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CpA containing oligoribonucleotides specifically inhibit protein synthesis in rabbit reticulocytes

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The diribonucleoside monophosphate CpA (and no others) inhibits polypeptide chain elongation in rabbit reticulocyte lysates at 10–50 μM. Furthermore, all the trinucleotides containing CpA, i.e., XpCpA and CpApX (X = U, C, A or G) block polypeptide chain elongation as well. At 10 μM the inhibition by XpCpA and not CpApX is transient because a 3′-exonucleolytic activity destroys the critical CpA moiety. The inhibitors do not appear to interfere with the aminoacylation of tRNAs or disrupt the interaction of aminoacyl-tRNAs with the protein synthetic machinery. High levels (200 μM) of CpA or the trinucleotides containing CpA have no effect on translation in a wheat germ cell-free system.

Oligoribonucleotide; Reticulocyte lysate; Protein synthesis; 3′-Exonucleolytic cleavage; Cell-free system; (Rabbit, Wheat germ)

1. INTRODUCTION

Numerous compounds that inhibit eukaryotic protein synthesis have been described (reviews [1–3]). Inhibitors are usually classified by the translational step they affect, i.e. initiation or elongation. Specific inhibitors of termination are unknown [3]. Although the effects of a large number of substances on protein synthesis have been thoroughly documented, the molecular basis for their mode of action remains unclear.

We have previously reported that the tetraribonucleotide GpApUpC, which both emulates and complements a unique sequence in the 'T-loop' of the eukaryotic initiator tRNAs, blocks protein synthesis at the initiation step [4]. This effect was subsequently traced to unidentified impurities in the original preparations. In a survey of highly purified 'control' oligoribonucleotides we noted several others to be strongly inhibitory to polypeptide synthesis in the rabbit reticulocyte lysate. This lysate is a very efficient cell-free system with a protein synthesizing rate close to that of the intact cells [5]. It has the advantage that more than 90% of the translation products consist of only two polypeptide chains, α- and β-globin [6]. Here we describe the inhibition of protein synthesis in a rabbit reticulocyte lysate by short oligoribonucleotides containing CpA. These oligomers have little effect on translation in the less active wheat germ cell-free system.

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2. EXPERIMENTAL

2.1. *Synthesis and purification of oligoribonucleotides*

All diribonucleoside 3′,5′-monophosphates (Sigma, St. Louis, MO) were purified by reverse-phase HPLC on a 4.6 × 250 mm Aquapore RP-300 column (Brownlee Labs, Santa Clara, CA) using a 0–7.5% acetonitrile gradient in 0.1 M triethylammonium acetate, pH 6.8 over 30 min at

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a flow rate of 1.5 ml per min. The fractions (0.75 ml) containing the respective dinucleotide as judged by UV absorbance at 260 nm were pooled and lyophilized. Trinucleotides and tetranucleotides were synthesized according to a slightly modified published procedure [7]. Reaction mixtures (0.2 ml) containing 150 mM Tris-HCl, pH 9.0, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM dinucleotide or 5 mM trinucleotide, 10 mM ribonucleoside 5′-diphosphate and 5 units (125 μg) polynucleotide phosphorylase (Sigma, St. Louis, MO) were incubated at 37°C overnight. The reaction products were separated by reverse-phase HPLC, and concentrated as described above for the dinucleotides. The oligoribonucleotides were characterized mainly by analyzing their nucleoside composition as described [8]. For the synthesis of CpCp[^{3}H]A, [^{3}H]ADP with a specific activity of 0.5 Ci/mmol (Amersham, Arlington Heights, IL) was used. Prior to their use the integrity of all oligomer preparations was checked by reverse-phase HPLC.

2.2. *Cell-free protein synthesis*

Protein synthesizing mixtures (55 μl) contained 25 μl rabbit reticulocyte lysate [9,10] and the following components: 75 mM KCl, 2 mM $MgCl_2$, 0.5 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 45 units/ml creatine kinase, 25 μM hemin, 0.3 mM L-[^{14}C]leucine (5 Ci/mol), 0.25–0.75 mM of the other 19 natural amino acids [11] and, where indicated, 10–200 μM oligoribonucleotide. The mixtures were incubated at 34°C and the incorporation of the radiolabeled amino acid into polymers (10 μl aliquots) was determined by a standard procedure [12].

2.3. *Analysis of reticulocyte lysates containing CpCp[^{3}H]A as inhibitor*

To test for a suspected 3′-exonucleolytic activity, reaction mixtures (88 μl) containing 40 μl of reticulocyte lysate and 10 or 50 μM CpCp[^{3}H]A (0.5 Ci/mmol) were incubated as described above. After 15 and 30 min two 10 μl aliquots were removed and each diluted into 200 μl ice-cold water. One set of these samples was used to determine the rate of protein synthesis by measuring the incorporation of radioactive leucine into polymers [12]. To the second set of samples 2 ml of ice-cold 10% aqueous trichloroacetic acid was added and the precipitates were collected by low speed centrifugation. The supernatants, containing all the ^{3}H radioactivity, were extracted twice with 1.5 ml ether to remove the acid and then lyophilized. Prior to their analysis by reverse-phase HPLC as described above for the purification of oligoribonucleotides, the samples were dissolved in 200 μl triethylammonium acetate, pH 6.8, and each received 0.2 A_{260} units nonradioactive CpCpA as carrier. After chromatography the radioactivity in each fraction (0.75 ml) was determined by homogeneous liquid scintillation counting of a 0.15 ml aliquot.

2.4. *Sucrose density gradient analysis*

Protein synthesizing mixtures (55 μl) were prepared as above, except that L-[^{14}C]leucine was replaced by nonradioactive L-leucine. Additional details are indicated in the legend to fig.4. After 15 min at 34°C the reaction mixtures were pulsed for 30 s with 2.5 pmol (2 μl) rabbit reticulocyte [^{35}S]Met-tRNA$_f$ (spec. act. 100 Ci/mol), prepared as described [13]. The reactions were stopped by diluting the samples with 150 μl of ice-cold 10 mM Tris-HCl, pH 7.5, 10 mM KCl and 1.5 mM $MgCl_2$, applied to linear 15–30% (w/v) sucrose gradients (in 10 mM Tris-HCl, pH 7.5, 10 mM KCl and 1.5 mM $MgCl_2$) and centrifuged for 4.5 h at 40000 rpm in the SW 41 rotor. The ribosome-bound [^{35}S]Met-tRNA$_f$ was determined after precipitation with cetyltrimethylammonium bromide [14].

3. RESULTS

3.1. *CpA is the unique moiety that inhibits translation*

Among the dinucleotides shown in table 1 only CpA inhibited protein synthesis (fig.1). At 200 μM, all the other dinucleotides that were tested, most notably those exhibiting a 5′-C and a 3′-nucleoside other than A or a 3′-A and a 5′-nucleoside other than C did not affect translation at all (table 1). The eight possible oligomers containing CpA, i.e. XpCpA and CpApX where X corresponds to U, C, A or G, also impaired protein synthesis in the reticulocyte lysate in the 10–200 μM range (fig.2). Again, a large number of other trinucleotides examined in control experiments failed to do so, even at 200 μM (table 1).

Table 1

Oligoribonucleotides (200 μM) in rabbit reticulocyte protein synthesis

Dimers	Trimers	Tetramers
(A) Oligoribonucleotides with no significant effect		
UpC	UpUpC	UpUpCpC
UpA	UpCpC	UpUpCpG
CpU	UpGpG	UpCpCpG
CpC	CpGpA	UpCpCpC
CpG	ApUpC	CpGpApU
ApC	GpApU	CpGpApA
ApA	GpGpA	ApUpCpC
GpA		ApUpCpG
		ApApApC
		ApApCpG
		ApGpCpU
		GpApUpU
		GpApUpC
		GpGpApA
(B) Inhibitors		
CpA	UpCpA	UpUpCpA
	CpCpA	UpCpCpA
	ApCpA	UpCpApA
	GpCpA	CpCpApA
	CpApU	
	CpApC	
	CpApA	
	CpApG	

Similar results were also obtained for various tetramers containing the CpA moiety at random positions (table 1).

Interestingly, in the case of the XpCpA trinucleotides (and not with CpApX) if tested at the 10 μM level the inhibition was transient; that is, normal polypeptide synthesis rates were almost completely restored after a lag of 30 min (fig.2). Assuming that CpA is the unique feature in the sequence of the inhibitory oligomers, this observation could be explained by invoking a 3′-exonucleolytic activity in the reticulocyte lysate that attacks oligonucleotides containing three or more nucleosides. The 3′-terminal cleavage of XpCpA would disrupt the CpA moiety, whereas CpApX oligomers would be converted to the stable inhibitory dinucleotide CpA.

Using CpCp[^{3}H]A, we demonstrated that the proposed 3′-exonucleolytic cleavage occurred during incubation in the lysate. After acid-precipitation of polymers, all ^{3}H radioactivity derived from the CpCp[^{3}H]A added to the reaction mixture was found in the supernatant. These soluble fractions were concentrated and analyzed by reverse-phase HPLC. The elution profiles obtained for such aliquots taken after 15 and 30 min of incubation showed progressive loss of the trinucleotide and transfer of the radioactivity to 5′-AMP (fig.3).

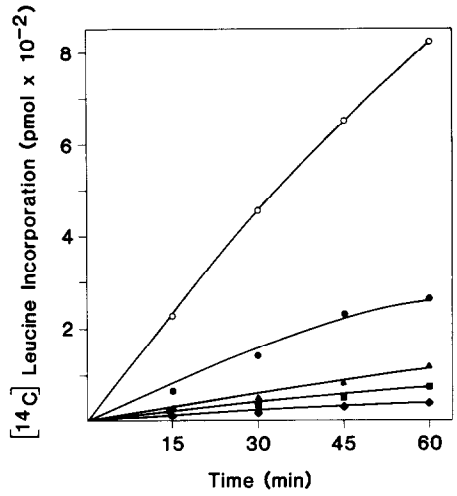


Fig.1. Inhibition of protein synthesis in the rabbit reticulocyte lysate by the dinucleotide CpA. The incorporation of radioactive leucine into acid-precipitable polymers was determined in the absence (—○—) and in the presence of 10 (—●—), 50 (—▲—), 100 (—■—), and 200 μM (—◆—) CpA.

3.2. *CpA-containing oligomers inhibit the elongation step*

The binding of [^{35}S]Met-tRNA$_i^{Met}$ to ribosomal particles in CpA containing oligoribonucleotide-inhibited reticulocyte lysates was analyzed by sucrose density gradient ultracentrifugation (fig.4). In the oligonucleotide-inhibited lysates about twice as much Met-tRNA$_i^{Met}$ was bound to small ribosomal subunits as in the control. This profile is typical of a block in elongation [15] and suggests that the formation of preinitiation complexes was not only unaffected, but that these complexes had also accumulated because of a stall in polypeptide synthesis. For contrast, fig.4 shows the characteristic reduction in 43 S complexes in

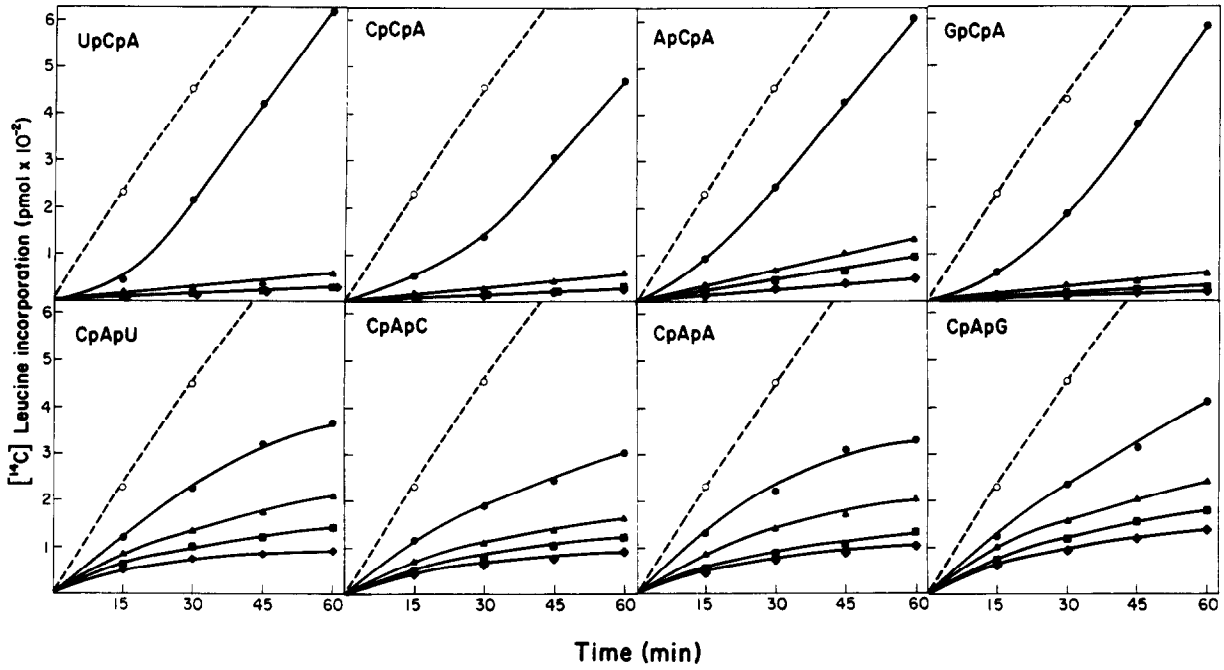
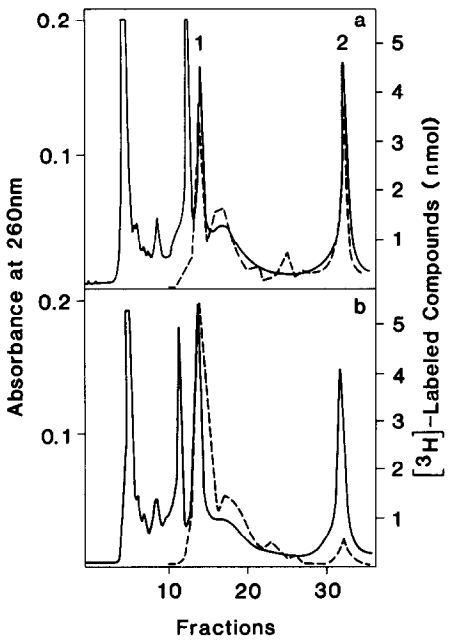


Fig.2. Inhibition of protein synthesis in the rabbit reticulocyte lysate by the trinucleotides containing CpA. The amount of radioactive leucine incorporated into acid-precipitable polymers was measured in the presence of 10 (—●—), 50 (—▲—), 100 (—■—), and 200 μM (—◆—) of the respective trinucleotide. The two time points for the noninhibited control curve (– –○– –) were taken from fig.1. Note that the scale for leucine incorporation is expanded as compared to fig.1.



the gradient profiles of lysates blocked at the initiation step by hemin depletion. When the time of centrifugation was reduced to permit examination of polysomes we found that CpA increased the peak polysome size from 4 to 6 ribosomes per mRNA (not shown), confirming that inhibition is at the level of elongation [16].

Fig.3. HPLC analysis of acid soluble components after inhibiting protein synthesis in the reticulocyte lysate with radiolabeled CpCpA. The components in the acid soluble supernatants of samples taken after 15 (a) and 30 min (b) of incubation were analyzed as described in section 2. The absorbance at 260 nm was recorded (solid line) and the ^{3}H radioactivity in the collected fractions was measured (broken line). Peak 1 corresponds to 5′-AMP and peak 2 to CpCpA; their elution volumes had been determined separately prior to this experiment by monitoring the UV absorbance of the pure compounds.

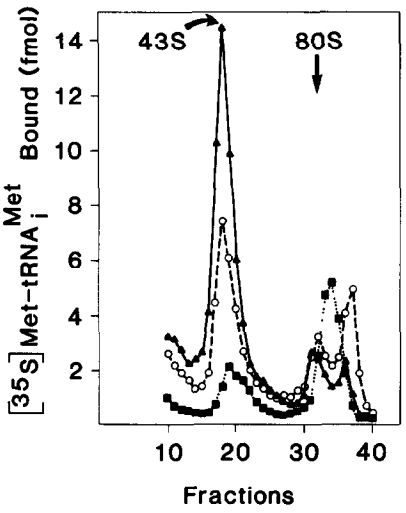


Fig.4. Sucrose gradient analysis of ribosomal complexes formed in inhibited and noninhibited reticulocyte lysates. The reaction mixtures were pulsed with [^{35}S]Met-tRNA$_i^{Met}$ and separated by sucrose density gradient ultracentrifugation as described in section 2. Shown are representative binding profiles of [^{35}S]Met-tRNA$_i^{Met}$ seen in the presence (—○—) and absence of hemin (—■—), and in the presence of hemin plus 50–200 μM CpA, CpCpA, UpCpA or CpApU (—▲—). 43 S indicates the position of the preinitiation complex.

4. DISCUSSION

We have shown that the only short (4 or less) oligoribonucleotides that inhibit elongation in protein synthesis in the rabbit reticulocyte lysate are those containing the moiety CpA, including the dinucleotide CpA. The oligomers inhibit polypeptide synthesis at surprisingly low concentrations (10 μM). This sensitivity seems to be characteristic of the reticulocyte lysate, since we do not observe any effect of up to 200 μM CpA (or XpCpA or CpApX) on the translation of viral or globin mRNA in a wheat germ cell-free system. On the other hand, Chroboczek and Jacrot [17], who have studied the effect of various diribonucleoside 3′,5′-monophosphates on translation in wheat germ, have found that CpA is the most inhibitory. However, the level of added dinucleotides was nearly 100-times higher than in our case and the effect was not specific but only strongest for CpA. These authors noted only marginal inhibition with 400 μM CpA, which is twice as high as the highest concentration used by us (without effect) in the wheat germ system. Thus our results are consistent with those of Chroboczek and Jacrot [17] and confirm the several hundred-fold greater sensitivity of the reticulocyte system to CpA-containing oligomers.

The loss of translational inhibitory activity after 30 min at low concentrations of XpCpA and not CpApX was traced to a 3′-exonuclease activity which disrupts the CpA moiety. Although this loss of inhibition was puzzling at first, it ultimately served to support the idea that the CpA moiety is the essential element for the inhibition of protein synthesis in the reticulocyte lysate.

We have found that the translation of non-globin mRNAs is also inhibited by CpA when the L-[^{35}S]methionine-labeled proteins were analyzed by polyacrylamide gel electrophoresis. The synthesis of a number of proteins was suppressed by 10 and 50 μM CpA to about the same degree as globin, including a major labeled product migrating with a molecular mass of approximately 65 kDa (not shown). We have also looked at the distribution of peptidyl-tRNA on polysomes in CpA-inhibited lysate labeled with [^{3}H]Ala-tRNA and found no evidence for the premature release of nascent peptide chains.

In our efforts to narrow down the target of the inhibitory oligomers we have considered that these compounds might interfere with reactions involving tRNA, since all tRNAs possess a 3′-terminal CpCpA [18]. First, we have not found any effect of CpA on aminoacylation, either directly in the lysate or in solutions containing isolated bulk tRNA and a reticulocyte enzyme fraction containing all the synthetases (not shown). Second, our attempts to detect an influence of CpA containing oligomers on the regeneration of the tRNA's 3′-terminus (ATP; CTP:tRNA nucleotidyltransferase reaction) [19] led us to the observation that such an activity cannot be detected in the reticulocyte lysate even in the noninhibited control. Third, we have been unable to measure any stable interaction of radiolabeled CpCpA (500 μM) with ribosomal particles.

We conclude that the inhibitory action exerted by CpA and oligonucleotides containing CpA is not obviously related to the proper functioning of tRNAs during elongation. It is conceivable that the

drastic effect on protein synthesis is secondary or indirect and that these inhibitors trigger a response that ultimately results in a translational shutoff. The reticulocyte lysate has a finely tuned and highly efficient protein synthetic machinery which is specifically regulated by hemin [20]. As such it is not surprising that it can be easily intoxicated. Although the precise molecular basis of their inhibitory action is still unclear, we feel it is important to point out how dramatically the CpA containing oligomers affect globin synthesis in the reticulocyte lysate and how astonishingly unique they are, among a large number of oligoribonucleotides, in doing so.

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REFERENCES

[1] Pestka, S. (1977) in: Molecular Mechanisms of Protein Biosynthesis (Weissbach, H. and Pestka, S. eds) pp.468–553, Academic Press, New York.
[2] Vazquez, D. (1979) Inhibitors of Protein Biosynthesis, Springer, Berlin.
[3] Vazquez, D., Zaera, E., Doelz, H. and Jimenez, A. (1982) in: Protein Biosynthesis in Eukaryotes (Perez-Bercoff, R. ed.) pp.311–337, Plenum, New York.
[4] Sigler, P.B., Schevitz, R.W., Gross, M., Kaplansky, A., Podjarny, A. and Sussman, J.L. (1981) in: Structural Aspects of Recognition and Assembly in Biological Macromolecules (Balaban, M. et al. eds) pp.631–643, Balaban ISS, Rehovot.
[5] Adamson, S.D., Herbert, E. and Godchaux, W. (1968) Arch. Biochem. Biophys. 125, 671–683.
[6] Lodish, H.F. and Desalu, O. (1973) J. Biol. Chem. 248, 3520–3527.
[7] Uhlenbeck, O.C., Baller, J. and Doty, P. (1970) Nature 225, 508–510.
[8] Sprinzl, M., Wagner, T., Lorenz, S. and Erdmann, V.A. (1976) Biochemistry 15, 3031–3039.
[9] Gross, M. (1979) J. Biol. Chem. 254, 2370–2377.
[10] Maxwell, C.R., Kamper, C.S. and Rabinovitz, M. (1971) J. Mol. Biol. 58, 317–327.
[11] Jackson, R., Campbell, E.A., Herbert, P. and Hunt, T. (1983) Eur. J. Biochem. 131, 289–301.
[12] Gross, M. (1976) Biochim. Biophys. Acta 447, 445–459.
[13] Kaplansky, D.A., Kwan, A. and Gross, M. (1982) J. Biol. Chem. 257, 5722–5729.
[14] Darnbrough, C.H., Legon, S., Hunt, T. and Jackson, R.J. (1973) J. Mol. Biol. 76, 379–403.
[15] Balkow, K., Mizuno, S., Fisher, J.M. and Rabinowitz, M. (1973) Biochim. Biophys. Acta 324, 397–409.
[16] Lodish, H.F. (1971) J. Biol. Chem. 246, 7131–7138.
[17] Chroboczek, J. and Jacrot, B. (1985) FEBS Lett. 184, 155–159.
[18] Sprinzl, M. and Gauss, D.H. (1984) Nucleic Acids Res. 12, r1–r57.
[19] Deutscher, M.P. (1982) in: The Enzymes, vol.15B (Boyer, P.D. ed.) pp.183–215, Academic Press, New York.
[20] Jackson, R. (1982) in: Protein Biosynthesis in Eukaryotes (Perez-Bercoff, R. ed.) pp.363–418, Plenum, New York.