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# PRESENCE OF 2′–5′ A SYNTHETASE IN DOG LIVER

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## 1. Introduction

Since the discovery of cyclic AMP by Sutherland [1], the concept that extracellular signals modulate the biology of their target cells by generating intracellular secondary signals has become well established. The effects of many hormones and neurotransmitters are mediated by intracellular calcium, cyclic AMP and cyclic GMP; the generation and action of these signals are now amongst the most investigated subjects in the field of cell regulation. However, the mechanism of action of several hormones and neurotransmitters and their control of fundamental cell processes such as growth, atrophy, or differentiation cannot be satisfactorily explained by the modulation of these signals. Therefore, our aim is to search for other general secondary intracellular signals. The existence of an unusual oligonucleotide, pppA2′p5′A2′p5′A (2′–5′ A) [2], discovered in interferon-treated cells [3,4] as a signal activating an endonuclease [5], and the occurrence of a 2′–5′ A synthetase activity in untreated cells* [6–8] led to the hypothesis that 2′–5′ A could be a general intracellular signal. Here, we have tested this hypothesis by investigating the presence of 2′–5′ A synthetase activity in a wide variety of tissues.

## 2. Materials and methods

Fibroblasts (Balb/3T3 clone A31) were grown as monolayer in Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 2% penstrep (5000 IU/ml) and 1% glutamine (200 mM); mouse erythroleukaemia Friend cells (clone M2) were cultured in suspension in Dulbecco's modified Eagle's medium with a supplement of vitamins, glutamine, amino acids and 10% foetal calf serum [9]; dog thyroid cubes (0.2 mm) were cultured in basal Eagle medium with Earles salts containing 2% penstrep, 1% fungizone, 1% glutamine and 20% dog serum. All the cultures were performed at 37°C in an atmostphere of 5% $CO_2$ and 95% air. Tissue samples obtained from animals used for physiological experiments were collected in phosphate-buffered saline and frozen at –70°C.

Cultured cells and finely minced frozen tissues were homogenized in a solution containing Hepes 10 mM (pH 7.5)–KCl 10 mM–$Mg(OAc)_2$ 2 mM–2 mercaptoethanol 7 mM with 1 ml of buffer for $10^8$ cells or 1 g frozen tissue. The homogenate was supplemented with glycerol (20% final) and KCl (50 mM final) and centrifuged at 40 000 rev./min with rotor type 65 in an ultracentrifuge spinco L50 (100 000 × $g$) for 60 min.

Throughout the preparation of the enzyme similar buffers were used: DBG; Hepes 10 mM (pH 7.5)–$Mg(OAc)_2$ 1.5 mM–2 mercaptoethanol 7 mM–glycerol 20%. DBG-50 is the same buffer supplemented with KCl 50 mM, DBG-90 with KCl 90 mM and DBG-50-15 with KCl 50 mM–$Mg(OAc)_2$ 15 mM. To avoid the action of inhibitory phosphodiesterases, the enzyme present in the supernatant was bound to poly(I)–poly(C) Sepharose 4B or to poly(I)–poly(C) paper. In the first case, columns of poly(I)–poly(C) Sepharose 4B (prepared using CNBr-activated Sepharose 4B (Pharmacia) and the method in [6]) charged with 100–200 μl supernatant were washed extensively with 5 ml DBG-50, 5 ml DBG-90 and 2 ml DBG-50-15, at room temperature. They were then incubated at 30°C for 17 h in the presence of

* The abbreviation 2′–5′ A designates a family of oligonucleotides characterized by 2′–5′-phosphodiester bonds

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[8-$^3$H]ATP (1.5 mM, spec. act. 22 Ci/mmol) in DBG-50-15 [6]. Poly(I)–poly(C) papers (prepared using diazobenzyloxymethyl paper [8]) 1 $cm^2$ were incubated with 100–400 $\mu l$ of supernatant for 1 h at room temperature, then washed 3 times with DBG-50 and once with DBG-90 and DBG-50-15 [8]. After drying, they were incubated 17 h at 30°C in the presence of [8-$^3$H]ATP (1.5 mM, spec. act. 22 Ci/mmol). 2′–5′ A prepared by the two methods was separated from ATP by chromatography on DEAE-cellulose. The columns (0.2 ml DEAE-cellulose) were washed with 35 ml of Hepes 20 mM pH 7.4–KCl 90 mM and eluted with 2 ml Hepes 20 mM pH 7.4–KCl 350 mM [3]. The latter fraction is tentatively called 'putative 2′–5′ A'.

Labelled 28 S RNA was prepared from V79 Chinese hamster lung fibroblasts ($10^6$ cells/ml) incubated with [$^{32}$P]phosphate. The RNA was isolated by phenol/chloroform extraction and centrifugation through a gradient of sucrose (5–30%) [10]. For the determination of 2′–5′ A-stimulated nuclease activity, postmitochondrial supernatant fractions from HeLa cells (2 $\mu l$) were incubated with [$^{32}$P]RNA (15 000 cpm) in the presence of KCl 75 mM for 1 h at 30°C, and the boiled samples were analyzed by SDS–polyacrylamide gel (7.5%) electrophoresis and autoradiography [11].

## 3. Results and discussion

The method utilized to detect the presence of 2′–5′ A synthetase activity is based on the assay developed in [8]. The enzyme retained on poly(I)–poly(C) paper generates the putative 2′–5′ A which is then separated as a peak II on DEAE-cellulose; its activity, expressed as equivalent AMP, is calculated from its radioactivity and the specific activity of its precursor ATP. As measured by this method, the enzyme from interferon-treated L929 cells produces 130 pmol . $10^8$ cells$^{-1}$ . 17 h$^{-1}$ and that from the reticulocyte 59 pmol . ml lysate$^{-1}$ . 17 h$^{-1}$. The results are in accordance to those in [8]. This method allows the detection of relatively low levels of 2′–5′ A synthetase; nevertheless, we decided to consider as a significant result a production of 2′–5′ A > 3 pmol . $10^8$ cells (1 ml lysate, 1 g tissue)$^{-1}$ . 17 h$^{-1}$; i.e., 2–5-times the value obtained for untreated L929 cells (control). We obtained the following results for the various growth phases of cultured cells and tissues studied: little activity (similar to the control) was detected in fibroblasts at various phases of growth (after 1 and 2 days of culture, growth phase; and 3 days, stationary phase), in thyroid organotypic fragments (after 1, 2 and 3 days of culture) or in M2 Friend mouse erythroleukaemia cells. In the latter case, an activity similar to the one found in another clone (F4N) would have been easily detected [8].

2′–5′ A synthetase activity was detected at a significant level in two dog tissues, the liver and the muscle. A lower but detectable activity was found in the supernatant of heart, brain and bladder. No activity (as the control) was found in kidney, lung and intestine (table 1). Beef and rat liver also contained 2′–5′ A synthetase but less than dog liver: 8.5 and 4 pmol . g tissue$^{-1}$ . 17 h$^{-1}$, respectively.

The peak II on DEAE-cellulose chromatography obtained with the rabbit reticulocyte lysate had been analyzed according to different criteria and identified as 2′–5′ A [12]. Our next step was to establish whether the putative 2′–5′ A generated by the dog liver enzyme was similar to that obtained in the same experimental conditions from the reticulocyte lysate. The different criteria utilized to establish the identity of the 2′–5′ A and putative 2′–5′ A were the following:

(i) Heat stability;
(ii) Existence of a series of products corresponding to the trimer, the tetramer, etc.;
(iii) Comigration on thin layer of polyethyleneimine cellulose;
(iv) Activation of a nuclease.

In both cases, the material generated by poly(I)–poly(C) bound enzyme was retained on the DEAE-cellulose column, whether boiled or not, for 5 min. Chromatography of both products on DEAE–Sephadex A25 eluted with NaCl gradients (100–350 mM) containing urea 7 M [13] showed a very similar series of peaks (fig.1). The small difference observed between the elution patterns results from the heterogeneity of the 2′–5′ A phosphate tails [14]. Nevertheless, we found in the two cases that the different species have apparent net charges included between –5 and ~–7.5 with the major species (probably the trimer) between –5.7 and –6.5 [2]. The enzymes from both the dog liver and the reticulocyte generated from ATP a mixture of products which separated on DEAE–Sephadex A25 according to their charge, i.e., the size of the oligomers. Whatever the solvent systems used in chroma-

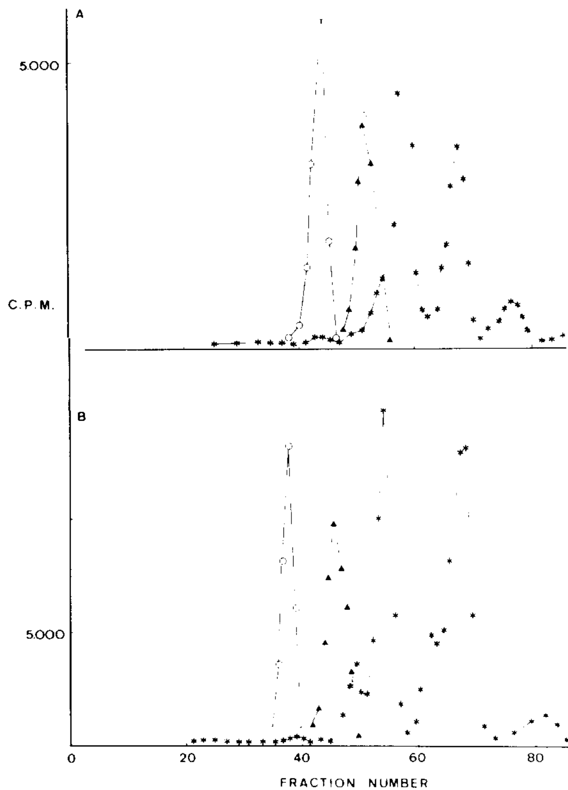


Fig.1. DEAE–Sephadex A25 chromatography of $^3H$ products generated by poly(I)–poly(C) retained enzyme from reticulocyte lysate and dog liver supernatant. Peaks II of DEAE-cellulose chromatography were applied to a column of DEAE–Sephadex A25 (0.5 × 17 cm) equilibrated with NaCl 100 mM in Tris–HCl 20 mM (pH 7.6)/urea 7 M (TU buffer). Elution (1 ml fractions, 5 ml/h) was with a gradient of 100 mM–350 mM NaCl (50 ml–50 ml in TU buffer) ATP (○) and $P_4A$ (▲) were included as markers: (A) the oligonucleotide derived from reticulocyte (∗); (B) the oligonucleotide derived from dog liver (∗).

tography on polyethyleneimine cellulose plates, the putative 2′–5′ A from the two sources migrated identically. Similarly, the products of putative 2′–5′ A digestion by alkali, bacterial alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) or snake venom phosphodiesterase (oligonucleate 5′-nucleotide hydrolase, EC 3.1.4.1) comigrated in these systems [15] (fig.2). In the 3 solvent systems, the products of snake venom phosphodiesterase digestion comigrated with 5′-AMP. The products from the alkali treatment migrated as 2′-AMP and 3′-AMP in acetic acid 1.0 M (not shown).

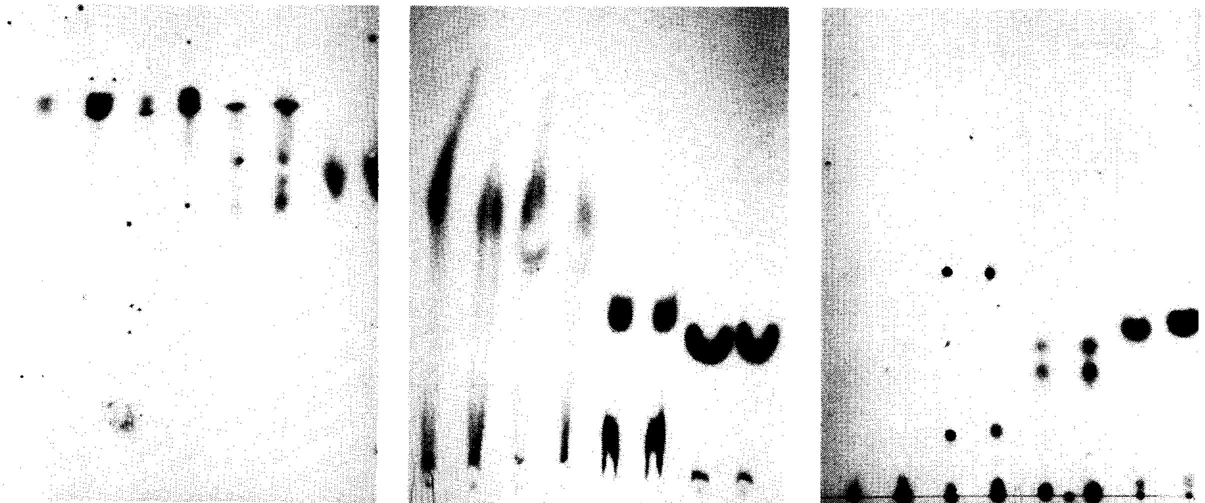

Fig.2. Thin-layer chromatography of the putative 2′–5′ A synthesized by the enzyme derived from reticulocyte and dog liver supernatant. The two products, labelled with $^{32}P$ (spec. act. 2000–3000 Ci/mmol) were partially purified on DEAE-cellulose (peaks II) precipitated with acetone and subjected to thin-layer chromatography on PEI-cellulose plates before and after enzymic digestion and alkali treatment. Chromatography solvents used were: (left) 0.75 M $KH_2PO_4$ (pH 3.4); (center) LiCl 1.0 M; (right) acetic acid 1.0 M: (1,2) Reticulocyte and liver preparation before treatment; (3,4) after digestion with bacterial alkaline phosphatase (BRL, 2 units, 37°C, 1 h); (5,6) after treatment with alkali (KOH 0.3 M, 37°C); (7,8) after digestion with snake venom phosphodiesterase (Boehringer, 80 μg, 37°C, 4 h).

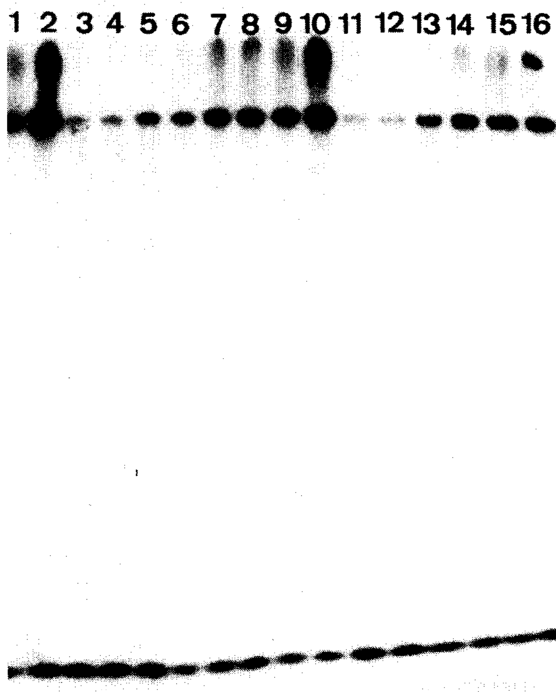


Fig.3. Activation of 28 S RNA hydrolysis in HeLa cells supernatant in the presence of the putative 2′–5′ A generated by reticulocyte lysate and dog liver supernatant. An autoradiograph of the dried gel is shown; (1,9) $^{32}$P-labelled RNA substrate without 2′–5′ A; (2) 2′–5′ A (0.3 μM without HeLa supernatant; (3–8) in the presence of peak II from reticulocyte lysate with, respectively, 0.3 μM, 30 nM, 3 nM, 1 nM, 0.3 nM and 0.1 nM of peak II (AMP equivalent); (11–16) as (3–8) but with peak II formed by dog liver supernatant.

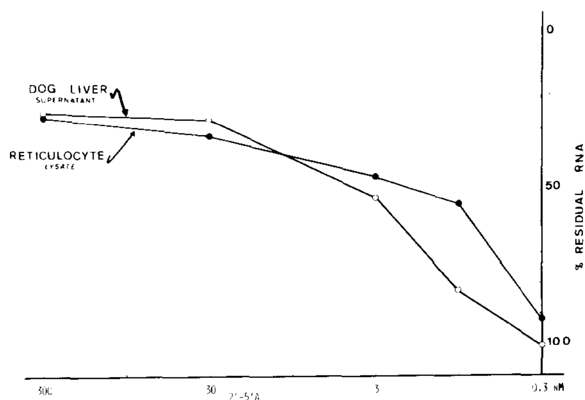


Fig.4. Effect of putative 2′–5′ A concentration on 28 S RNA hydrolysis by postmitochondrial supernatant of HeLa cells. The dried gel and autoradiograph were superimposed and the area of the gel corresponding to the amount of [$^{32}$P]RNA remaining at the origin were cut out and counted.

The existence of multiple components in a 2′–5′ A preparation as shown by chromatography on DEAE–Sephadex A25, was confirmed in both cases by the results obtained by bacterial alkaline phosphatase digestion. The resulting products were indeed resolved into several species by chromatography in 1.0 M acetic acid. Treatment with that enzyme released the core of 2′–5′ A: i.e., in the case of the trimer pppApApA: ApApA; and for the tetramer and the pentamer according to the same mechanism, ApApApA, ApApApApA, which were well separated using 1.0 M acetic acid [16].

The postulated antiviral action of 2′–5′ A seems to be mediated by the activation of an endonuclease [5] which degrades RNA. Indeed 2′–5′ A generated by reticulocyte lysate and putative 2′–5′ A activated the hydrolysis of $^{32}$P-labelled 28 S RNA (fig.3). At ~3 nM (in AMP equivalents) >50% of the $^{32}$P-labelled 28 S RNA in the medium was hydrolyzed. Hydrolysis was dependent on 2′–5′ A at ⩽30 nM (fig.4).

It appears thus that the putative 2′–5′ A generated from ATP by the dog liver supernatant is similar according to our proposed criteria to the reference reticulocyte lysate 2′–5′ A. The behaviour of the native products of their hydrolyzed derivatives were similar whether they were chromatographed on

Table 1
Generation of 2′–5′ A by supernatant of different tissues and cells

| Origin | Putative 2′–5′ A (pmol) = 1 ml lysate or 1 g tissue in 17 h |
|---|---|
| Dog heart | 2 ± 0.2 |
| Dog liver | 18 ± 2 |
| Dog brain | 2.3 ± 0.2 |
| Dog kidney | 0.5 ± 0.1 |
| Dog lung | 0.9 ± 0.1 |
| Dog bladder | 1.7 ± 0.2 |
| Dog muscle | 3.5 ± 0.3 |
| Dog intestine | 0.7 ± 0.1 |
| Rabbit reticulocyte lysate | 59 ± 5 |
| Control (untreated L929 cells) | 0.6 –1.4 |

The enzyme present in the different supernatants was bound on poly(I)–poly(C) Sepharose 4B and the amount of 2′–5′ A synthesized was calculated from its radioactivity and the specific activity of its precursor ATP

DEAE–Sephadex A25 or on a thin layer of polyethyleneimine cellulose, as was their activity in the activation of ribonuclease.

In conclusion, dog liver supernatant contains in the presence of dsRNA and enzyme synthesizing from ATP a product which has the properties of 2′–5′ A. The possibility that the enzyme could be derived from liver blood is unlikely as neither serum [17] nor red cells could account for the activity recovered: 31 pmol . g tissue$^{-1}$ . 17 h$^{-1}$ for dog liver, 3 pmol . ml lysate$^{-1}$ . 17 h$^{-1}$ for dog red cells and undetectable for dog serum.

pppA2′p5′A2′p5′A synthetase could possibly result from liver viral infection, but the demonstration of the activity in different healthy animals of two species bears against this hypothesis. Nevertheless, little enzyme was found in mouse liver [8]. Although these present data, obtained in an acellular system may not reflect the true in vivo activity of 2′–5′ A synthetase, and the presence of dsRNA in intact cells is unproven, we are now studying the possible role of 2′–5′ A synthetase in cell regulation in the liver.

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