of RNAs as follows: RNA 1+2, 4.2 µg; RNA 3, 2 µg; and RNA 4, 1 µg.*

the translation of total BMV RNA indicated that upon the addition of increasing amounts of $m^7G^{5'}p$ to the system, the proportions of products were changed (Fig. 6). When equal amounts of hot trichloroacetic acid-precipitable material from the incorporation performed in the presence of increasing amounts of analog were analyzed by gel electrophoresis and subsequent fluorography, it could be seen that the synthesis of nonstructural polypeptides 1a, 2a, and 3a decreased, with a parallel increase in the synthesis of coat protein. At high potassium ion concentrations, the complete exclusion of longer polypeptides synthesized upon the addition of increasing amounts of $m^7G^{5'}p$ could be observed. The restriction of initiation, which was due to the presence of the cap analog, resulted in the exclusion of the translation of nonstructural genes. The effect was reinforced by supraoptimal potassium ion concentrations, the factor which was additionally restrictive toward initiation. In conclusion, it seems that the addition of an inhibitor of initiation to the system affects strongly not only the overall translation but also the type of polypeptide synthesized in the system primed with a population of messages.

This supposition was reinforced by experiments performed with another inhibitor of initiation, ATA. It is believed that at low concentrations, ATA acts as a selective inhibitor of the initiation of polypeptide synthesis (11) by blocking the binding of mRNA to ribosomes (5). We observed that upon the addition of increasing amounts of ATA, the synthesis of product 1a+2a decreased to 42.3%, and later to 5.8%, of the initial synthesis. Simultaneously, the formation of product 3a decreased to 18.3 and 4.7%, respectively; on the other hand, coat protein synthesis was diminished early to 74% and later to 21.8%, respectively (Fig. 7, lanes 1, 2, and 3). The restriction of initiation caused by ATA therefore led to the same effect as the addition of potassium ions or the cap analog or both to the system.

On the other hand, the differentiation of BMV polypeptide synthesis was not induced by the presence of cycloheximide in the system. Cycloheximide is a potent inhibitor of protein synthe-

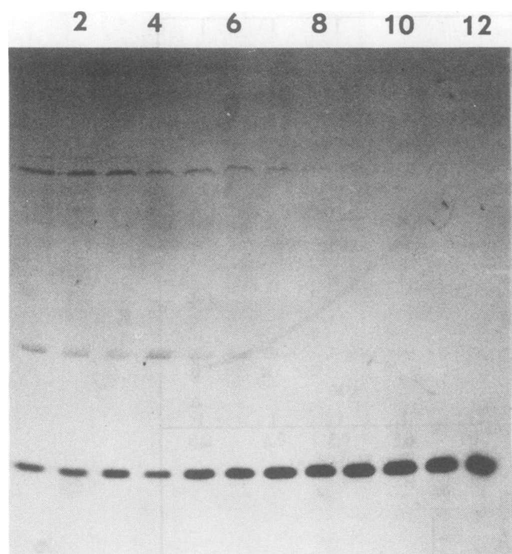

FIG. 6. *Effect of $m^7G^{5'}p$ on the translation products directed by total BMV RNA. The incubation was performed as described in the legend to Fig. 1. Equal amounts of hot trichloroacetic acid-precipitable radioactive material were subjected to electrophoresis and fluorography. Potassium ion concentrations were 87 mM for lanes 1 to 6 and 127 mM for lanes 7 to 12. Concentrations of $m^7G^{5'}p$ were as follows: lanes 1 and 7, none; lanes 2 and 8, 0.09 mM; lanes 3 and 9, 0.18 mM; lanes 4 and 10, 0.27 mM; lanes 5 and 11, 0.36 mM; lanes 6 and 12, 0.45 mM.*

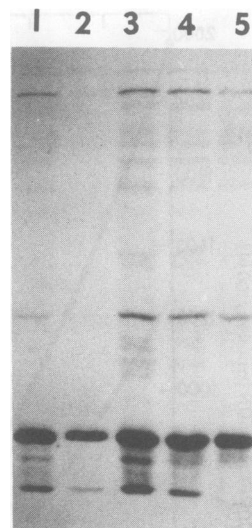

FIG. 7. *Effect of ATA and cycloheximide on translation products directed by total BMV RNA. The translation was performed as described in the legend to Fig. 1, at a 90 mM potassium ion concentration and with 3 μg of total BMV RNA per 50 μl. Lane 1, 80 μM ATA; lane 2, 120 μM ATA; lane 3, no ATA and no cycloheximide added; lane 4, 24 μM cycloheximide; lane 5, 28 μM cycloheximide.*

sis, which arrests translocation (2) and therefore diminishes the rate of elongation. The addition of cycloheximide to the wheat germ system primed with total BMV RNA resulted in an even inhibition of the formation of all BMV polypeptides (Fig. 7, lanes 4 and 5), reaching about 50% at higher cycloheximide concentrations.

It is clear from the above results that upon restriction of initiation, nonstructural viral messages are excluded from translation, whereas the coat protein gene is expressed at a high rate. We observed that such a restriction can also be imposed by the specific component(s) of the plant extract, e.g., the regulatory protein kinase isolated by us that is able to phosphorylate some ribosome-associated proteins, presumably initiation factors (12). The addition of this protein kinase to the incorporation system diminished the rate of association of nonstructural messengers with ribosomes, but did not affect the association of BMV RNA 4 with ribosomes (14). The wheat germ cell-free system supplemented with protein kinase still expresses all four BMV messengers, but whereas the formation of the coat protein is not inhibited, the formation of longer polypeptides in the presence of the enzyme is diminished to about 50% (Fig. 3 from reference 12).

DISCUSSION

The differential translation of structural versus nonstructural viral messengers is observed under conditions restrictive for chain initiation. This means that the mechanism of initiation for these two classes of messengers should be significantly different.

According to Kozak and Shatkin (7), the initiation of capped messengers occurs in two steps. First, a ribosome recognizes the 5′-terminal cap structure and then moves along the mRNA strand, searching for an AUG initiation codon. If the distance between the cap structure and the AUG codon is large enough, a second ribosome can recognize the cap structure before the translation starts and can enter the untranslated region. This results in the formation of an initiation complex with two ribosomes. It is clear that when the cap structure is near the AUG initiation codon, both regions will be covered by one ribosome, and the initiation complex will contain a single ribosome.

It is known that in BMV RNA 4 the 5′-terminal cap structure is separated by 10 nucleotides from the initiation codon (6), and we found that, as is expected, this RNA forms initiation complexes with single ribosomes (data not

shown). In contrast to that, the initiation complexes formed with RNA 1+2 at low messenger concentrations contain not only monosomes but also disomes (data not shown). This is in agreement with a recent communication from Ahlquist et al. in which they demonstrated that in initiation complexes formed with RNA 3, two ribosomes are simultaneously present on one RNA molecule (1).

Therefore, it may be that the translation of nonstructural viral messages (RNA 1, RNA 2, and RNA 3) is initiated by a mechanism different from that operating during the initiation of coat protein synthesis. It is probably this difference which is revealed by the use of inhibitors of initiation, such as $m^7G^{5'}p$, K^+ ions and ATA.

This difference may also account for the specific effect of the wheat germ protein kinase on the translation of viral mRNA's (12). It can be speculated that protein kinase is a natural host cell inhibitor, which plays a part in turning on and off the expression of viral genes at different stages of the viral life cycle.

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LITERATURE CITED

1. **Ahlquist, P., R. Dasgupta, D. S. Shih, D. Zimmern, and P. Kaesberg.** 1979. Two-step binding of eukaryotic ribosomes to brome mosaic virus RNA 3. Nature (London) **281**:277-281.
2. **Barbacid, M., M. Fresno, and D. Vazquez.** 1975. Inhibitors of polypeptide elongation on yeast polysomes. J. Antibiot. **28**:453-462.
3. **Benicourt, C., and A.-L. Haenni.** 1978. Differential translation of Turnip Yellow Mosaic Virus mRNAs *in vitro*. Biochem. Biophys. Res. Commun. **84**:831-839.
4. **Bonner, W. M., and R. A. Laskey.** 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur. J. Biochem. **46**:83-88.
5. **Huang, M. T., and A. P. Grollman.** 1972. Effects of aurintricarboxylic acid on ribosomes and the biosynthesis of globin in rabbit reticulocytes. Mol. Pharmacol. **8**:111-112.
6. **Kaesberg, P.** 1976. Structure and function of the RNAs of Brome Mosaic Virus. Prog. Nucleic Acid Res. Mol. Biol. **19**:465-471.
7. **Kozak, M., and A. J. Shatkin.** 1978. Migration of 40 S ribosomal subunits on messenger RNA in the presence of edeine. J. Biol. Chem. **253**:6568-6577.
8. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.
9. **Lane, L. C.** 1974. The bromoviruses. Adv. Virus Res. **19**: 152-220.
10. **Lodish, H. F.** 1974. Model for the regulation of mRNA translation applied to haemoglobin synthesis. Nature (London) **251**:385-388.
11. **Marcus, A., J. D. Bewley, and D. P. Weeks.** 1970. Aurintricarboxylic acid and initiation factors of wheat embryo. Science **167**:1735-1736.
12. **Rychlik, W., E. Kupidłowska, E. Nowak, and W. Zagórski.** 1980. Wheat germ protein kinase affects the translation of Brome Mosaic Virus RNA *in vitro*. Biochemistry, in press.
13. **Rychlik, W., and W. Zagórski.** 1978. Protein synthesizing system from wheat germ. Efficient translation of synthetic and natural messages. Acta Biochim. Pol. **25**: 129-146.
14. **Rychlik, W., and W. Zagórski.** 1980. Purification and characterization of cyclic AMP-independent protein kinase from wheat germ, differentiating translation of natural templates. Eur. J. Biochem., in press.
15. **Saborio, J. L., S. S. Pong, and G. Koch.** 1974. Selective and reversible inhibition of initiation of protein synthesis in mammalian cells. J. Mol. Biol. **85**:195-211.
16. **Shatkin, A. J.** 1976. Capping of eukaryotic mRNA. Cell **9**:645-653.
17. **Shih, D., and P. Kaesberg.** 1976. Translation of the RNAs of Brome Mosaic Virus: the monocistronic nature of RNA 1 and RNA 2. J. Mol. Biol. **103**:77-88.
18. **Stubbs, J. D., and P. Kaesberg.** 1967. Amino acid incorporation in an *Escherichia coli* cell-free system directed by Bromegrass Mosaic Virus ribonucleic acid. Virology **33**:385-397.
19. **Weber, L. A., E. D. Hickey, and C. Baglioni.** 1978. Influence of potassium salt concentration and temperature on inhibition of mRNA translation by 7-methylguanosine 5'-monophosphate. J. Biol. Chem. **253**:178-183.
20. **Weber, L. A., E. D. Hickey, P. A. Maroney, and C. Baglioni.** 1977. Inhibition of protein synthesis by Cl^-. J. Biol. Chem. **252**:4007-4010.
21. **Weber, L. A., E. D. Hickey, D. L. Nuss, and C. Baglioni.** 1977. 5'-Terminal 7-methylguanosine and mRNA function: influence of potassium concentration on translation in vitro. Proc. Natl. Acad. Sci. U.S.A. **74**:3254-3259.
22. **Zagórski, W.** 1978. Preparation and characteristics of a wheat embryo cell-free extract active in the synthesis of high molecular weight viral polypeptides. Anal. Biochem. **87**:316-332.
23. **Zagórski, W.** 1978. Translational regulation of expression of the Brome Mosaic Virus RNA genome *in vitro*. Eur. J. Biochem. **86**:465-472.