

Effect of 5'-Terminated (2'-5')-Oligoadenylylates on Cap Degrading Activities in Rat Liver Nuclei

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Dedicated to Professor Friedrich Cramer on the Occasion of His 60th Birthday

(2'-5')-oligoadenylylates, 5'Capped-(2'-5')-adenylylates, Decapping Activities, Cap-Type Dinucleoside Triphosphatase, Rat Liver Nuclei

(2'-5')-oligoadenylylates bearing a 5'-terminal triphosphate or a 5'GTP-group inhibit the activity of a dinucleoside triphosphatase in rat liver nuclei thereby protecting mRNA against 5'-exonucleolytic degradation. (2'-5')-oligoadenylylates, on the other hand, were known to enhance the activity of an endoribonuclease, RNaseF. Thus a synergistic effect may be assumed *in vivo*, i.e. cellular metabolism seems to be protected twice in virus-infected cells.

1. Introduction

Addition of double stranded RNA(dsRNA) to extracts of interferon-treated cells markedly inhibits ribosomal protein synthesis [1–3]. This is due to the formation of $p_3(A_2'p)_nA$ ($n = 1–14$) from ATP by an (2'-5') A_n -synthetase which is activated by dsRNA. These unusually linked (2'-5')-oligoadenylylates enhance, on the other hand, the activity of an endoribonuclease (RNase F). This RNase F degrades single-stranded RNAs as mRNA and rRNA [4–7] and thereby impedes virus replication.

The 5'-terminal cap structures in eucaryotic mRNA [8–11] are known to impart stability to these molecules against the attack of 5'-exoribonucleases [12–18]. However, in different vegetable and mammalian tissues [17, 19–22] decapping activities were found.

The enzymatic cleavage of the cap from either mRNA [19, 23], capped mRNA-fragments with a chain length shorter than three [24] or ten nucleotides [14], respectively or of cap-structured dinucleotides [25] follows the mode of action of a dinucleoside triphosphatase. This is confirmed by the cleavage pattern as m^7GDP [17], m^7GMP [19, 24] and GMP [25] could be detected as degradation products.

Based on our recent report on the enzymatic degradation of cap-structured dinucleotides in isolated rat liver nuclei [25], our point in question in

the present paper is: Do(2'-5')-oligoadenylylates affect the enzymatic cleavage of cap-structures in rat liver nuclei; and do they influence exonucleolytic mRNA degradation? Some of our findings have been published in part as a poster contribution [26].

2. Materials and Methods

The synthesis of $p(A_2'p)_nA$ ($n = 1, 2$) was performed by condensation of imidazole-activated AMP in aqueous solution by using lead nitrate as a catalyst according to [27]. $pA_3'pA-2',3'-iso-prop$ -[(adenylyl(3'-5')-2',3'-isopropylideneadenosine)-5'-monophosphate] was prepared using the phosphite method [28]. $G_5'p_3(A_2'p)_nA$, $[^3H]G_5'p_3(A_2'p)_nA$, $p_3(A_2'p)_nA$ ($n = 1, 2$) as well as $Gp_3A_3'pA-2',3'-iso-prop$ were prepared by reaction of carbonyl-dimidazole-activated [29] $p(A_2'p)A$ and $pA_3'pA-2',3'-iso-prop$ with GDP, $[^3H]GDP$ and pyrophosphate. The preparation of $G_5'p_3[^{14}C]A$ was described elsewhere [30]. All the compounds used were characterized by ^{31}P -NMR spectroscopy (Bruker WP 250 F8). Product analysis after enzymatic degradation was done by convenient chromatographic techniques including h.p.l.c. as in [31]. The ^{31}P -NMR (D_2O , pH 7) signals centered at: $\delta = +1.2$ (3'-5'P), -0.1 (2'-5'P), -11 (α -, γ -P), -22 (β -P) ppm upfield to 85 percent H_3PO_4 (see Figs. 2, 3).

Liver nuclei were isolated from male rats (Wistar rats Bor: WISW, SPF TNO, 150–200 g) in analogy to a method of Jungblut *et al.* [32]. The isolation procedure shown in Fig. 1 was published recently [25].

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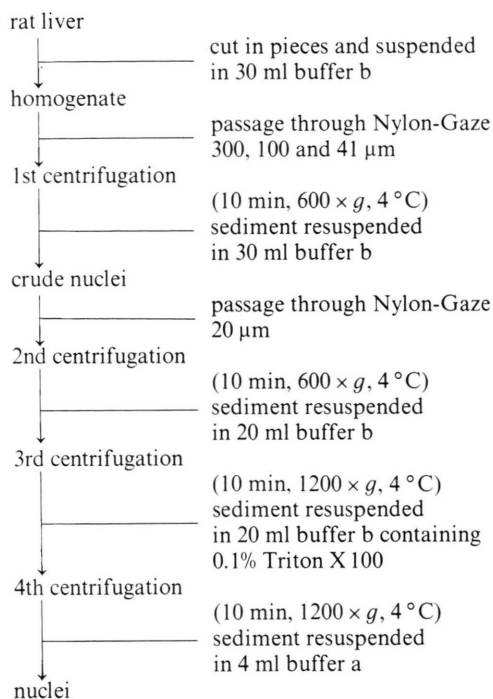
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Fig. 1. Isolation procedure for rat liver nuclei.



Buffer systems: (a) 0.01 M K_2HPO_4 , 0.003 M $MgCl_2$, 0.005 M NaN_3 ; (b) 0.44 M sucrose in buffer a.

DNA was determined as described [33] and protein according to the Biuret-method.

The degradation studies of cap-structured nucleotides were performed using the following assay conditions (for details compare [25]): 50 μ l suspension of rat liver nuclei (4 mg protein \times ml $^{-1}$; 0.7 mgDNA \times ml $^{-1}$) in buffer b, 100 μ l 0.05 M triethanolamine \times HCl buffer, pH 7.2, 215 μ M of the employed compound(s) in a total volume of 150 μ l at 25 °C. All the degradation experiments were carried out at saturating conditions over a period of 30 min. 20 μ l-aliquots of the incubation mixture taken during assaying were denaturated with 5 μ l perchloric acid, and neutralized after centrifugation. The determination of the 3H - or ^{14}C -labeled reaction products was performed by thin-layer chromatography on PEI-cellulose plates (Macherey and Nagel, Düren) using 0.5 M KNO_3 , pH 5.7, as the mobile phase. The radioactive spots of the catabolites guanosine, adenosine, GMP and AMP as well

as of non-degraded cap-structured nucleotides were detected by a TLC-Linear Analyzer LB 2821 (Berthold, Wildbad).

For further details in assaying compare legend in Table I. The compounds employed are listed in the Figs. 2 and 3.

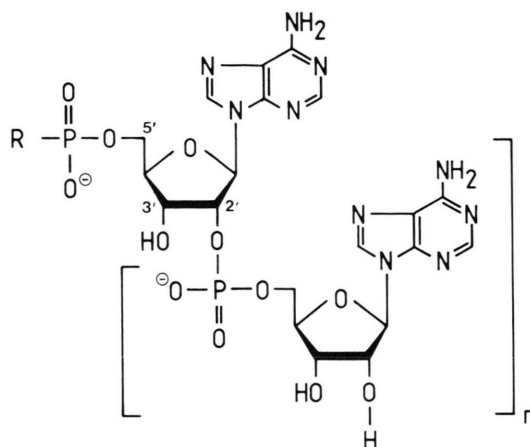
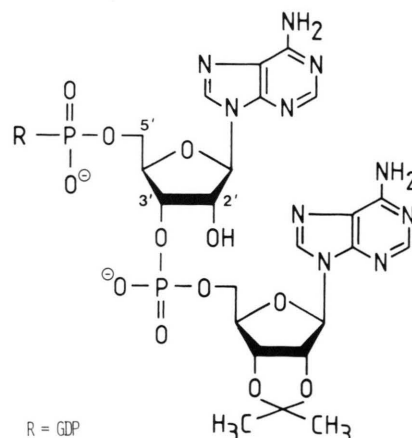


Fig. 2. 5'-terminated adenylates.

| | | | |
|------------|---------------------------|---------|--------------------|
| I | Gp ₃ A | $n = 0$ | R = GDP |
| II | Gp ₃ A2'pA | $n = 1$ | R = GDP |
| III | Gp ₃ A2'pA2'pA | $n = 2$ | R = GDP |
| IV | pA2'pA | $n = 1$ | R = OH |
| V | p ₃ A (ATP) | $n = 0$ | R = p ₂ |
| VI | p ₃ A2'pA | $n = 1$ | R = p ₂ |
| VII | p ₃ A2'pA2'pA | $n = 2$ | R = p ₂ |

Fig. 3. P₁,P₃-(5''-Guanosyl-5'-adenylyl-(3'-5')-2',3'-isopropylideneadenosyl)-triphosphate.

Compound **VIII**.

3. Results and Discussion

The non-methylated parent compound Gp₃A of cap-type dinucleoside triphosphates is cleaved by a dinucleoside triphosphatase (EC 3.6.1. x) of rat liver nuclei [25]. This enzyme may be involved, in principle in the decapping of mRNA. It is unknown, so far, if this nuclear enzyme requires capped oligonucleotides of defined length as described for example for a decapping nuclease detected in HeLa-cell extracts. The HeLa-cell-enzyme failed to cleave caps in capped oligonucleotides with a length longer than ten nucleotides [14].

As (2'-5')-oligoadenylates are known to enhance the activity of a cellular endoribonuclease, it was of interest to see if these unusually linked oligonucleotides affect decapping as well as exonucleolytic activities in rat liver nuclei. Table I shows degradation rates obtained for the inhibition of Gp₃A-cleavage by the aforementioned enzymatic decapping activities, *i.e.* nuclear dinucleoside triphosphatase, in the presence of equimolar amounts of different (2'-5')-oligoadenylates. The degradation rate of Gp₃[¹⁴C]A alone was found to be 11 nmol × min⁻¹ per mg nuclei protein under the conditions applied. Addition of p₃A (ATP) does not affect Gp₃A-cleavage at all whereas pA2'pA (expt. 3) inhibits slightly and p₃A2'pA (expt. 4), p₃A2'pA2'pA (expt. 5) as well as Gp₃A2'pA (expt. 6) reduce the Gp₃A-degradation rate markedly.

[³H]Gp₃A3'A-2',3'-*iso*-prop (compd. VIII, Fig. 3) was used as a 5'-capped mRNA fragment. This compound is not a substrate for 3'-exonucleases due to the 2',3'-isopropylidene group which mimics a 3'-poly (A) sequence. Degradation of compound

Table II. Cap-degradation rates of [³H]Gp₃(A2'p)_nA (n = 1,2).

| Compound (compare Fig. 2) | nmol × min ⁻¹ per mg nuclear protein |
|---------------------------------|--|
| Gp ₃ A (I) | 11.0 ^a |
| Gp ₃ A2'pA (II) | 2.5 |
| Gp ₃ A2'pA2'pA (III) | 2.5 |

^a The degradation rate was determined during the linear phase of assaying (up to 5 min); range of confidence (95%): ± 2.0.

VIII by the nuclear triphosphatase (expt. 7) results in a [³H]GMP-liberation rate of 7 nmol × min⁻¹ per mg nuclei protein. Addition of equimolar amounts of p₃A2'pA2'pA (expt. 8) inhibits the decapping of compound VIII markedly.

Additional studies have been performed with [³H]Gp₃A2'pA and [³H]Gp₃A2'pA2'pA in the absence of Gp₃A in order to investigate the stability of the Gp₃A-terminus in mRNA-like capped (2'-5')-oligoadenylates during assaying (Table II). Product analysis of the incubation assay demonstrates that the degradation rate of [³H]Gp₃(A2'p)_nA (n = 1,2) is about five-fold slower compared to Gp₃[¹⁴C]A. Furthermore, as Gp₃A is formed as an intermediate product during the course of Gp₃(A2'p)_nA incubation with rat liver nuclei, it is necessary to correct for Gp₃A cleavage which amounts to 10 percent (Table I, expt. 6). This degradation is obviously caused by a (2'-5')A_n-phosphodiesterase activity present in rat liver nuclei. This enzyme, which has been detected in L cells [34], mouse reticulocytes [35] and HeLa cells [36] attacks (2'-5')-oligonucleotides from the free 2'(or3')-end.

Table I. Cap-degradation rates of Gp₃[¹⁴C]A and [³H]Gp₃A3'pA-2',3'-*iso*-prop. in the presence of various (2'-5')-oligoadenylates.

| Expts. | Compound (compare Figs. 2, 3) | | nmol × min ⁻¹ per mg nuclear protein |
|--------|---|--------------|--|
| 1 | Gp ₃ A | (I) | 11.0 ^a |
| 2 | Gp ₃ A + p ₃ A(ATP) | (I + V) | 12.0 |
| 3 | Gp ₃ A + pA2'pA | (I + IV) | 8.0 |
| 4 | Gp ₃ A + p ₃ A2'pA | (I + VI) | 5.5 |
| 5 | Gp ₃ A + p ₃ A2'pA2'pA | (I + VII) | 3.5 |
| 6 | Gp ₃ A + Gp ₃ A2'pA | (I + II) | 4.0 ^b |
| 7 | Gp ₃ A3'pA-2',3'- <i>iso</i> -prop. | (VIII) | 7.0 |
| 8 | Gp ₃ A3'pA-2',3'- <i>iso</i> -prop. + p ₃ A2'pA2'pA | (VIII + VII) | 2.0 |

^a The degradation rate was determined during the linear phase of assaying (up to 5 min); range of confidence (95%): ± 2.0.

^b Corrected by the amount of Gp₃A liberated from Gp₃A2'pA.

Furthermore, a 3'(or2')-terminal phosphate group protects against enzymatic cleavage whereas esterification of the 5'-terminus does not [7]. Capped (2'-5')-oligoadenylates, *i.e.* $Gp_3(A2'p)_nA$, thus can be degraded in rat liver nuclei yielding Gp_3A . However, the level of phosphodiesterase activity is low because the inhibitory action of the (2'-5')-oligoadenylates remains unaltered during assaying up to 15 min. This was confirmed by preincubation studies of the (2'-5')-oligoadenylates with rat liver nuclei over a period of 30 min.

The results obtained show that (2'-5')-oligoadenylates bearing either a 5'-terminal triphosphate (expt. 4, 5) or a 5'GTP-group (expt. 6) inhibit the activity of a nuclear dinucleoside triphosphatase. On the other hand, these (2'-5')-oligoadenylates do not enhance exonucleolytic activities. This was demonstrated by preincubation (30 min) of rat liver nuclei with the (2'-5')-oligoadenylates which do not alter the rate of $Gp_3[^{14}C]A$ -degradation (see expts. 4, 5). It is known from different reports [4-7] that the (2'-5')-oligoadenylate activated

endoribonuclease cleaves single-stranded RNA primarily at the 3' side of UA-, UG- and UU-sequences. Due to this specificity the nuclease preferentially acts on viral mRNA [7] and thereby "protects" cellular mRNA of virus infected cells. According to our studies with intact rat liver nuclei which show an inhibition of cap degrading enzyme activities by different (2'-5')-oligoadenylates a synergistic effect may be assumed *in vivo*. This means, cellular mRNA is a poor substrate for the (2'-5')-oligoadenylate induced endoribonuclease. In addition, cellular mRNA is protected against 5'-exonucleolytic activities because a degradation of its cap structure is inhibited by (2'-5')-oligoadenylates. Thus, cellular metabolism seems to be protected twice in virus infected cells.

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