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Wheat Germ Protein Kinase Affects the Translation of Brome Mosaic Virus Ribonucleic Acid in Vitro†

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ABSTRACT: Wheat germ protein kinase inhibits in vitro translation of Brome Mosaic virus (BMV) RNA 1 and 2, without affecting the translation of RNA 4. Inhibition of formation of BMV polypeptides 1a and 2a is due to the arrest of initiation of polypeptide synthesis. It was found that protein kinase inhibits the formation of the 80S initiation complex with BMV RNA 1 and 2, without affecting the formation of the initiation complex with BMV RNA 4. Inhibition of protein synthesis by wheat germ protein kinase is accompanied by the phosphorylation of two ribosome-associated polypeptides, with molecular weights of 32 000 and 76 000, respectively. Both polypeptides are readily dephosphorylated by the enzyme(s) present in the cell-free extract. Their dephosphorylation is accompanied by restoration of the translational capacity of the system.

Recently, we isolated cAMP-independent protein kinase[1] from wheat germ (Rychlik & Zagórski, 1980), which exerts an inhibitory effect on translation of some natural messengers in vitro. Here we describe in detail the effect of purified protein kinase on in vitro protein synthesis. The experiments were carried out in a wheat germ cell-free system which is homologous for the enzyme. BMV template was used since wheat is one of the natural hosts of this virus.

BMV is a multicomponent virus, and its total RNA consists of four monocistronic messengers, called in the order of their decreasing length RNA 1, 2, 3, and 4 (Lane, 1974). These messengers can be separately translated in vitro into three nonstructural polypeptides, designated 1a, 2a, 3a, and the coat protein, referred to as polypeptide 4a (Shih & Kaesberg, 1976). A mixture of all BMV RNAs, total BMV RNA, also induces in vitro the translation of these polypeptides. In this case, however, the messengers are translated with different efficiencies (Zagórski, 1978a).

In the system primed with total BMV RNA as well as in that directed by separate BMV RNAs, we observed a different expression of viral genes in the presence of wheat germ protein kinase. It is known that protein kinases (ATP:protein phosphotransferases, EC 2.7.1.37) control a variety of cellular processes [for review, see Rubin & Rosen (1975)] and are also involved in the regulation of protein synthesis. This regulatory activity depends on the phosphorylation of specific proteins. Therefore, in order to understand the mechanism by which protein kinase affects the synthesis of some BMV polypeptides, we studied the phosphorylation of various components of the protein-synthesizing machinery in the presence of enzyme.

It is known that the reticulocyte system contains a cAMP-independent protein kinase, called the hemin-controlled repressor, which inhibits translation of natural messages by phosphorylating the small subunit of the initiation factor eIF-2 [for review, see Safer & Anderson (1978)]. This leads to a diminished formation of the 40S initiation complex. Analyzing the formation of initiation complexes in our system, we found that wheat germ protein kinase arrests the formation of the 80S initiation complex in the presence of mRNAs for nonvirion proteins (BMV RNAs 1, 2, and 3), without inhibiting the formation of the 40S complex. These results allow the supposition that the enzyme acts differently than the hemin-controlled repressor. The observed inhibition of initiation complex formation with RNAs 1, 2, and 3 but not with RNA 4 results in a differential in vitro expression of viral genes.

Materials and Methods

General. The methods of isolation of Brome Mosaic virus, BMV RNA, wheat germ cell-free extract, wheat germ tRNAs, and wheat germ protein kinase and the conditions for translation of BMV RNA as well as for aminoacylation of tRNA were described previously (Rychlik & Zagórski, 1980; Zagórski, 1978a,b). Wheat germ initiation factor eIF-2 was kindly given by Dr. A. Legocki, University of Agriculture, Poznań. Subcellular fractions were incubated with [γ-^{32}P]ATP under salt conditions optimal for translation in the presence as well as in the absence of protein kinase under conditions described previously (Rychlik & Zagórski, 1980). Dodecyl

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[1] Abbreviations used: BMV, Brome Mosaic virus; DTT, dithiothreitol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; eIF, eukaryotic initiation factor. Enzymes: protein kinase or ATP:protein phosphotransferase, EC 2.7.1.37; creatine kinase or ATP:creatine *N*-phosphotransferase, EC 2.7.3.2.

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sulfate/polyacrylamide gel electrophoresis was carried out on gels 10% in acrylamide, according to Laemmli (1970). Products of protein synthesis were visualized by fluorography according to Bonner & Laskey (1974). Molecular weight values were calculated by comparing the electrophoretic mobility of polypeptides under study with that of marker polypeptides (cytochrome *c*, M_r 12400; chymotrypsinogen A, M_r 25000; ovalbumin, M_r 45000; bovine serum albumin, M_r 67000; *Escherichia coli* RNA polymerase, M_r of subunits 39000, 155000, and 165000). RNA polymerase was a gift of Dr. B. Mazuś from this institute. Radioactivity measurements and protein estimation were performed as earlier described (Rychlik & Zagórski, 1980; Zagórski, 1978a).

Chemicals. Sepharose 2B was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). ^{3}H-Labeled L-methionine (8.8 Ci/mmol), ^{14}C-labeled L-phenylalanine (60 Ci/mol), and ^{14}C-labeled L-leucine (211 Ci/mol) were Amersham/Searle (Arlington, IL) products. Sparsomycin (Upjon) was kindly given by Dr. P. Kaesberg, University of Wisconsin, Madison, WI. [γ-^{32}P]ATP was prepared as described previously (Rychlik & Zagórski, 1980).

Isolation of Ribosomes and the High-Speed Supernatant. Wheat germ cell-free extract (30–40 mL, 30–35 mg of protein per mL) prepared in extraction buffer (10 mM Tris–acetate, pH 7.6, 50 mM potassium acetate, 3 mM magnesium acetate, and 1 mM DTT) was filtered through a Sepharose 2B column (4.5 × 35 cm). The protein material eluted in the void volume and containing inhibitors of protein synthesis was discarded. The ribosomal fraction (V_e/V_t equal to 0.51–0.71) was collected, clarified by centrifugation at 23000*g* for 20 min, and concentrated by ultracentrifugation (3 h at 105000*g*). This preparation, referred to as "crude ribosomes", was washed with buffer composed of 50 mM Tris–acetate, pH 7.6, 12 mM magnesium acetate, 800 mM potassium acetate, and 20 mM mercaptoethanol and collected by centrifugation as above. The ribosomal pellet was suspended in extraction buffer and designated as "washed ribosomes". Under the salt conditions used for washing, the ribosomes dissociate into subunits, but upon lowering the potassium and magnesium ion concentrations they readily reassociate into 80S couples.

The ribosomal wash was salted out with ammonium sulfate. The fraction that precipitated between 20 and 70% ammonium sulfate saturation was dialyzed against buffer composed of 50 mM Tris–acetate, pH 7.6, 250 mM potassium acetate, and 20 mM mercaptoethanol. This preparation is referred to as "crude initiation factors" (S. Perzyński and W. Zagórski, unpublished results).

The high-speed supernatant was obtained from the cell-free extract by 3-h centrifugation at 105000*g*. The upper three-fourths of the supernatant was collected and extensively dialyzed against extraction buffer or passed through a Sephadex G-25 column (Rychlik & Zagórski, 1978) to remove low molecular weight inhibitors of translation.

All subcellular fractions were prepared at 0–4 °C and stored under liquid nitrogen.

Translation of BMV RNA. The standard incorporation mixture (25 μL) contained 10 μL of cell-free extract (300 μg of protein), 0.25 μCi of [^{14}C]leucine, 0.5 mM DTT, 5 mM Tris–acetate buffer, pH 7.6, 20 mM Hepes–KOH buffer, pH 7.6, 5 nmol each of 19 nonlabeled amino acids, 3.1 mM magnesium acetate, 95 mM potassium acetate, 1.0 mM ATP, 0.375 mM GTP, 20 mM creatine phosphate, 1.5 μg of creatine kinase, and BMV RNA in concentrations indicated in the figures. Incubation was carried out at 31 °C for 120 min or as indicated. In some experiments the cell-free extract was replaced by ribosomes (up to 80 μg/25 μL of incorporation mixture) and high-speed supernatant (up to 300 μg of protein per 25 μL). When stated, incorporation mixtures were supplemented with an appropriate amount of purified wheat germ protein kinase and preincubated for 5 min at 31 °C before addition of template.

Translation of Poly(U). The incorporation mixture (50 μL) contained 20 μL of the cell-free extract (600 μg of protein), 0.02 μCi of [^{14}C]phenylalanine, 0.5 mM DTT, 5 mM Tris–acetate buffer, pH 7.6, 20 mM Hepes–KOH buffer, pH 7.6, 8.9 mM magnesium acetate, 100 mM potassium acetate, 1 mM ATP, 0.375 mM GTP, 20 mM creatine phosphate, 2.5 μg of creatine kinase, and 24 μg of poly(U). Incubation was carried out at 31 °C for 30 min. Hot trichloroacetic acid percipitable radioactivity was measured by standard methods (Zagórski, 1978b).

Formation of Initiation Complexes. The procedure described by Hickey et al. (1976) was generally followed. The incubation mixture (25 μL) contained 23 μg of crude ribosomes, high-speed supernatant (80 μg of protein), 3 μg of $tRNA_f^{Met}$, 1 μCi of [^{3}H]methionine 0.5 mM DTT, 5 mM Tris–acetate buffer, pH 7.6, 20 mM Hepes–KOH buffer, pH 7.6, 1 mM magnesium acetate, 100 mM potassium acetate, 1 mM ATP, 0.375 mM GTP, 20 mM creatine phosphate, 1.25 μg of creatine kinase, and, when indicated, 6 μg of purified wheat germ protein kinase. The mixture was preincubated at 31 °C for 2 min, the Mg^{2+} concentration was adjusted to 3 mM, and the mixture was supplemented with either BMV RNA 1 + 2 (1.75 μg) or BMV RNA 4 (2.35 μg) and sparsomycin (final concentration 0.25 mM). The mixtures were incubated at 31 °C for 5 min, layered over 5-mL linear sucrose gradients (5–25% sucrose in extraction buffer), and centrifuged at 2 °C in a Spinco SW-50 rotor at 150000*g* for 170 min. Fractions (160 μL) were collected from the bottom of the tube, and 150 μL of each was transferred onto Whatman glass fiber paper disks. The disks were washed 3 times for 10 min in 5% cold trichloroacetic acid and then in ethanol/ethyl ether (1:1) and ether. After the samples were dried, the radioactivity was counted in a toluene scintillator.

Results

Effect of Protein Kinase on Polypeptide Synthesis. When added to the protein-synthesizing wheat germ cell-free system, the purified enzyme did not affect translation of poly(U) and only slightly diminished the overall translation of total BMV RNA (Figure 1). It was observed in a series of experiments in which the concentrations of enzyme as well as that of cell-free extract were varied that the polypeptide synthesis directed by total BMV RNA was inhibited usually by the enzyme up to about 30%. Taking into account the fact that the total BMV RNA consists of four different templates, which are expressed in vitro with different efficiencies (Zagórski, 1978a; Shih & Kaesberg, 1976), we decided to compare the pattern of polypeptide synthesis in the presence as well as in the absence of the enzyme (Figure 1 insert).

Total BMV RNA stimulates the formation of four classes of products. The fastest migrating polypeptide is the product of translation of BMV RNA 4 (coat protein gene). Polypeptide 3a is the product stimulated by BMV RNA 3, and BMV RNA 1 + 2 stimulates the synthesis of heavy polypeptides (1a and 2a) with molecular weights around 100000. Polypeptides migrating between the bands 1a + 2a and 3a represent unfinished products of translation of BMV RNA 1 and 2 (Shih & Kaesberg, 1976; Davies & Kaesberg, 1974). After addition of the enzyme to the system, the pattern of polypeptide synthesis is distinctly changed. The formation of

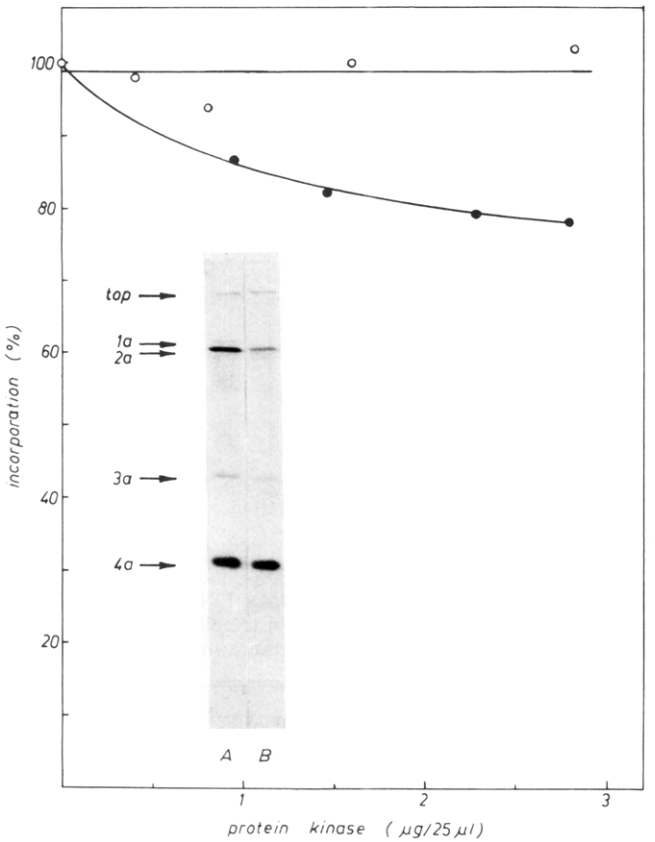

FIGURE 1: Effect of purified wheat germ protein kinase on the translation of total BMV RNA and poly(U) in the wheat germ cell-free extract. (○) Poly(phenylalanine) synthesis. 50-μL incorporation mixtures were supplemented with 24 μg of poly(U). Incorporation without enzyme was equal to 31 pmol of [^{14}C]phenylalanine per 25 μL. Background incorporation [no poly(U)] was equal to 2 pmol of [^{14}C]phenylalanine. (●) Total BMV RNA translation. 25-μL incorporation mixtures each contained 2 μg of total BMV RNA and the indicated amounts of enzyme. Incorporation without enzyme was 384 pmol of [^{14}C]leucine per 25 μL, and background incorporation (no mRNA) was 6 pmol of [^{14}C]leucine. (Insert) 50-μL incorporation mixtures contained 4 μg of total BMV RNA. (Track A) Incorporation with no enzyme added; (track B) incorporation mixtures were supplemented with 10 μg of enzyme. Incubation was carried out at the standard concentration of potassium ions (95 mM) for 8 min at 31 °C, and then the potassium ion concentration was raised to 170 mM and the incubation was continued for 2.5 h. Incorporation without enzyme was equal to 216 pmol of [^{14}C]leucine and that with enzyme to 130 pmol. Background incorporation (no mRNA) was 3 pmol of [^{14}C]leucine. 10-μL aliquots of incubation mixtures were submitted to electrophoresis.

long, nonstructural viral proteins was almost completely inhibited, whereas that of coat protein remained unchanged. It should be pointed out that protein kinase does not possess any nucleolytic activity since BMV RNA incubated with enzyme at 31 °C for 1 h remained undegraded (results not shown).

These results show that a rather slight inhibition of total BMV RNA translation profoundly affects the expression of genes for nonvirion proteins. This was confirmed by experiments in which individual BMV RNAs were translated in the presence of protein kinase (Figure 2). Protein kinase added to the cell-free system primed with total BMV RNA diminished protein synthesis by about 30% (Figure 2A). Translation of RNA 1 + 2 and of RNA 3 exhibited a higher sensitivity to the presence of the enzyme (parts A and B of Figure 2). On the other hand, translation of RNA 4 was almost totally unaffected by protein kinase (Figure 2B). It should be noted that the inhibitory effect of protein kinase was directly proportional to the concentration of enzyme. We observed that in the systems supplemented with 10 μg of enzyme per 50 μL of incorporation mixture, inhibition of BMV RNA 1 + 2 translation was up to 80%.

Differential inhibition of translation of BMV templates may result either from diminished initiation or from slowed down elongation of individual BMV polypeptides. Lack of inhibition of poly(phenylalanine) synthesis by protein kinase (Figure 1) could suggest that the enzyme does not affect polypeptide elongation. This was supported by the analysis of the products of translation on polyacrylamide gel (Figure 3).

Upon addition of the enzyme, the formation of full-length polypeptides 1a + 2a was inhibited, but this was not accompanied by the appearance of an additional amount of prematurely terminated polypeptides (Figure 3B). This shows that protein kinase inhibits initiation of nonstructural viral polypeptide synthesis but not elongatin of these polypeptides. Furthermore, we observed that protein kinase did not affect the formation of a variety of aminoacyl-tRNAs (Met-tRNA$_f^{Met}$, Met-tRNA$_m^{Met}$, and His-, Leu-, Lys-, and Phe-tRNA) (results not shown).

Inhibition of translation was dependent on the time and on the order of addition of the components to the incorporation mixture. The inhibition was strong when the incorporation system was preincubated with the enzyme for 5 min before addition of the template, but was nil when the enzyme was added after the system had been incubated with the template for 20 min. It shows that protein kinase does not affect the formation of already initiated polypeptide chains; this, together with the observed lack of inhibition of poly(U) translation, also indicates that the enzyme affects the initiation of polypeptide synthesis.

Effect of Protein Kinase on the Formation of Initiation Complexes. The effect of protein kinase on the early steps of protein synthesis prompted us to analyze the initiation complexes formed in the presence and absence of protein kinase. Indeed, wheat germ protein kinase does inhibit the formation of the 80S initiation complex stimulated by BMV RNA 1 + 2 (Figure 4A), but not that stimulated by BMV RNA 4 (Figure 4B).

Enzyme-induced inhibition of the 80S initiation complex formation in the presence of BMV RNA 1 + 2 was followed by accumulation of [^{3}H]methionine label in the region of the 40S initiation complex. It therefore appears that the enzyme does not affect the eIF-2-mediated formation of the 40S initiation complex, but inhibits the BMV RNA 1 + 2 stimulated "shift" of radioactivity from the 40S to the 80S region. Hence, it can be assumed that the enzyme inhibits initiation of BMV RNA 1 + 2 translation at the stage preceding the formation of the fully active 80S initiation complex. The enzyme probably arrests the binding of RNA 1 + 2 to the 40S subunit, or it modifies the 40S initiation complex, slowing down the coupling of the 40S complex and 60S subunits. In contrast, the enzyme does not inhibit formation of the 80S complex in the presence of BMV RNA 4 (Figure 4).

Phosphorylation of Ribosome-Associated Polypeptides by Wheat Germ Protein Kinase. In view of the foregoing results and of the enzymatic specificity of the protein kinase, it could be assumed that the differences in translation of BMV RNAs in the presence of protein kinase are due to phosphorylation of some component(s) essential for initiation. Therefore, phosphorylation of the high-speed supernatant and that of ribosomes were analyzed in the absence and presence of the enzyme.

The enzyme did not stimulate to an appreciable extent overall phosphorylation of polypeptides present in the high-

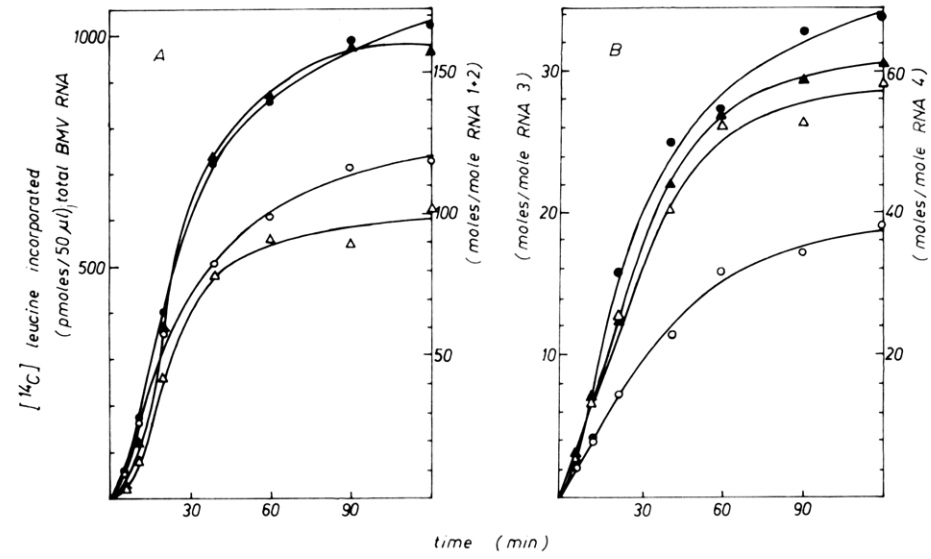

FIGURE 2: Effect of wheat germ protein kinase on the translation of BMV RNAs in wheat germ cell-free extract. 50-µL incorporation mixtures were supplemented with (A) 8 µg of total BMV RNA (●) or 3 µg of BMV RNA 1 + 2 (▲) and (B) 2 µg of BMV RNA 3 (●) or 0.75 µg of BMV RNA 4 (▲). The amounts of individual species of RNAs were adjusted to obtain equimolar amounts of templates. Open symbols in (A) and (B) refer to mixtures incubated in the presence of 6.6 µg of protein kinase. 2-µL aliquots were withdrawn at indicated times.

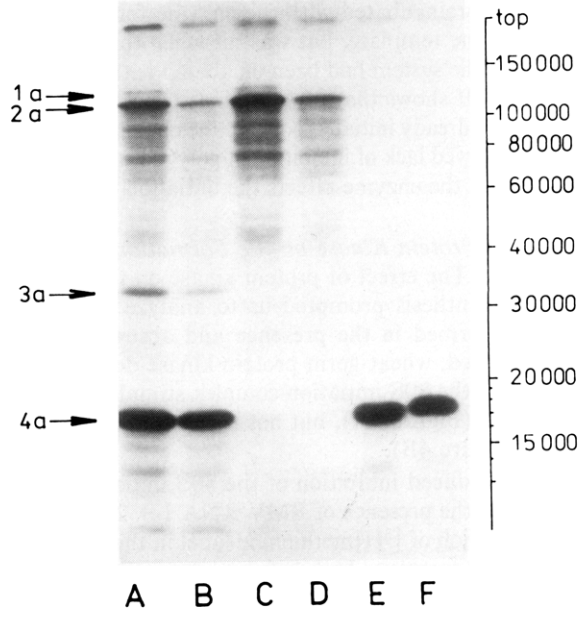

FIGURE 3: Effect of wheat germ protein kinase on in vitro synthesis of BMV polypeptides. 50-µL incorporation mixtures contained respectively 4 µg of total BMV RNA (tracks A and B), 3 µg of BMV RNA 1 + 2 (tracks C and D), and 4 µg of BMV RNA 4 (tracks E and F). In tracks B, D, and F, the incorporation mixtures contained 10 µg of enzyme each. Incorporation mixtures were incubated in the standard concentration of potassium ions (95 mM) for 8 min at 31 °C, and then the potassium ion concentration was raised to 170 mM and the incubation was continued for 2.5 h. Without enzyme, in the presence of total BMV RNA, BMV RNA 1 + 2, and BMV RNA 4; 200, 118, and 332 pmol of [^{14}C]leucine were incorporated respectively. In the presence of the enzyme 120, 64, and 315 pmol of [^{14}C]leucine were incorporated respectively. 10-µL aliquots of incubation mixtures were submitted to electrophoresis. Details of incorporation and autoradiography are described under Materials and Methods.

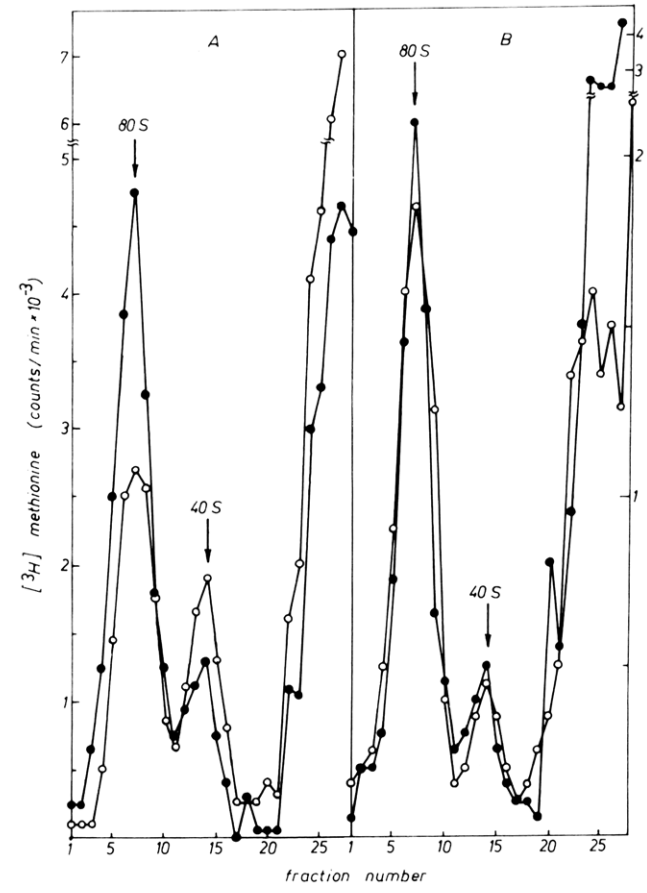

FIGURE 4: Effect of purified wheat germ protein kinase on formation of initiation complexes in the presence of different BMV templates. (A) Mixtures supplied with 1.75 µg of BMV RNA 1 + 2 incubated without enzyme (●) or with 6 µg of wheat germ protein kinase (○). (B) Mixtures supplied with 2.34 µg of BMV RNA 4 incubated as in (A). Fractions were collected from the bottom of the tubes. The positions of 80S and 40S ribosomes were established by centrifugation of 80S ribosomes and 40S subunits under identical conditions.

speed supernatant; neither did it exert any effect on the pattern of the ^{32}P-labeled proteins (results not shown). It was observed, however, that the enzyme does phosphorylate the crude ribosomal preparation, introducing up to 750–1000 pmol of ^{32}P per mg of ribosome proteins (Figure 5). After addition of high-speed supernatant, the preparation of enzyme-phosphorylated ribosomes became partially dephosphorylated, owing to the presence of protein phosphatases in the supernatant. Irrespective of the amount supernatant added and the time of incubation, only partial dephosphorylation was observed, suggesting the existence of two classes of ^{32}P-labeled poly-

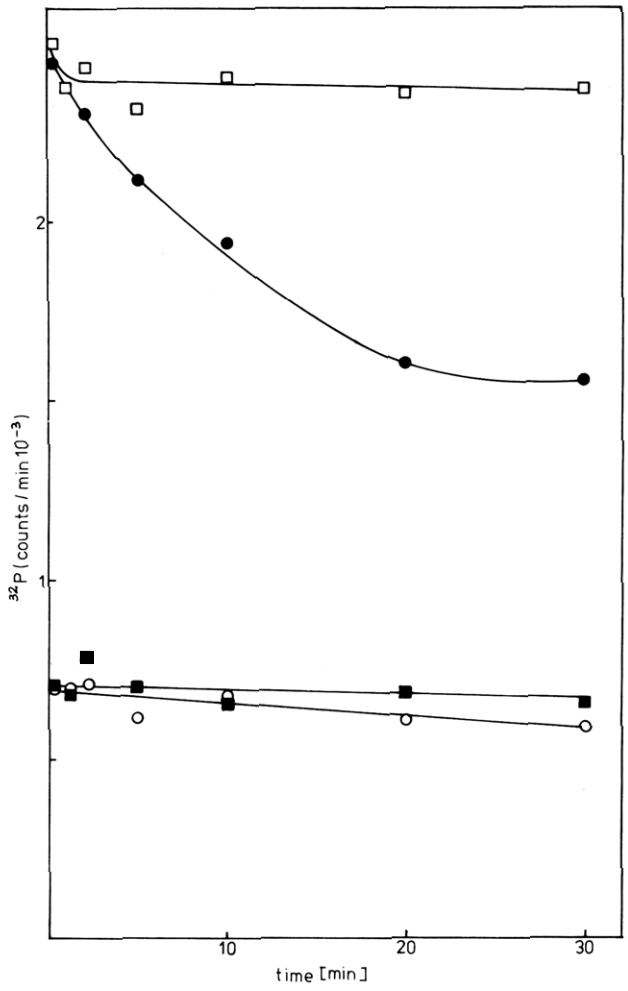

FIGURE 5: Effect of high-speed supernatant on the degree of phosphorylation of wheat germ crude ribosomes. Two portions of crude ribosomes (480 μg) were incubated for 20 min at 31 °C under salt conditions optimal for translation (2.1 mM magnesium acetate, 95 mM potassium acetate, 5 mM Tris–acetate buffer, pH 7.6, 20 mM Hepes–KOH buffer, pH 7.6, and 0.5 mM DTT) with 220 μM [γ-^{32}P]ATP (specific activity 2200 cpm/pmol). One incubation mixture was supplemented with 8 μg of wheat germ protein kinase. Ribosomes were recovered by ultracentrifugation. Incorporation of ^{32}P was measured. 28-μg portions of phosphorylated ribosomal preparation were incubated in 25 μL of incubation mixture at 31 °C under the same salt conditions, but in the presence of 1 mM unlabeled ATP with or without 20 μg of high-speed supernatant. 3-μL portions were withdrawn at the indicated times, and the radioactivity was measured. (□) Ribosomes phosphorylated in the presence of wheat germ protein kinase, incubated without supernatant; (●) ribosomes phosphorylated in the presence of wheat germ protein kinase, incubated with supernatant; (■) ribosomes phosphorylated by ribosome-associated protein kinase, incubated without supernatant; (○) ribosomes phosphorylated by ribosome-associated protein kinase, incubated with supernatant.

peptides: one class of which was dephosphorylated upon addition of high-speed supernatant and another of which was resistant to dephosphorylation. This was confirmed by polyacrylamide gel electrophoresis of a ribosomal preparation phosphorylated under different conditions (Figure 6).

Autophosphorylation of ribosomal polypeptides is shown in Figure 6, lane A. Several ribosome-associated polypeptides are phosphorylated by endogenous protein kinases. Addition of protein kinase isolated as described above enhanced the phosphorylation of these polypeptides and also induced phosphorylation of two additional polypeptides of M_r 32 000 and 76 000 (Figure 6, lane B). These two polypeptides underwent dephosphorylation upon addition of high-speed supernatant, whereas the labeling of polypeptides phosphorylated

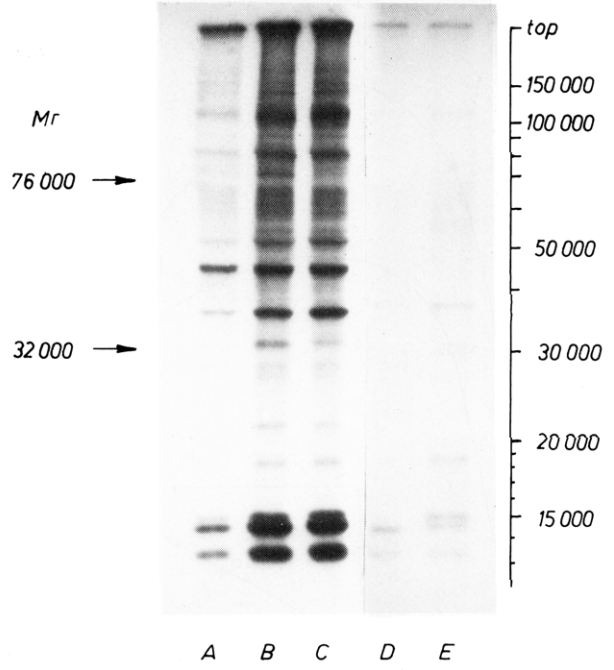

FIGURE 6: Polypeptide patterns of phosphorylated ribosomes. Conditions of phorphorylation were as in Figure 5. 20 μg of phosphorylated ribosomes was submitted to polyacrylamide gel electrophoresis. (Track A) Crude ribosomes phosphorylated, no exogenous enzyme; (track B) crude ribosomes phosphorylated in the presence of purified wheat germ protein kinase; (track C) conditions as in track B and phosphorylated ribosomes, incubated for 1 min with 40 μg of high-speed supernatant; (track D) washed ribosomes autophosphorylated, no exogenous enzyme; (track E) washed ribosomes phosphorylated in the presence of purified wheat germ protein kinase.

by ribosomal protein kinase remained unaffected by the supernatant (Figure 6, lane C). Polypeptides of M_r 32 000 and 76 000 as well as other peptides phosphorylated by wheat germ protein kinase do not seem to be structural ribosomal proteins as they were not detected in high salt washed ribosomes active in poly(U) translation (Figure 6, lanes D and E). Presumably, these polypeptides belong to the fraction of initiation factors.

An important question was whether the phosphorylation of two polypeptides of M_r 32 000 and 76 000 or that of other ribosome-associated polypeptides could be linked to the protein kinase induced inhibition of BMV RNA 1 + 2 translation. We therefore tested the effect of high-speed supernatant on the translation of BMV RNA 1 + 2 in a fractionated cell-free system supplemented with protein kinase (Figure 7). At each concentration of supernatant the amount of radioactivity incorporated without enzyme addition was taken for 100%. At low concentrations of supernatant, inhibition by enzyme reached 50%. With the addition of supernatant the inhibition decreased, and at high supernatant concentrations protein synthesis was the same in the presence as well as in the absence of enzyme (see Figure 7 insert).

Taking into account that the enzyme simultaneously inhibits translation and catalyzes de novo phosphorylation of two polypeptides and that the high-speed supernatant simultaneously reduces the enzyme-dependent inhibition of translation and the enzyme-induced phosphorylation of the two ribosome-associated polypeptides, we suggest that the inhibition of BMV RNA 1 + 2 translation by the wheat germ protein kinase may result from phosphorylation of the two polypeptides, M_r 32 000 and 76 000, found in the crude ribosomal preparation.

Discussion

The results presented in this paper demonstrate that, upon addition of protein kinase to the in vitro translational system,

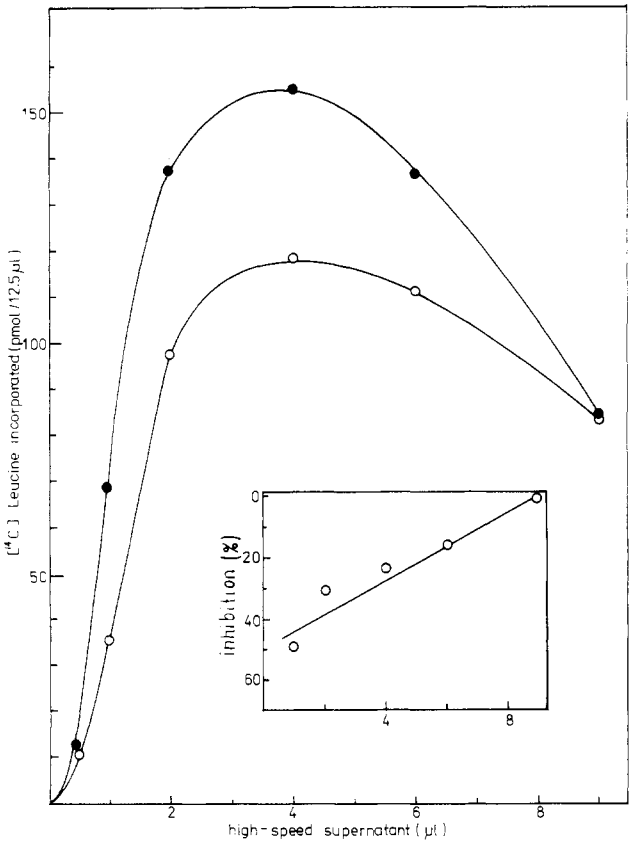

FIGURE 7: Effect of purified wheat germ protein kinase and concentration of high-speed supernatant on translation of BMV RNA 1 + 2 in a fractionated incorporation system. 12.5-μL incorporation mixtures containing 33 μg of crude ribosomes were supplemented with 1 μg of BMV RNA 1 + 2 and high-speed supernatant (20 μg of protein per 1 μL). (●) Incorporation without enzyme; (○) incorporation in the presence of 0.9 μg of enzyme.

the expression of BMV messengers is influenced in such a way that nonstructural genes are excluded from translation, whereas the coat protein gene remains fully expressed. It seems that such a differential effect results from the different affinity of the protein kinase modified initiation apparatus toward different mRNAs present in total BMV RNA.

We are aware that our results do not prove directly that the phosphorylation of some ribosome-associated proteins is the cause of wheat germ protein kinase induced inhibition of BMV RNA translation. One could assume that both processes are not interrelated but simply concurrrent. However, there seems to be a strong correlation between the degree of phosphorylation of crude ribosome-associated polypeptides of M_r 32000 and 76000 and the translational capacity of the system (compare Figures 6 and 7).

Phosphorylation of these polypeptides is followed by the arrest of translation, whereas their dephosphorylation restores protein synthesis. Thermally inactivated protein kinase neither phosphorylates crude ribosomes nor inhibits protein synthesis (results not shown).

The cell-free extract which promotes translation of BMV RNA contains a variety of compounds which strongly reduce the activity of isolated enzyme (Rychlik & Zagórski, 1980). Owing to that and to the high activity of specific phosphatases, we were able to observe the inhibition of translation only in a system overloaded with protein kinase, which competes with phosphatase(s) for ribosome-associatied proteins and constantly preserved their phosphorylated state. This may explain why the inhibition of translation has become visible at a quite high concentration of enzyme. On the other hand, our enzyme inhibits translation when added to the system in concentrations roughly the same as those of other protein kinases involved in translational repression (Clemens et al., 1976; Dealaunay et al., 1977; Pinphanichakarn et al., 1977). Attempts to reveal inhibition in a translational system supplemented with phosphorylated ribosomes deprived of kinase failed because of instant dephosphorylation of the ribosomal preparation by the high-speed supernatant.

The existence of enzymatic systems which are actively phosphorylating and dephosphorylating the same subset of ribosome-associated proteins provides an argument for the possible functional role of modification of these polypeptides. It seems that the cell developed a system of enzymes which controls the level of phosphorylation of several ribosome-associated proteins but not of others. Perhaps the phosphorylation status of these particular polypeptides is of importance for the regulation of protein biosynthesis.

The transiently phosphorylated polypeptides are associated with the crude ribosomal preparation. However, they disappear from the ribosomes on dissociation into subunits and can be found in the fraction of crude initiation factors (results not shown). It therefore appears that these polypeptides are not structural ribosomal proteins but rather, at least in part, belong to factors involved in the initiation of protein synthesis.

It is known that factor eIF-2 is a target for the action of regulatory protein kinases from other sources (Safer & Anderson, 1978). However, none of the ribosome-associated polypeptides phosphorylated by our protein kinase form a part of the wheat germ factor, eIF-2, isolated by Benne et al. (1980). Indeed, the protein kinase we studied phosphorylated the heavy M_r 42000 subunit of purified wheat germ eIF-2 factor (results not shown), which is not found among the crude ribosome polypeptides phosphorylated by the enzyme. Moreover, it seems that in the crude extract, wheat germ eIF-2 does not undergo phosphorylation by the isolated enzyme. Neither does addition of purified eIF-2 from wheat germ to a system inhibited by protein kinase restore translation of RNA 1 + 2. This shows that in the wheat germ cell-free extract eIF-2 is not the target of our protein kinase and that the system of regulation we have described seems to differ from those based on phosphorylation of the light subunit of eIF-2. Perhaps the enzyme affects the functions of other initiation factors known for differential stimulation of mRNA binding to ribosomes (Revel, 1977). This assumption remains to be tested in a fractionated system dependent on purified factors.

In conclusion it appears that wheat germ protein kinase acting in vitro on the translational apparatus may diminish translation of certain classes of templates without affecting the translation of others. This kind of regulatory effect may be a general phenomenon, not necessarily restricted to the BMV model.

Acknowledgments

The authors are indebted to General Mills Inc., Golden Valley, MN, for constantly and patiently supplying us with raw wheat germ highly active in protein synthesis. Discussions with Drs. P. Szafrański, A. Legocki, and W. Filipowicz are gratefully acknowledged.

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Complexes of Alfalfa Mosaic Virus RNA 4 with One and Three Coat Protein Dimers†

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ABSTRACT: RNA 4, the subgenomic coat protein messenger of alfalfa mosaic virus, was loaded with small amounts of coat protein in a reaction in which complete virions were the protein donor. In such a reaction the protein subunits attach to the high-affinity binding sites near the 3′ end of RNA 4 [Houwing, C. J., & Jaspars, E. M. J. (1978) *Biochemistry 17*, 2927–2933]. At a ratio of up to 13 coat protein subunits to 1 mol of RNA 4, complexes with one and three protein dimers, designated complex I and complex III, respectively, were formed. These complexes were isolated by preparative electrophoresis in 4% polyacrylamide gel. At a large excess of the protein donor (280 protein subunits/mol of complex), both complexes I and III were converted into uniform complexes with 10 protein dimers. There were no indications for stable intermediate complexes. A model is suggested for the structure of the complexes which is based on the model proposed for the protein coat of alfalfa mosaic virus [Mellema, J. E., & Van Den Berg, H. J. N. (1974) *J. Supramol. Struct. 2*, 17–31]. The complexes possibly serve as successive stages in virion assembly. More intriguingly, the complexes could be of regulatory significance. Since the four RNA species of alfalfa mosaic virus have an extensive 3′-terminal homology, and since 3′-terminal interaction with coat protein subunits is thought to be a process leading to recognition of the viral genome by the viral replicase and thus to infectivity, complexes analogous to complexes I and III could represent the infectious forms of the genome RNAs.

In no other class of simple RNA viruses is a dual role of the coat protein more prominent than in the class of alfalfa mosaic virus (AMV)[1] and related viruses. With these viruses no infection is possible in the absence of the coat protein or of its messenger, the small subgenomic RNA 4 [see Van Vloten-Doting & Jaspars (1977) for a review], despite the positive strandedness of their tripartite genomes (consisting of RNAs 1, 2, and 3). There is evidence that the coat protein has to attach to all three parts of the genome in order to induce infection (Smit & Jaspars, 1980). All four species of RNA possess high-affinity binding sites for the coat protein, as is shown by their ability to withdraw protein subunits from intact virions (Verhagen et al., 1976). The high-affinity binding sites are likely to be located close to the 3′ ends of the molecules, since they have been found in a 3′-terminal fragment of 88 nucleotides of RNA 4 (Houwing & Jaspars, 1978) that has been sequenced (Koper-Zwarthoff & Bol, 1979) and since virtually the same sequence is present at the 3′ termini of the genome RNAs (Pinck & Pinck, 1979; Koper-Zwarthoff et al., 1979; Gunn & Symons, 1980). Binding of a few coat protein molecules is an endothermic reaction (Srinivasan et al., 1977) and induces a conformational change, at least in RNA 4 (Srinivasan & Jaspars, 1978, and results to be published). It has been postulated that a conformational change at the 3′ ends of the genome RNAs induced by the coat protein would be necessary for RNA replicase recognition, which would explain the essential role of the coat protein at the start of the infection (Houwing & Jaspars, 1978).

Our primary interest now is to see what is the exact nature of the complexes that are formed when small amounts of coat protein attach to AMV-RNAs. The reaction in which coat protein is withdrawn by viral RNA from AMV virions is highly specific in that only the RNAs of AMV and related viruses are active (Van Vloten-Doting & Jaspars, 1972; Van Boxsel, 1976). The reaction has been applied to test fragments of RNA 4 with regard to protein binding (Houwing & Jaspars, 1978). It is used here to obtain RNA 4/protein complexes with the protein exclusively on high-affinity sites. We succeeded in isolating, by means of gel electrophoresis, complexes

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[1] Abbreviations used: AMV, alfalfa mosaic virus; TYMV, turnip yellow mosaic virus; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/80/0419-5255$01.00/0