Volume 8 Number 14 1980 Nucleic Acids Research

2'5' Oligoadenylate synthetase, an interferon induced enzyme: direct assay methods for the products, 2'5' oligoadenylates and 2'5' co-oligonucleotides

J.Justesen, D.Ferbus and M.N.Thang

Institut de Biologie Physico-Chimique, 13, rue Pierre et Marie Curie, 75005 Paris, France

Received 3 June 1980

ABSTRACT

The interferon induced enzyme 2'5' oligoadenylate synthetase produces 2'5' $pppA(pA)_n$ the first discovered natural nucleotide with a 2'5' linkage. We describe a direct assay of this enzyme based on separation by thin layer chromatography (TLC) of the substrate ATP and the products 2'5'$pppA(pA)_n$ ($n \geq 1$). This technique presents obvious advantages compared to the currently used methods. Moreover the enzyme uses other nucleotides as substrates forming co-oligonucleotides 2'5'$pppA(pA)_n pN$ (N = U,G,C,dA,dG,dT and dC). Additional procedures are described using different developing solvent systems for the separation of the core-2'5' oligonucleotides (2'5'$A(pA)_n pN$) containing AMP-residues entirely and those with another nucleotide at the 2'end.

INTRODUCTION

The 2'5' oligoadenylates are low molecular weight presumed mediators of the interferon induced inhibition of protein synthesis. The oligoadenylates are formed after activation by double stranded RNA of the 2'5' oligoadenylate synthetase. This enzyme is present not only in interferon treated cells (review by Baglioni (1)) but also in reticulocyte lysate (2) and all mamalian cells tested (3). The 2'5' oligoadenylate synthetase catalyzes the reaction

$$(n+1)\ ATP \rightleftharpoons ppp5'A(2'p5'A)_n + n\ PPi \quad n \geq 1$$

The products of the reaction have been shown to be the same for the enzyme from either source (4). Intact 2'5' oligoadenylates can be assayed by their inhibiting effect on translation (5). So far the analysis of the oligonucleotides by TLC (6,7) or paper electrophoresis (8) has only been performed after digestion with alkaline phosphatase leaving the core nucleotide without the 5' terminal triphosphate group. In this report we present a direct method for separating ATP and 2'5' $pppA(pA)_n$ by PEI-cellulose thinlayer chromatography without any treatment of the products.

Incubating 2'5' oligoadenylate synthetase with ATP plus another ribo-

or deoxyribonucleoside triphosphate results in formation of the 2'5' pppA $(pA)_n$, n = 1,2,3,... plus co-oligonucleotides 2'5'ppp$A(pA)_n$pN, n = 0,1,2,3,.., N=G,U,C,dG,dC,T, 2' or 3' dA (9). The core nucleotides from these cooligonucleotides after alkaline phosphatase treatment can be analyzed by different types of PEI-cellulose chromatography. We present 3 systems that complement each other so that all differences are revealed.

MATERIALS AND METHODS

Materials

Radioactive nucleotides were obtained from the Radiochemical Centre (Amersham). PolyI.polyC and polyI.polyC-agarose were from Choay, France. Calf intestine alkaline phosphatase from Boehringer. Polyethylene imine (Polymin P) was a gift from BASF, France. Cellulose MN 300 from Macherey Nagel & Co. Nuclease P1 and 2'5'ApA from Ymosa Shogo Co. Tokyo, Japan. Nuclease U2 from Sigma. Snake venom phosphodiesterase (SVPD) from Boehringer. 3'5'ApApA and 3'5'$A(pA)_4$ were prepared in our laboratory.

The PEI-cellulose thin layer plates are produced according to the procedure thoroughly described by Randerath and Randerath (10).

Assay for 2'5' adenylate synthetase

The incubation mixture (20 µl) contains : 20 mM Tris-HCl pH 8.0 20 mM $Mg(OAc)_2$, 0.1 mg/ml Bovine Serum Albumin, 10% glycerol (v/v), 4 µg/ml polyI.polyC, 1 mM dithiothreitol, 1 mM ATP and (ca.) 0.02 µCi $\alpha(^{32}P)$ATP besides an aliquot of extract containing the enzyme. After 1 or 2 hours at 37°C the reaction is stopped by heating to 72°C for 2 minutes or by immediate application onto PEI-cellulose thin layer plates.

Chromatography of pppA$(pA)_n$

2-8 µl of the incubation mixture are spotted directly onto a PEI-cellulose thin layer plate (18 x 18 cm) 2 cm from the bottom. After drying the spots, the plate is washed in water (10 min) and in methanol (5 min) drying after each wash. A 4cm broad paperwick (DESAGA paper bridge) is attached to the top with staples. The chromatogram is developed in methanol to 2 cm above the application line, transferred directly to 2 M Tris-HCl pH 8.6 and developed to the top of the paperwick in this solvent. The procedure takes 4 to 6 hours. The chromatogram is dried and exposed to X-Ray film overnight. When tritium compounds are used the spots are localized by fluorography : the thin

layer sheet is treated with 1 g PPO (2,5 Diphenyl oxazole) in 15 ml ether, that evaporates quickly. The plate is exposed to Kodak X-omat film at -80°C.

Chromatography of $A(pA)_n$

To 10 µl of the incubation mixture is added 3 µl of a solution of 25 µg/ml alkaline phosphatase from calf intestine in 0.1 M Tris-HClpH 9.0, and this is incubated at 37°C for 2 hours. 5-10 µl of this is applied onto PEI-cellulose plates. The plates are dried, then washed in methanol (5 min) and dried.

Three solvent systems are used for the development :

- A : Chromatography in a gradient of Tris-HCl in Urea.

This procedure is a modification of a method described by Gupta and Randerath (11).

The chromatogram is developed in a serie of solvents. The chromatogram is moved to the next solvent when the liquid front has reached the level that is indicated in centimeter (cm) above the application line : methanol (1 cm), water (2 cm), 0.05 M Tris-HCl pH 8.0 (5 cm), 0.25 M Tris-HCl pH 8.0 7.2 M urea (9 cm), 0.50 M Tris-HCl pH 8.0 7.2 M urea (12 cm), 0.75 M Tris-HCl pH 8.0 7.2 M urea (15 cm), 1 M Tris-HCl pH 8.0 7.2 M urea (to the top). There is no drying in between. Finally the plate is dried and washed in methanol to remove the urea. The plate is exposed to X-ray film as described above.

- B : Chromatography in acetic acid and LiCl.

The chromatogram is developed in 1 M Acetic acid to 6.5 cm above application line, transferred directly to 0.3 M LiCl and developed to the top. The plate is dried and exposed as described above.

- C : Chromatography in LiCl and Boric acid.

A paperwick is attached to the top of the chromatogram which is developed in 0.2 M LiCl, 5% Boric acid pH 7.0 to 8 cm above application line, transferred to 0.5 M LiCl, 5% Boric acid pH 7.0, and developed to the top. The plate is dried and treated as above.

Hydrolyses

The oligonucleotides were treated with nucleases P1 and U2 and with KOH as described by Kerr and Brown (6).

Purification of 2'5' oligoadenylate synthetase

Rabbit reticulocyte lysate (12) was passed through a DEAE-cellulose column (5 x 25 cm) equilibrated with buffer D (20 mM Tris-HCl pH 8.0, 5 mM $Mg(OAc)_2$, 25 mM KCl, 1 mM dithiothreitol, 10% glycerol (v/v)). The flow-through fraction is applied to a polyI.polyC-agarose column (1 x 3 cm). After washing with buffer D plus 0.1 M KCl the 2'5' oligoadenylate synthetase activity is eluted by 0.2 M KCl in buffer D. To the active fractions is added $(NH_4)_2SO_4$ to 46% and the precipitate is removed. By addition of 50 µg/ml polyI.polyC the synthetase activity precipitates out. The precipitate is redissolved in buffer D, dialyzed against buffer D and stored at -80°C. Results from a purification are given in table 1. 1 unit of enzyme activity is defined as the incorporation of 1 nanomole of AMP residue into $pppA(2'p5'A)_n$, n = 1,2,3,... per minute.

Preparation of 2'5' oligoadenylates.

2'5' oligoadenylate synthetase (4-8 units) is incubated in 0.5-1.0ml with ATP in a mixture with the same composition as in the enzyme assay except that the ATP concentration is varied between 1 and 3 mM. After overnight incubation, 5 volumes of acetone at -20°C is added to precipitate the oligonucleotides. After at least 2 hours the precipitate is collected by centrifugation, dried and redissolved in 0.2 M NH_4HCO_3(0.5 ml). This is applied onto a DEAE-

TABLE 1

Purification of 2'5' oligoadenylate synthetase.

	Volume ml	Protein conc. mg/ml	Specific activity units/mg protein	Total activity units
Reticulocyte lysate	85	160	-	-
DE-52 pass-through	105	32	0.13	440
PolyI.polyC-agarose chromatography	9.7	0.35	-	-
Precipitation with 50 µg/ml polyI.polyC in 46% ammonium sulfate sodium	0.90	2.7	32	80

The purification of 2'5' oligoadenylate synthetase was performed as described in Methods. The activity was measured, under the standard assay conditions with 1 mM ATP.

Sephadex (A-25) column (1 x 20 cm). After washing with 20 ml 0.2 M NH_4HCO_3 the nucleotides are eluted with a gradient of 0.2 M to 0.8 M NH_4HCO_3 (2 x 50 ml) (figure 1A). This is a slight modification of the method of Martins et al. (4). The fractions containing the oligoadenylates are pooled after beeing tested by chromatography on PEI-cellulose plates as described (figure 1B). The pooled fractions are lyophilized twice and redissolved in H_2O.

RESULTS

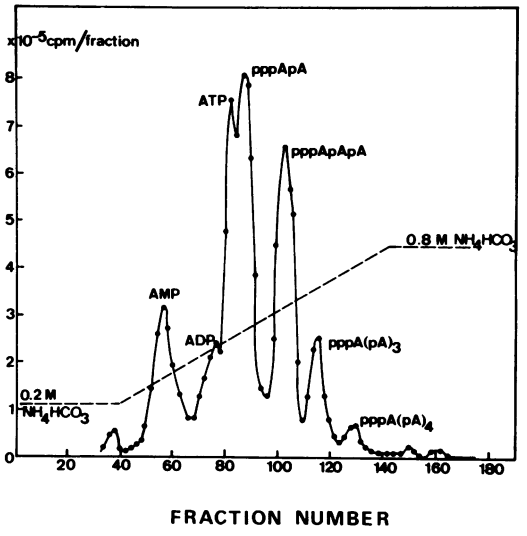
Separation of 2'5' $pppA(pA)_n$

A direct separation of the substrate ATP and the products 2'5' $pppA(pA)_n$ n = 1,2,3,4,5 of the 2'5' oligoadenylate synthetase reaction was achieved by PEI-cellulose chromatography with 2 M Tris-HCl pH 8.6 as developer (figure 1B and 2). The pH value is very critical for a good resolution. Small changes in pH modify significantly the R_f values and diminishes the separation effect. At pH values higher than 8.6 the migration of the oligoadenylates is too small, and at pH lower than 8.4 the R_f values do not differ significantly. Moreover a good resolution was obtained with the PEI-cellulose plates prepared as described, while commercially obtainable plates had a lower capacity and an inferior separation presumably because they are thinner and have a lower concentration of PEI.

Confirmation of the structure of the 2'5' $pppA(pA)_n$

The presumed 2'5' oligoadenylates were treated with nucleases P1 and U2, known to be inactive against 2'5' linkages in nucleotides (2,13). The preparations of oligonucleotides were resistant to these nucleases under conditions where 3'5'$A(pA)_2$ and 3'5'$A(pA)_4$ were totally degraded (data not shown). Alkaline hydrolysis of 2'5' oligoadenylates yielded 2'AMP and 3'AMP although apparently at a lower rate than hydrolysis of the 3'5' oligoadenylates.

The presence of the triphosphate group was demonstrated by the retention of the γ-phosphate of ATP in oligoadenylates : Incubations in the presence of $\gamma(^{32}P)$ATP gave rise to labelled pyrophosphate and also labelled oligoadenylates (data not shown). Double labelling with $\gamma(^{32}P)$ATP and (^{3}H)ATP confirmed that 2'5' pppApA contains two and 2'5'pppApApA contains three adenosine groups per terminal phosphate group (data not shown). The 2'5'$A(pA)_n$ also have different R_f values compared to two 3'5' $A(pA)_n$ tested in the chromatography system A (Tris gradient as solvent) (figure 3).



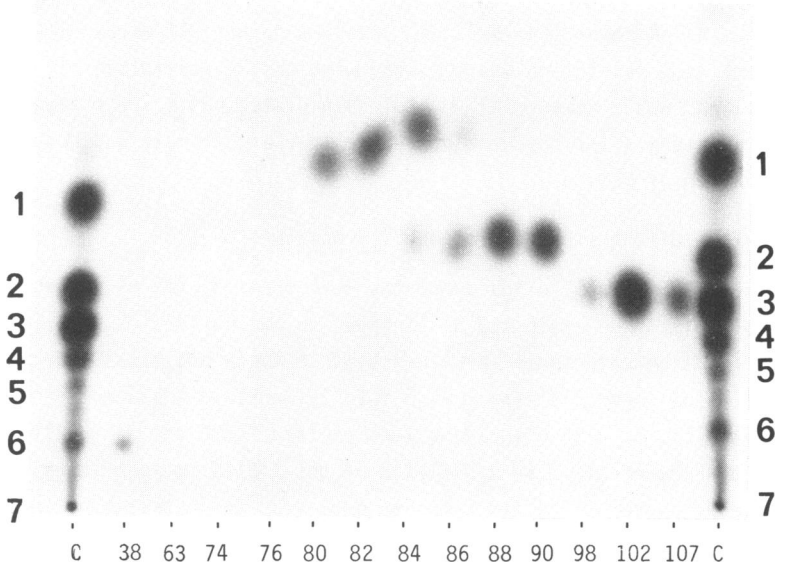


Fig. 1 - Preparation of 2'5' pppA(pA)$_n$, n = 1,2,3,4.

Purified 2'5' oligoadenylate synthetase was incubated as described in Methods in 500 µl, 1 mM ATP and 10 µCi $\alpha(^{32}P)$ATP overnight. A : The synthetized oligonucleotides were separated on a DEAE-sepharose column as described in Methods. B : From the peak fractions 5 µl were analyzed by 2 M Tris-HCl chromatography. The reference sample is an aliquot of the total incubation mixture (C). (1) ATP, (2) 2'5' pppApA,(3) 2'5' pppApApA, (4) 2'5' pppA(pA)$_3$, (5) 2'5' pppA(pA)$_4$, (6) contaminant in the $\alpha(^{32}P)$ATP, (7) application line. The lower half of the chromatogram is shown.

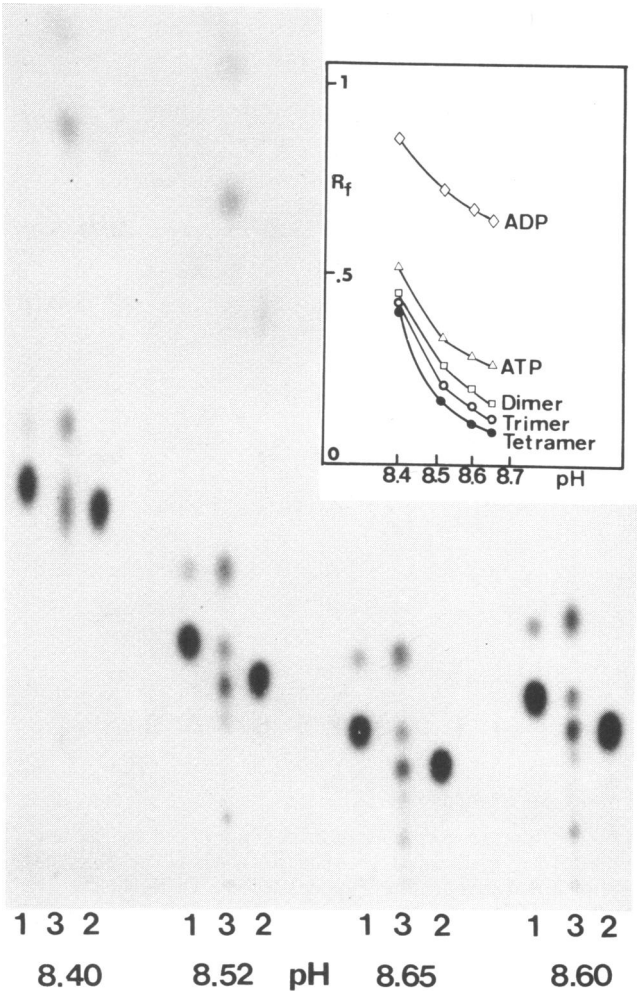


Fig. 2 - The effect of pH variations in solvent

5 µl samples of ^{32}P-labeled nucleotides were applied on PEI-plates. (1) 2'5' pppApA, (2) 2'5' pppApApA, (3) total incubation mixture. The plates were developed as described in Methods with 2M Tris-HCl at the pH indicated. The inset shows a plot of R_f values from the chromatograms versus pH.

2'5' co-oligonucleotides, $pppA(pA)_npN$.

2'5' oligoadenylate synthetase can catalyze the formation of 2'5' co-oligonucleotides as described previously (9). These co-oligonucleotides are formed either by incubating the 2'5' oligoadenylate synthetase with ATP and

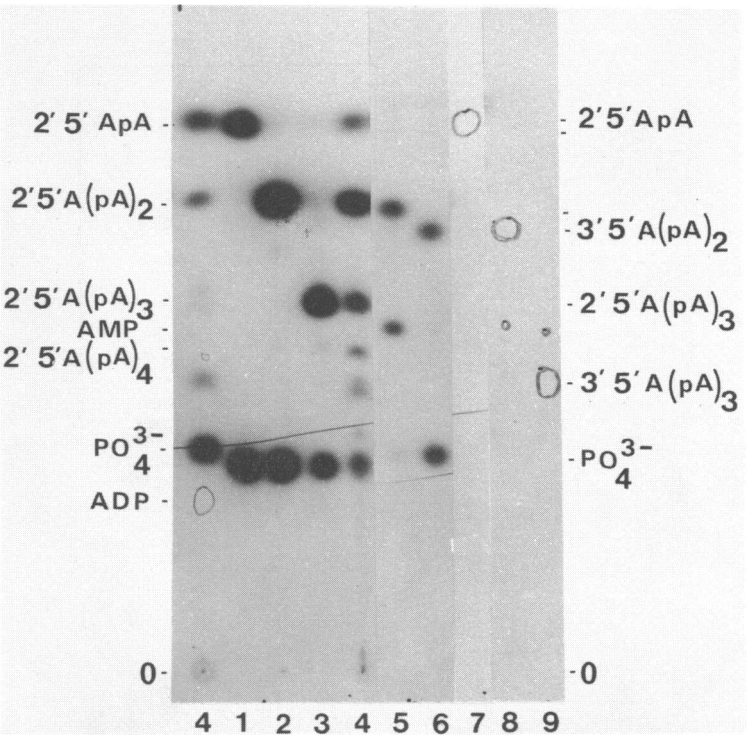


Fig. 3 - Chromatography of $A(pA)_n$

10 µl samples of ^{32}P-labeled oligonucleotides purified by column chromatography as described in Fig. 1 were treated with alkaline phosphatase, then chromatographed and autoradiographed as described in Methods. The samples were (1) 2'5' pppApA, (2) 2'5' pppApApA, (3) 2'5' pppApApApG, (4) total incubation mixture, (5) 2'5' pppApApA and 2'5' pppApApApG, (6) 2'5' pppApApG. Non-radioactive nucleotides : (7) 2'5' ApA, (8) 3'5' $(Ap)_2A$, (9) 3'5' $(Ap)_4A$. The non-radioactive compounds were localized by U.V.-light.

NTP (N stands for one of the normally occurring ribo- and deoxyribonucleosides or cordycepin) or by incubating the enzyme with a mixture of preformed 2'5' oligoadenylate primer and NTP. We have shown previously that the chain is growing in the 2' direction and that the NTP are chain terminators (9), thus suggesting that the structure of the co-oligomers is $pppA(pA)_npN$.

The separation of these co-oligonucleotides from the oligo-adenylates is performed by chromatography with one of the three solvent systems A, B and C described in methods. With system A (Tris-HCl gradient in urea) there are additional spots to the oligoadenylate series when GTP or UTP is present in the

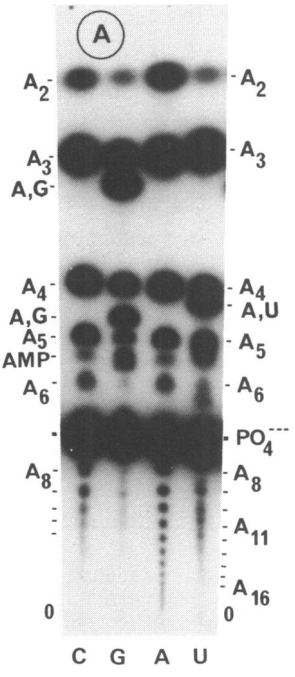


Fig. 4 - Chromatography of co-oligonucleotides.

Purified 2'5' oligoadenylate synthetase was incubated under assay conditions with 1 mM NTP, 1 mM ATP and for A : 60 µCi/ml $\alpha(^{32}P)$ATP, for B and C : 5 µCi/ml (^{14}C) ATP. The different NTP's used are indicated : (A) ATP, (G) GTP, (C) CTP, (U) UTP, (2'dA) 2'dATP, (3'dA) 3'dATP, (εA) εATP, (dG) 2'dGTP, (dC) 2'dCTP, (T) 2'dTTP. After treatment with alkaline phosphatase 5 µl was applied on PEI-cellulose plate.

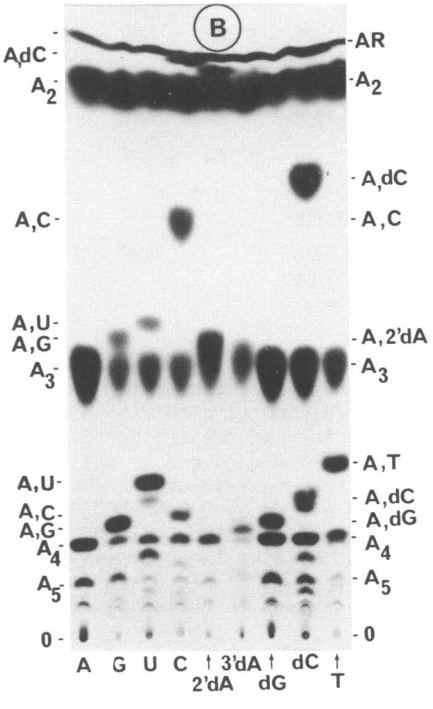
The chromatograms were developed in the different solvent systems as described in Methods

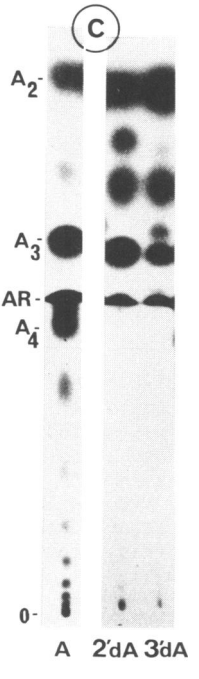
A : a Tris-HCl gradient in urea.

B : 1M acetic acid followed by 0.3M LiCl.

C : 0.25 M and 0.50 M LiCl in 5% Boric acid.

After chromatography the PEI-cellulose plates were autoradiographed. The co-oligonucleotides appearing are marked by A,N designating $(Ap)_nN$, n = 1, 2, 3... The oligoadenylates are designated A_n for $A(pA)_{n-1}$, n = 2,3,4,... . The application line is marked by O.





mixture. These spots are indicated in figure 4A. When the incubation contains a mixture of GTP and ATP three extra spots are visible : ApG, ApApG and ApApApG. The other co-oligonucleotides are not well separated in this system. The traces of the longer chains of oligonucleotides ($A(pA)_n$, $n > 7$) are clearly absent when an extra nucleoside triphosphate is added.

With system B (Acetic acid; LiCl) the resolution of the oligoadenylates is not as high as for system A, but extra spots are found in incubations of nearly all mixtures (figure 4B) corresponding to ApApN, $A(pA)_2N$, $A(pA)_3N$ (N stands for G,U,C,dC,dG and T). System C (LiCl + Boric acid) was used particularly to separate ribo- and deoxyribonucleotides, because the boric acid chelates the ribonucleotide but not the deoxyribonucleotides (figure 4C).

TABLE 2

Composition of co-oligonucleotides

1 spot	2 picomoles eluted G	A	3 ratios A/G	G/A	4 alk. hydrol. products		5 SVPD dig. products		6 deduced structure
1	157	4	0	39	GR		GR		GR
2	101	111	1.1	0.9	GR	2'AMP 3'AMP	AR	5'GMP	2'5' ApG (+2'5'ApA)
3	1	126	126	0	AR	2'AMP 3'AMP	n.d.		2'5' ApApA
4	72	154	2.1	0.5	GR	-	AR	5'GMP	2'5' ApApG
5	1	59	59	0		2'AMP 3'AMP	n.d.		2'5' ApApApA
6	12	40	3.3	0.3		2'AMP 3'AMP		5'GMP	2'5' ApApApG
7	2	19	9.5	0.1	-		-		2'5' ApApApApA

The mixture of 2'5' oligonucleotides after incubation of (^{3}H)ATP (14 cpm/picomole) and (^{14}C)GTP (3.2 cpm/picomole) with 2'5' oligoadenylate synthetase was heated to 72°C for 2 minutes and treated for 2 hours with alkaline phosphatase and heated again to eliminate enzyme activity. This was chromatographed with system A (Tris-HCl gradient in urea). The spots were detected by fluorography (Column 1) and eluted by 2 M NH_4HCO_3 (3 x 50 µl). An aliquot was counted and the contents were calculated (column 2). The remaining eluate was lyophilized and digested by alkaline hydrolysis or SVPD (columns 4 and 5). The deduced structures (column 6) are based on these results and on the observation that they are resistant to nucleases P1 and U2 showing the 2'5' linkage.
GR is guanosine
AR is adenosine
n.d. not determined.

Structure of $pppA(pA)_npG$

The structure of the oligonucleotides formed by incubation of ATP and GTP were examined more closely. After treatment with alkaline phosphatase the 2'5' oligonucleotides were separated by chromatography on PEI-cellulose plates as shown in figure 4A. The spots were eluted from the PEI-cellulose with 2 M NH_4HCO_3 and lyophilized. Quantitation of the two labels in the eluted co-oligonucleotides KOH and with Snake Venom Phosphodiesterase (SVPD) revealed that the structure really was 2'5'pppApG and 2'5'pppApApG (Table 2). Digestion with KOH produces 2' and 3' monophosphates hence the terminating base is found as a nucleoside, whereas digestion with SVPD produces 5' monophosphates and hence the terminating base is found as a 5' nucleoside monophosphate and the 5' terminal base is found as a nucleoside. 2'5' pppApApG was difficult to degrade by alkaline hydrolysis compared to 2'5'pppApApA and $3'5'A(pA)_3$.

DISCUSSION

The low molecular weight inhibitors of protein synthesis in interferon treated cells have been identified as $2'5'pppA(pA)_n$ n = 2,3,4,...(6,13,14). However no direct nor quantitative assay method for them has been available. The separation of ATP and $2'5'pppA(pA)_n$ by PEI-cellulose thin layer chromatography with 2 M Tris-HCl pH 8.6 as eluant is a simple, quick and quantitative method that makes it possible to assay for 2'5' oligoadenylate synthetase in cell extracts as well as in purified systems. An assay method for its products permits a characterization of the 2'5' oligoadenylate synthetase. An analysis of this type is particularly important for this enzyme where all the products $2'5'pppA(pA)_n$ can also be substrates. The direct separation makes it possible to use $\gamma(^{32}P)$ATP for studying the composition of co-oligonucleotides and the direction of chain growth (9).

The treatment with alkaline phosphatase which removes the 5' terminal triphosphate group allows several variations in chromatography on PEI-cellulose plates. The separations are perfect when ATP and one other NTP is present. These systems evidently can not show whether the 5' triphosphate group remains intact or not after the 2'5' oligonucleotide synthesis. The formation of co-oligonucleotides (9) has lead us to search for systems to analyze mixtures of oligoadenylates and (A,N) co-oligonucleotides. PEI-cellulose chromatography with a Tris-HCl-gradient in urea separates oligoadenylates ($2'5'A(pA)_n$) very well and can distinguish oligonucleotides of up to a chain length

of 16. However, when (A,N) co-oligonucleotides are present they are only well separated from $A(pA)_n$ in the case of $(Ap)_nG$. Development with acetic acid and LiCl separates so that incubation of ATP with each NTP produces extra spots on the chromatogram. Ball (15) has demonstrated using a similar chromatography system that the chick embryo 2'5' oligoadenylate synthetase 2'adenylates co-oligonucleotides 2'5' NpA and 3'5'NpA, where N is G, U or C.

The present analysis system will help to elucidate the mechanisms of this enzyme that synthesizes 2'5' oligonucleotides of varying composition but which exhibits a very stringent requirement to the acceptor for 2' addition. The acceptor must be adenosine with free 2' and 3' hydroxyl groups.

Abbreviations used : TLC, thin layer chromatography. PEI, polyethyleneimine. dsRNA, double stranded RNA. εATP, $1,N^6$-Ethenoadenosine 5'-Triphosphate.

ACKNOWLEDGEMENTS

We thank H. Bertrand from Choay Institute, Paris for his generous help preparing rabbit reticulocytes. J. Justesen holds a long term European Molecular Biology Organization fellowship and D. Ferbus, a fellowship from the French Delegation Générale à la Recherche Scientifique et Technique.

This work was supported by grants from Centre National de la Recherche Scientifique (G.R. n°18), Institut National de la Santé et de la Recherche Médicale (C.R.L. 77.7.04), Délégation Générale à la Recherche Scientifique et Technique (G.B.M. 78.7.0693) and Commissariat à l'Energie Atomique.

REFERENCES

1. Baglioni, C., (1979)
Cell. 17, 255-264.
2. Hovanessian, A.G. and Kerr, I.M. (1978)
Eur. J. Biochem. 81, 149-159.
3. Stark, G.R., Dower, W.J., Schimke, R.J., Brown, R.E. and Kerr, I.M. (1979)
Nature, 278, 471-473.
4. Martin, E.M., Birdsall, N.J.M., Brown, R.E. and Kerr, I.M. (1979)
Eur. J. Biochem. 95, 295-307.
5. Clemens, M.J. and Williams, B.R.G. (1978)
Cell. 13, 565-572.
6. Kerr, I.M. and Brown, R.E. (1978)
Proc. Natl. Acad. Sci. USA, 75, 256-260.
7. Ball, A.L. (1979)
Virology, 94, 282-296.
8. Schmidt, A., Zilberstein, A., Shulman, L., Federman, P., Berissi, H. and Revel, M. (1978)
FEBS Letters, 95, 257-264.

9. Justesen, J., Ferbus, D. and Thang, M.N. (1980)
Proc. Natl.Acad. Sci. USA, in press.
10. Randerath, E. and Randerath, K. (1967)
J. of Chromatography, 31, 485-499.
11. Gupta, R.C. and Randerath, K. (1977)
Nucl. Acid Res. 4, 1957-1978.
12. Borsook, H., Deasy, C.L., Haagen-Smit, A.L., Keighley, G. and Lowy, P.H. (1952)
J. Biol. Chem. 196, 669-694.
13. Kerr, I.M., Brown, R.E. and Hovanessian, A.G. (1977)
Nature, 268, 540-542.
14. Hovanessian, A.G., Brown, R.E. and Kerr, I.M. (1977)
Nature, 268, 537-540.
15. Ball, A. (1979)
In Low molecular weight mediators of macro-molecular synthesis, Koch and Richter eds., Academic Press Inc. (New York), in press.