Effect of dinucleotides on wheat germ translation system

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The effect of ribodinucleoside monophosphates on total protein synthesis was studied in a wheat germ cell-free system, using brome mosaic virus (BMV) RNA as a messenger. Dinucleotides inhibit total protein synthesis to different extents. Of those tested the most inhibitory is CpA. The inhibitory effect of dinucleotides is due to their adverse effect on initiation and not on elongation of polypeptide synthesis. It seems that the dinucleotides complementary to the initiation codon are able to compete with the initiator tRNA during initiation of protein synthesis. The comparison of the effect exerted by different dinucleotides suggests that under conditions of the in vitro protein synthesis RNA 4 is an mRNA molecule with the initiation codon and its immediate neighbourhood being exposed.

Ribodinucleoside monophosphate *In vitro protein synthesis* *BMV RNA* *Initiation complex*

1. INTRODUCTION

A number of naturally occurring cellular factors other than protein factors are implicated in polypeptide chain initiation, elongation and termination. Studies on the effects of polyamine [1], oligonucleotides [2,3] as well as ADP and AMP [4,5], have contributed to our knowledge of the mechanism and the regulation of protein synthesis.

Here we evaluate the effect of different dinucleotides on protein synthesis. These compounds being ubiquitous in the cells of all living organisms are in permanent contact with the protein synthesizing machinery. The studies were carried out with the in vitro protein synthesizing system derived from wheat germ. As a messenger, RNA isolated from brome mosaic virus (BMV) was used. It was found that ribodinucleoside monophosphates inhibit predominantly initiation of polypeptide chain.

2. MATERIALS AND METHODS

CpU was purchased from PL Biochemicals and the other (3′-5′)-ribodinucleoside monophosphates from Sigma. Stock solutions (20 mM) of nucleotides were stored at −20°C. Brome mosaic virus was grown and harvested according to Shih et al. [6]. Total BMV RNA was isolated from virus by phenol extraction, RNA 4 was isolated from the sucrose density gradient of total RNA [7] and further purified by elution from agarose/polyacrylamide gels [8]. Poly(U) and wheat germ tRNA were purchased from Sigma. Raw, untoasted wheat germ was kindly donated by Grands Moulins de Paris.

A protein synthesizing system and post-ribosomal supernatant from wheat germ were isolated as described by Rychlik and Zagorski [9]. Incorporation of [^{35}S]methionine (spec. act. 1460 Ci/mmol) in the presence of BMV RNA was performed as in [10]. Incorporation of [^{14}C]phenylalanine (spec. act. 508 mCi/mmol) in the presence of poly(U) (6 μg/25 μl incubation mixture) was carried out at 12 mM Mg^{2+}. It was checked that the presence of nucleotides in the incubation mixtures did not affect the pH of the mixtures.

Wheat germ tRNA (6 μg) was charged in 12 μl incubation mixtures containing 25 mM Tris (pH

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7.6), 5 mM ATP, 10 mM magnesium acetate, 120 mM potassium acetate, 0.5 mM spermidine, 2 mM dithiothreitol, 2.5 μl ^{14}C-labelled protein hydrolysate (spec. act. 56 mCi/matom); 3 μl post-ribosomal supernatant from wheat germ as a source of aminoacyl-tRNA synthetases. The incubation was at 30°C for 20 min.

Formation of initiation complexes was performed for 5 min at 30°C [11] at 2 mM Mg^{2+} to minimize the elongation of polypeptide chain and in the presence of 2 mM sparsomycin which has the ability to arrest the movement of ribosomes past the initiator codon [12]. The incubation mixtures (25 μl) were layered on 5 ml linear sucrose gradients (10–34% sucrose in 20 mM Tris buffer, pH 7.6, containing 3 mM magnesium acetate, 100 mM potassium acetate and 2 mM dithiothreitol) and centrifuged for 95 min at 2°C in a Beckman SW55 rotor at 50000 rpm. Fractions of 5 drops (about 170 μl) were collected from the top of the gradients with aid of a Buchler Auto-Densi Flow II. The absorbance profile was monitored in an ISCO density gradient monitor. Portions of 150 μl of each fraction were transferred to Whatman 3 MM paper disks and the cold trichloroacetic acid-precipitable radioactivity measured.

3. RESULTS

The effect of dinucleotides on the protein synthesis directed by BMV RNA 4 was variable (fig.1). When total BMV RNA was used for priming wheat germ cell-free system, comparable results were obtained for a given dinucleotide. For the dinucleotides containing cytidine it was observed that dinucleotides containing cytidylyl in the 5′-position were always more inhibitory when compared with their counterparts with C in the 3′-position.

The dinucleotides exert only a small effect on tRNA charging (fig.2). To characterize the level at which the dinucleotide acts the following kinetic studies were performed. When the dinucleotides were added 10 min after the beginning of protein synthesis (fig.3a) the inhibitory effect exerted on total protein synthesis was significantly lower than when the nucleotide was already present in the system before the onset of protein synthesis (fig.3b). This demonstrates that if the initiation of protein synthesis was allowed for 10 min in the absence of the dinucleotide, the effect was certainly weaker. These results suggested that the inhibitory dinucleotides may act at the level of initiation of polypeptide chain.

The efficiency of the formation of 80 S initiation complexes was tested in the system containing sparsomycin (example in fig.4). The comparison of the amount of initiation complexes formed in the presence of various dinucleotides is presented in table 1. These values follow in general the results of total protein synthesis inhibition presented in fig.1. They show that the dinucleotides inhibitory for protein synthesis intervene predominantly at the level of initiation of the polypeptide chain. In addition, it was found that the dinucleotides most inhibitory in the assay of total protein synthesis do not affect the rate of polypeptide chain elongation as measured by the formation of polyphenylalanine in the presence of poly(U) (not shown).

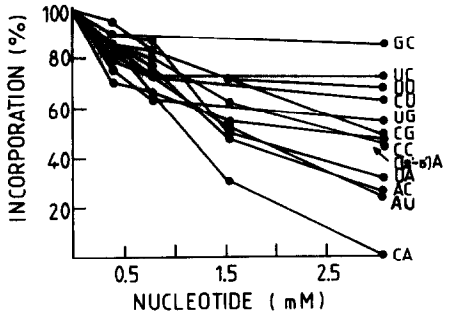

Fig.1. Effect of dinucleotides on the translation of RNA 4 in the wheat germ cell-free system. 100% value refers to the hot trichloroacetic acid-precipitable cpm obtained for incorporation in the absence of nucleotide.

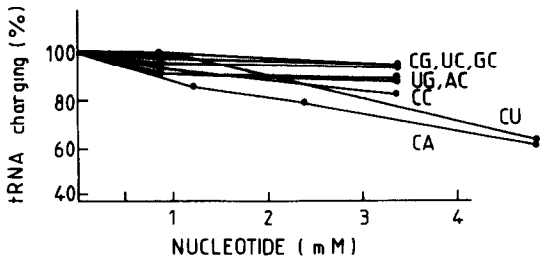

Fig.2. Effect of dinucleotides on the total wheat germ tRNA charging. 100% value refers to the cold trichloroacetic acid-precipitable cpm obtained for tRNA charging in the absence of nucleotides.

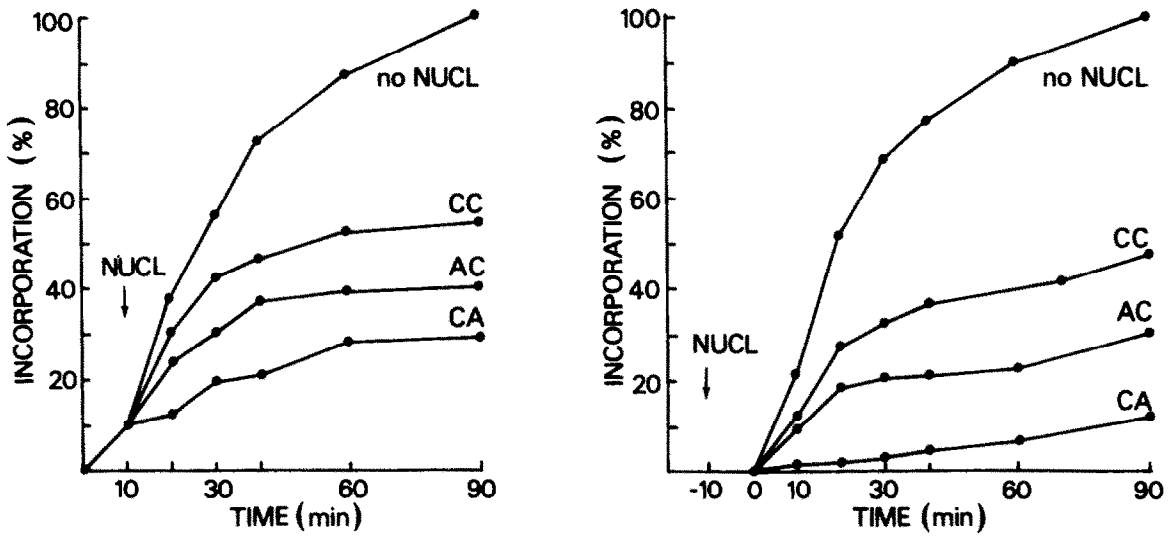

Fig.3. Effect of the time of nucleotide addition on the translation of RNA 4. Nucleotides at 3 mM were added at indicated times. Portions of incubations mixtures (3 μl) were withdrawn at the times shown and hot trichloroacetic acid-precipitable radioactivity was measured.

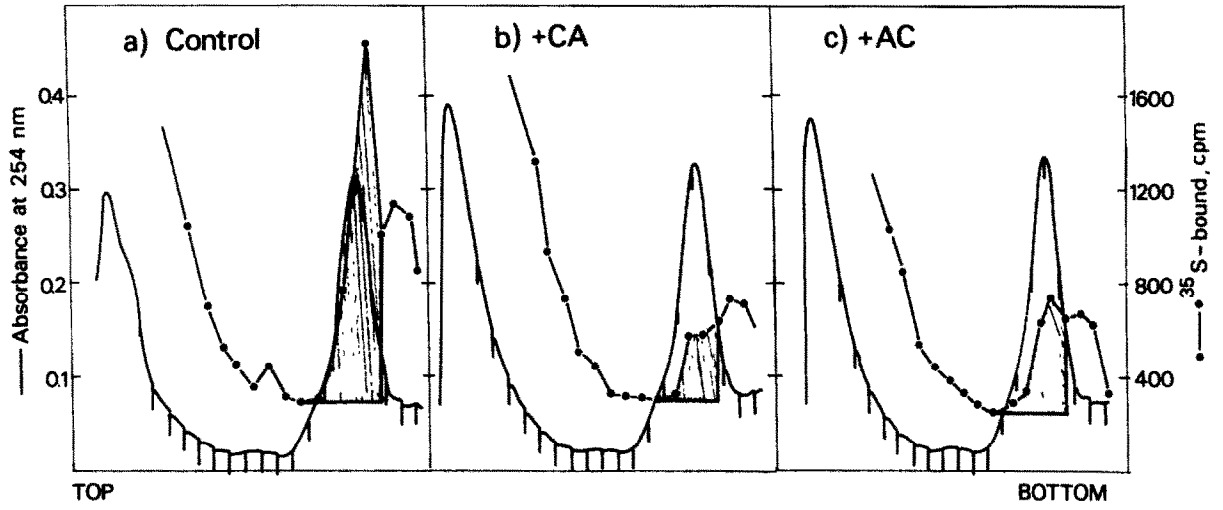

Fig.4. Formation of initiation complexes with RNA 4. Nucleotides at 3 mM were used. Counts in the shaded area were used for the calculations presented in table 1.

For some inhibitory dinucleotides we carried out an experiment in which the inhibition of total protein synthesis was monitored in the presence of elevated concentrations of mRNA. It was observed, for example, that at 3 mM AU or AC, which usually resulted in incorporation at a level of about 25% of control for 0.5 μg mRNA/incubation mixture (see fig.1), increase in the amount of messenger to 0.75 μg leads to incorporation of 60–70% of control. This suggests that inhibitory dinucleotides might interact directly with messenger RNA.

Table 1

Formation of initiation complexes with BMV RNA 4 in the presence of nucleotides

Nucleotide	Exp.I	Exp.II	Exp.III	Exp.IV
–	100	100	100; 98.3[a]	100
CA	23.6		25.7	24.5
AC	38.1			
C(2′-5′)A		83.3	86.0	
UA			71.3	
AU			74.1	

[a] This value refers to the second control gradient processed at the end of experiment (about 1 h later) Radioactivity present in the 80 S ribosome peak is expressed as the percent of radioactivity present in the absence of nucleotides. The choice of integrated values is somewhat arbitrary (see shaded areas in fig.4) which might result in some lack of precision. Exp. I is presented in fig.4

4. DISCUSSION

3′,5′-Dinucleoside phosphates are the smallest molecules in which nucleosides are connected through the same phosphodiester bonds which exist in nucleic acids. They contain only two bases which implies a high probability of a base-pairing with the nucleic acids necessary for protein synthesis. This would be augmented since they were used in a high molar excess during these studies (10^8 vs mRNA concentration, and 10^5 vs tRNA concentration). It was, therefore, surprising that not all dinucleotides used were inhibitory.

The nucleotides inhibitory in the assay with RNA 4 (fig.1) exerted a comparable level of inhibition of overall protein synthesis when total BMV RNA was used (4 different mRNA species) but detailed reactions were studied only with RNA 4. It can be seen that the inhibitory nucleotides have the complementary sequences in or adjoining the initiation codon of BMV RNA 4 (fig.5). Although base-pairing between initiation codon and inhibitory dinucleotides has not been directly demonstrated we showed that the increase in the amount of mRNA available in the system reverses the inhibitory effect of dinucleotides and it seems that occurrence of such an interaction is a reasonable hypothesis. Thus dinucleotides able to

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          5'    -1 123 4      3'
RNA4   ... AUA AUG UCG ...
          3'     U UAC A      5'
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Fig.5. Nucleotide sequence at the initiation codon of BMV RNA 4 [15] and its complementary sequence.

compete effectively against the anticodon of initiator tRNA for the initiation codon of mRNA will inhibit formation of initiation complexes and thereby total protein synthesis.

The assay of the formation of initiation complexes provides a direct measure of the effect of inhibitory dinucleotides on the interaction of mRNA and the anticodon of initiator tRNA under the conditions of protein synthesis. The dinucleotides which are complementary to the initiation codon and its two 5′- and 3′-neighbouring nucleotides are AC, CA, AU, and UU (see fig.5). The first 3 are indeed the most inhibitory in the assay of total protein synthesis (fig.1) as well as during initiation complexes formation (table 1). The fact that UU has only very weak effect suggests that the 3′-part of the initiator codon might interact more easily with dinucleotides. It also shows that U at position 4 of RNA 4, the 5′-base of the serine codon UCG (fig.5), may be exposed to the same extent as 5′-A of the initiation codon (cf. inhibitory effect of AC and AU on initiation complex formation). In conclusion, it seems that under conditions of in vitro protein synthesis, BMV RNA 4 is an RNA molecule with the initiation codon and its immediate neighbourhood being exposed.

The results obtained with C(2′-5′)A merit some comments. The proton magnetic resonance data for dinucleotides in solution indicate that the bases overlap much more extensively in (2′-5′) linked dinucleoside monophosphate than in their (3′-5′) linked counterparts [13]. Conformational energy calculations indicate that (2′-5′) linked dinucleoside monophosphates occur generally in the left-handed folded conformations whereas (3′-5′) linked counterparts occur generally in the right-handed folded conformations [14]. These differences in structure between (2′-5′) and (3′-5′) linked dinucleotides indicate the possible three-dimensional unsuitability of C(2′-5′)A for base-pairing with UG of initiation codon AUG. During these studies C(2′-5′)A inhibited total protein syn-

thesis by 50% (fig.1) and to a certain extent the formation of initiation complexes. This might signify that it can base-pair with UG under conditions of in vitro protein synthesis, however, significantly weaker than C(3′-5′)A.

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