STEPWISE PHOSPHOROLYSIS WITH POLYNUCLEOTIDE PHOSPHORYLASE: A NOVEL METHOD FOR SEQUENCE ANALYSIS OF OLIGORIBONUCLEOTIDES

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1. Introduction

Snake venom phosphodiesterase, bovine spleen phosphodiesterase and polynucleotide phosphorylase are currently used for sequence analysis of oligoribonucleotides.

These exonucleases remove mononucleotide residues in a sequential order from either the 3′- or 5′-end of an oligonucleotide. The nucleotide sequence of a given oligonucleotide is then derived by analyzing the partially degraded oligonucleotides which appear in the course of the reaction (cf. [1–3]). These procedures, while very useful for analyzing labeled oligonucleotides, are inadequate when only trace amounts of non-labeled material are available.

In the present report we describe a novel and sensitive method for sequence analysis of non-labeled oligoribonucleotides. The method is based on the property of polynucleotide phosphorylase to phosphorolyze short oligonucleotides from the 3′-end in a stepwise fashion by a nonprocessive mechanism [4–6]. The final products of the reaction consist of a mixture of nucleoside diphosphates (NDP's) and a limit oligonucleotide that is not further degraded by the enzyme. Depending on its base composition, this oligonucleotide is a di- or trinucleotide. By following the order in which the released NDP's appear during the phosphorolysis of a given oligonucleotide, it is possible to determine the nucleotide sequence from the 3′-end up to 2–3 residues from the 5′-terminus.

The high sensitivity of this method is achieved by including $^{32}P_i$ in the phosphorolysis medium. Upon digestion, the label is incorporated in the β-phosphate moieties of the generated NDP's which can be easily separated and counted. As little as 0.005 A_{260} units of a nonlabeled hexanucleotide sufficed to establish the sequence of the first three nucleotide residues at its 3′-end. It will also be shown that this method can be extended to the analysis of dodecanucleotides.

2. Experimental

The oligonucleotides C–C–C–C–U, C–C–C–U–C, C–C–C–U–C–A, U–U–U–G–A, U–U–U–G–A–A and U–U–U–G–A–A–G were obtained by stepwise syntheses using monofunctional substrates of polynucleotide phosphorylase [7–9]. ^{32}P-labeled $tRNA^{Tyr}_{su_3^+}$ was isolated from ϕ80psu$_3^+$ phage-infected *E. coli* cells [10] by phenol extraction, followed by polyacrylamide gel electrophoresis. The RNA extract was dissolved in 0.1 M Tris-borate buffer, pH 8.3, containing 0.2% sodium dodecyl-sulfate and 30% glycerol and applied to a 5% gel made up in 0.1 M Tris-borate buffer, pH 8.3; containing 2.5 mM Na_4EDTA and 7 M urea. This procedure separated $tRNA^{Tyr}$ from the bulk tRNA (plate 1A). The $tRNA^{Tyr}$-containing band was then excised from the 5% gel, transplanted to the origin of a 16% gel and subjected to a second electrophoretic run. In this step, $tRNA^{Tyr}$ was further purified from two faster migrating components (plate 1B) (J.S. Beckmann and H. Grosfeld, unpublished). Finally, the $tRNA^{Tyr}$ was eluted from the gel with 0.5 M NaCl, 0.01 M Na_4EDTA, 0.1 M Tris-HCl, pH 8.8 and the eluate was dialyzed against H_2O. Transfer RNA^{Tyr} thus obtained was judged to be at least 95% pure by fingerprint analysis of its T_1 ribonuclease digest [10] and

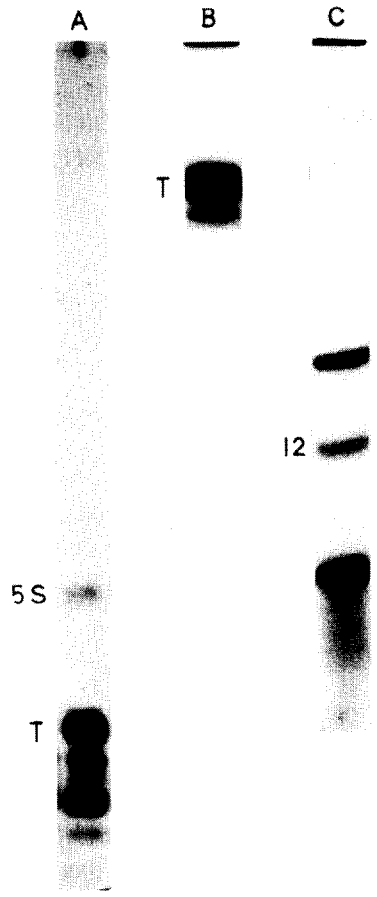

Plate 1. Isolation of $tRNA^{Tyr}$ and its dodecanucleotide fragment. A) Isolation of $tRNA^{Tyr}$ by 5% polyacrylamide gel electrophoresis. B) Further purification of $tRNA^{Tyr}$ by 16% polyacrylamide gel electrophoresis. C) Isolation of the dodecanucleotide from a T_1 ribonuclease digest of $tRNA^{Tyr}$. 5 S, 5 S RNA; T, $tRNA^{Tyr}$; 12, dodecanucleotide.

by comparing the ratio, cpm/nucleotide residue, of the various oligonucleotide spots to that of T–ψ–C–Gp. [^{32}P]A–C–U–C–U–A–$\overset{*}{A}$–A–ψ–C–U–Gp ($\overset{*}{A}$ stands for 2-methylthio N^6-γ-γ-dimethylallyl adenylic acid) was isolated from a T_1 ribonuclease digest of $tRNA^{Tyr}_{su_3}$ by polyacrylamide gel electrophoresis (16% gel) (plate 1C). The dodecanucleotide was eluted from the gel as described above and desalted by passage through a Sephadex G-10 column. The 3′-terminal phosphate of the dodecanucleotide was removed by incubation with *E. coli* alkaline phosphatase and the progress of this reaction was monitored by the disappearance of Gp from an alkaline hyrolysate of the product. The preparation used in the experiment cited here still contained 35% of the phosphorylated species, as exhaustive phosphatase treatment led also to phosphodiester breakage due to contaminating nucleases. Carrier free [^{32}P]inorganic phosphate was purchased from Nuclear Research Center-Negev, Israel, and was treated for 1 hr in 1 N HCl at 100° prior to its use. This treatment was needed to destroy trace amounts of polyphosphates that could otherwise interfere with the analysis of the phosphorolysis products. Highly purified *E. coli* polynucleotide phosphorylase was prepared as previously described [11]. We thank Dr. Y. Kimhi and Mr. Y. Tichauer for supplying the enzyme.

2.1. *Phosphorolysis of oligonucleotides*

From 0.02 to 0.005 A_{260} units of oligonucleotide were digested in reaction mixtures (20–50 μl) containing 0.1 M Tris-HCl buffer, pH 8.2; 0.5 mM $MnCl_2$ (or 1 mM $MgCl_2$), 0.2–1.0 mM ^{32}P-labeled potassium phosphate buffer, pH 8.2 (about 10^8 cpm/μmole) and 0.004–0.1 units [11] of purified polynucleotide phosphorylase. The mixtures were incubated at 25° and at various intervals aliquots of 1–2 μl were transferred to DEAE-cellulose paper sheets (Whatman DE-81, 24 × 46 cm). A mixture of cold ADP, GDP, UDP and CDP (0.05 μmole each) in 2 μl of 1 N acetic acid was subsequently applied to each spot. The paper was then developed by descending chromatography with 40 ml of 0.25 M NH_4HCO_3 for 16 hr, by which time the solvent was completely drained from the paper. By this procedure the various NDP's were separated from one another and from inorganic phosphate. The distances migrated relative to the dye, xylene cyanol FF were: GDP – 0.53; ADP – 0.74; UDP – 0.91; CDP – 1.14 and P_i – 1.70. The NDP spots were then excised and counted in 3 ml of toluene scintillation fluid. To establish the required length of the incubation period, the time course of the overall phosphorolysis was first determined by transferring, at various intervals, several aliquots of 1 μl from the above reaction mixtures to 1 ml of 1% perchloric acid. The amount of $^{32}P_i$ incorporated into NDP was then determined [10]. To distinguish between the successive NDP residues appearing in the course of the phosphorolysis, it sufficed to take 3 to 4 aliquots per phosphorolyzed NMP residue.

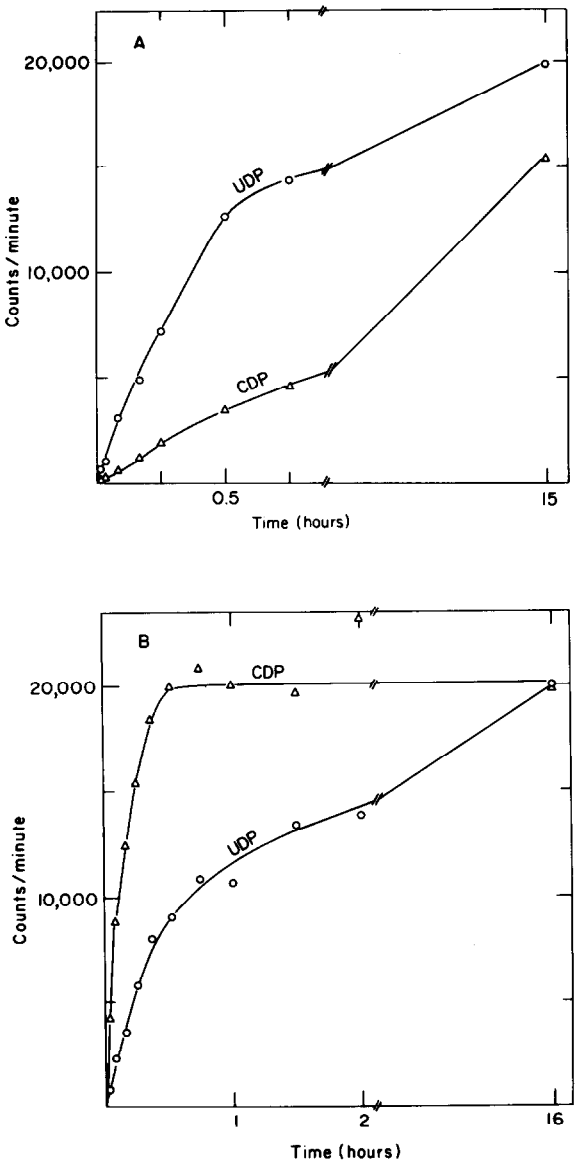

Fig. 1. A) Phosphorolysis of C–C–C–C–U. The reaction mixture (20 μl) contained: 0.005 A_{260} units of C–C–C–C–U; 100 mM Tris-HCl, pH 8.2; 0.2 mM ^{32}P-labeled potassium phosphate buffer, pH 8.2 (3.5×10^8 cpm/μmole); 0.5 mM $MnCl_2$ and 0.004 units of polynucleotide phosphorylase. Incubation was at 25°. Aliquots of 2 μl were transferred to DEAE-cellulose paper at the indicated intervals. The samples were chromatographed and radioactivity in the NDP spots (see Experimental) was determined. (△—△—△) CDP; (○—○—○) UDP. B) Phosphorolysis of C–C–C–U–C. The experiment was carried out as in fig. 1A except that the reaction mixture contained 0.005 A_{260} units of C–C–C–U–C.

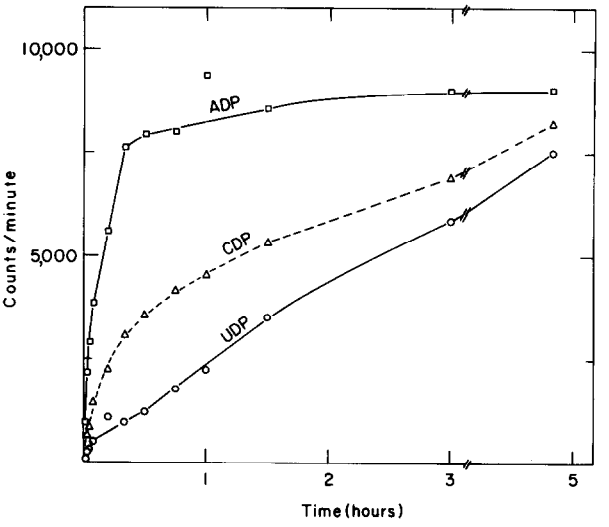

Fig. 2. Phosphorolysis of C–C–C–U–C–A. The experiment was carried out as in fig. 1A except that the reaction mixture contained 0.006 A_{260} units of C–C–C–U–C–A. (□—□—□) ADP; (△—△—△) CDP; (○—○—○) UDP.

3. Results and discussion

The sequential phosphorolysis procedure was first examined with two isomeric pentanucleotides of known sequence, C–C–C–C–U and C–C–C–U–C. Fig. 1A and B show the time course of their phosphorolysis. It was found that C–C–C–C–U yielded first UDP and then CDP, while C–C–C–U–C yielded first CDP and then UDP. As expected, the final level of the two released NDP's was the same (fig. 1A, B). Thus, both pentanucleotides were sequentially phosphorolyzed in a pattern corresponding to their base sequence at their 3′-end (C–C–C was not phosphorolyzed by the enzyme). Next, we analyzed the base sequence of a hexanucleotide that was obtained by monoaddition of 2′(3′)-*O*-isovaleryl-ADP to C–C–C–U–C [8]. Fig. 2 shows that the order of NDP appearance was ADP; CDP; UDP, and that the final amounts of all three NDP's reached about the same level. Since C–C–C is not phosphorolyzed we deduced C–C–C–U–C–A to be the correct sequence of the analyzed oligonucleotide. Similar stepwise phosphorolysis patterns were obtained with the pentanucleotide U–U–U–G–A (fig. 3) and with the hexanucleotide U–U–U–G–A–A (fig. 4). Again the

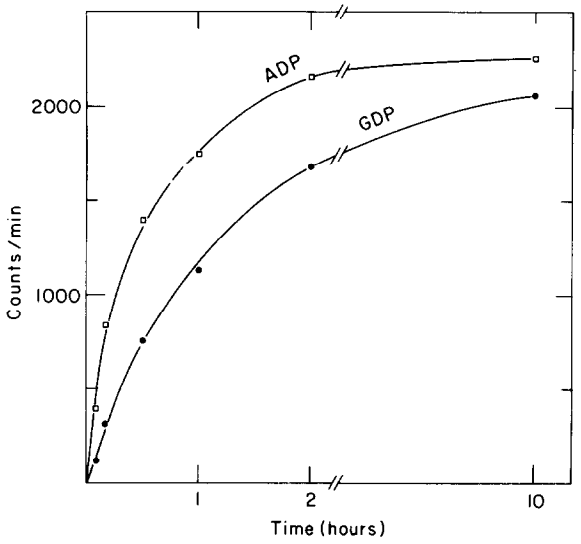

Fig. 3. Phosphorolysis of U–U–U–G–A. The experiment was carried out as in fig. 1A except that the reaction mixture contained 0.005 A_{260} units of U–U–U–G–A. (□—□—□) ADP; (●—●—●) GDP.

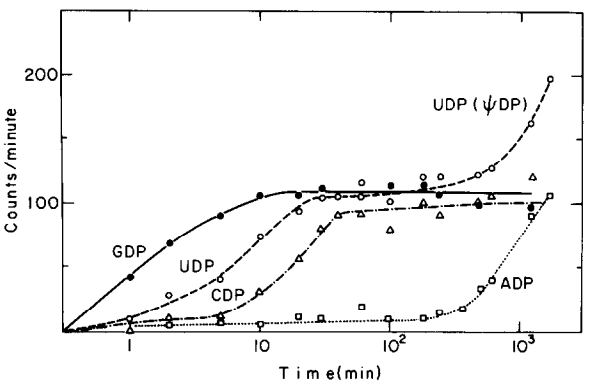

Fig. 5. Phosphorolysis of A–C–U–C–U–A–A*–A–ψ–C–U–G. The reaction mixture (40 μl) contained ^{32}P-labeled A–C–U–C–U–A–A*–A–ψ–C–U–G (60,000 cpm; 1.5 × 10^7 cpm/μg); 10 mM potassium phosphate buffer, pH 8.0; 20 mM Tris-HCl buffer; pH 8.3; 1 mM $MgCl_2$ and 4 units of polynucleotide phosphorylase. Incubation was at 25°. Aliquots of 1 μl were transferred to DEAE-cellulose paper at the indicated intervals. (●—●—●) GDP; (□--□--□) ADP; (△-·-△-·-△) CDP; (○--○--○) UDP (and ψDP).

order and the level of NDP appearance reflected the base sequence at the 3′-end of these oligonucleotides.

It is known that polynucleotide phosphorylase degrades short oligonucleotides by a nonprocessive mechanism [4–6]. On the other hand, long polynucleotides are phosphorolyzed by a processive mechanism, i.e. the enzyme tends to degrade a single chain to completion before initiating phosphorolysis of another chain [5, 12, 13]. The chain length at which transition occurs from nonprocessive to processive phosphorolysis is unknown since kinetic studies [5, 6] were confined to oligonucleotides with chain lengths up to 7 or to homopolymer fractions with average chain lengths of 26 or higher. It is obvious that sequential phosphorolysis of an oligonucleotide can only be performed when the degradation mechanism is nonprocessive. To determine whether the sequential phosphorolysis procedure can be applied for sequencing oligonucleotides of chain lengths greater than 7, we subjected the dodecanucleotide A–C–U–C–U–A–A*–A–ψ–C–U–G to this digestion procedure. (The phosphorolysis was carried out in the presence of non-labeled P_i since the dodecanucleotide was already highly labeled with ^{32}P.) Fig. 5 shows the time course of NDP appearance during the partial phosphorolysis of A–C–U–C–U–A–A*–A–ψ–C–U–G. The pattern obtained clearly reflects a nonprocessive mechanism since the appearance of the various NDP's corresponds to the base sequence from the 3′-end. Thus, GDP appeared first, followed by UDP and CDP. The further increase in the UDP curve which

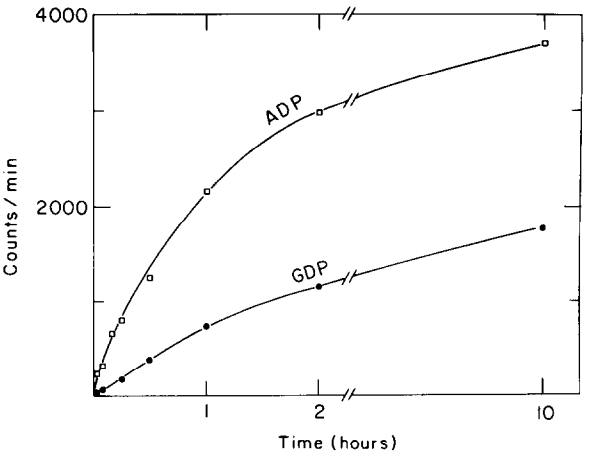

Fig. 4. Phosphorolysis of U–U–U–G–A–A. The experiment was carried out as in fig. 1A except that the reaction mixture contained 0.005 A_{260} units of U–U–U–G–A–A.

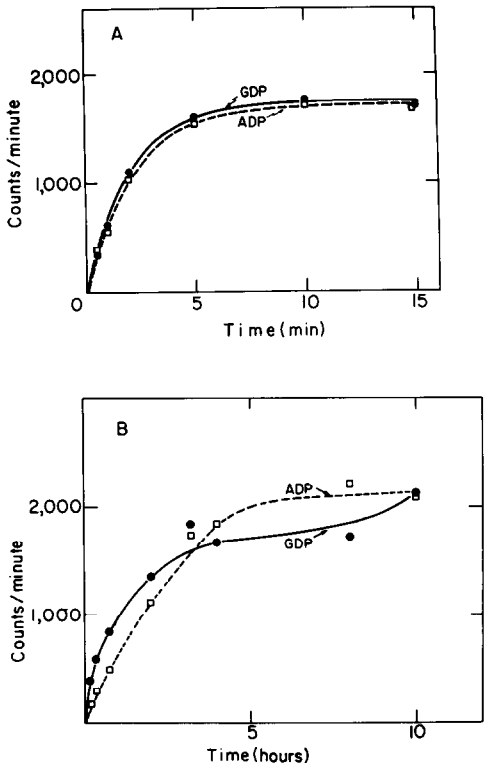

Fig. 6. Phosphorolysis of U–U–U–G–A–A–G. A) The incubation was carried out at 25°. The reaction mixture (50 μl) contained: 0.006 A_{260} units of U–U–U–G–A–A–G; 100 mM Tris-HCl buffer, pH 8.2, 1 mM ^{32}P-labeled potassium phosphate buffer (10^8 cpm/μmole); 0.5 mM $MnCl_2$ and 0.1 units of polynucleotide phosphorylase. Aliquots of 2 μl transferred as indicated to DEAE-cellulose paper. (•—•—•) GDP; (□--□--□) ADP. B) The incubation was carried out at 60°. The composition of the reaction mixture similar to that described in (A) except that the enzyme's concentration was 5-fold lower. Reaction mixture samples corresponding to time points up to 60 min were incubated separately in 0.2 ml polypropylene tubes. In these tubes all the ingredients of the reaction mixture except $MnCl_2$ were applied in a 7 μl droplet while $MnCl_2$ was applied as a separate 3 μl droplet. The tubes were preincubated for 1 min at 60° and the reaction was initiated by mixing the two drops. The reaction was stopped as indicated by rapidly adding 50 μl of 1 N acetic acid. Later time points were withdrawn from a 100 μl reaction mixture and diluted with 50 μl of 1 N acetic acid.

occurs after the appearance of CDP, is presumably due to the release of ψDP which migrates together with UDP in the chromatography system. The appearance of the final product, ADP, closely followed the appearance of ψDP probably because of the sluggish phosphorolysis of the ψMP residue (the polymerization of ψDP proceeds much slower than that of UDP [14]). It should be noted that the rate of phosphorolysis of the labeled dodecanucleotide was extremely slow and we were unable to follow the reaction beyond the 5th residue. The reason for this slow reaction rate is not clear and it could be due to the presence of inhibitory substances. In any event, it is apparent from fig. 5 that this procedure allows at least a partial analysis of the sequence of rather long oligonucleotides. In addition, the result demonstrates that a dodecanucleotide can still be digested by polynucleotide phosphorylase in a nonprocessive mechanism.

A different pattern of phosphorolysis was found with the heptanucleotide U–U–U–G–A–A–G. At 25° (fig. 6A), its phosphorolysis was strictly processive. However, at 60° (fig. 6B) the pattern of NDP appearance reflected the base sequence at the 3′-end of the molecules, albeit not in as clear a fashion as seen with the other oligonucleotides (figs. 1–5). It should be noted that the same results were obtained when $MnCl_2$ was replaced by $MgCl_2$. The processive phosphorolysis observed with U–U–U–G–A–A–G (fig. 6A) could be related to its capacity to form dimers, viz:

$$\begin{array}{l} 3' \rightarrow 5'\ \mathrm{G{-}A{-}A{-}G{-}U{-}U{-}U} \\ \qquad\qquad\quad | \ \ | \ \ | \ \ | \ \ | \ \ | \\ 5' \rightarrow 3'\quad \mathrm{U{-}U{-}U{-}G{-}A{-}A{-}G} \end{array}$$

However, this explanation does not fit the nonprocessive phosphorolysis found with U–U–U–G–A–A (fig. 4) which is also capable of dimerization. Another possible explanation is that increased stacking due to an additional G residue brings about the processive phosphorolysis of this particular heptanucleotide. It seems from the above results that the shift from a processive to a nonprocessive mechanism of phosphorolysis does not depend solely on the length of the oligonucleotide.

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