ENZYMIC POLYNUCLEOTIDE SYNTHESIS PRIMED BY POLYVINYLALCOHOL LINKED OLIGOTHYMIDYLATE*

Ranajit ROYCHOUDHURY**, Siegfried KÜHN, Herbert SCHOTT*** and Hans KÖSSEL
Institut für Biologie III der Universität Freiburg, D-78 Freiburg i. Br., Schänzlestrasse 9-11, BRD

Received 15 September 1974

1. Introduction

The chemical preparation of mono- and oligonucleotides covalently bound to polyvinyl alcohol+ by 5′-phosphodiester linkages has been reported recently [1,2]. The availability of this kind of water soluble polymer has prompted us to test, whether the coupled oligothymidylate chains can serve as primers for the enzymic synthesis of polynucleotides. In this communication we wish to present evidence that polyvinyl alcohol bound oligothymidylate effectively primes the addition of nucleotide residues catalyzed by terminal deoxynucleotidyl transferase, and that the extended chains can act as templates in the presence of DNA polymerase I. From this a new route to polynucleotide synthesis on polymer support seems possible, which is based on enzymic reactions in homogeneous liquid phase.

2. Materials and methods

[^{3}H] dATP, [^{14}C] dTTP, [α^{32}P] rUTP and alkaline phosphatase were obtained from commercial sources. Heptathymidylic acid [3] and $d(T)_{30}$ [4] were prepared as described earlier. PV-pU_r and PV-$(pT_d)_x$ were prepared by reaction of 2′, 3′-di-*O*-acetyluridine 5′-phosphate or thymidine 5′-phosphate with polyvinyl alcohol according to the published procedures [1,2]. PV-$(pT_d)_x pU_r$ was prepared by the same procedure using a mixture of 3′-ribouridine terminated oligothymidylate $(pT_d)_x pU_r (x>3)$ [5] for the condensation with polyvinyl alcohol. Removal of unreacted oligonucleotides not linked to polyvinyl alcohol was achieved by chromatography of up to 3 A_{260} units of material on a combination column with Sephadex G-50 (0.7 × 27 cm) at the top and Bio-Gel A 1.5 m (0.7 × 86 cm) at the bottom [4]. The polyvinyl alcohol linked nucleotidic material is eluted within the void volume (0.05–0.1 M TEAB as eluent). The same column was used for the preparative and analytical separation of the polymeric products from unreacted nucleoside triphosphates or from uncoupled oligonucleotides (figs. 1B and 3B). Terminal deoxynucleotidyl-transferase and *E. coli* DNA polymerase I were applied in homogeneous form as reported earlier [4,6].

3. Results and discussion

The terminal addition of ribouridylic acid residues

* Support of this work by the Deutsche Forschungsgemeinschaft (Schwerpunkt Synthese Makromolekularer Naturstoffe and SFB 46) is gratefully acknowledged.

** Present address, Section of Biochemistry, Molecular and Cell Biology, Wing Hall, Cornell University, Ithaca, New York 14850, USA.

*** Present address, Institut für Organische Chemie, D-74 Tübingen-1, Auf der Morgenstelle, BRD.

+ PV is used as abbreviation for polyvinylalcohol; thus, PV-pU_r symbolizes 5′-uridylic acid covalently attached to polyvinyl alcohol, PV-$(pT_d)_x$ represent oligothymidylate covalently bound to polyvinyl alcohol by a 5′-phosphodiester linkage. One A_{260} unit is defined as the amount of nucleotide giving an absorbance of 1 at 260 nm when dissolved in 1 ml of solvent and measured in a 1-cm light path quartz cell. Triethyl ammonium bicarbonate buffer is abbreviated as TEAB. p*designates a ^{32}P phosphate residue.

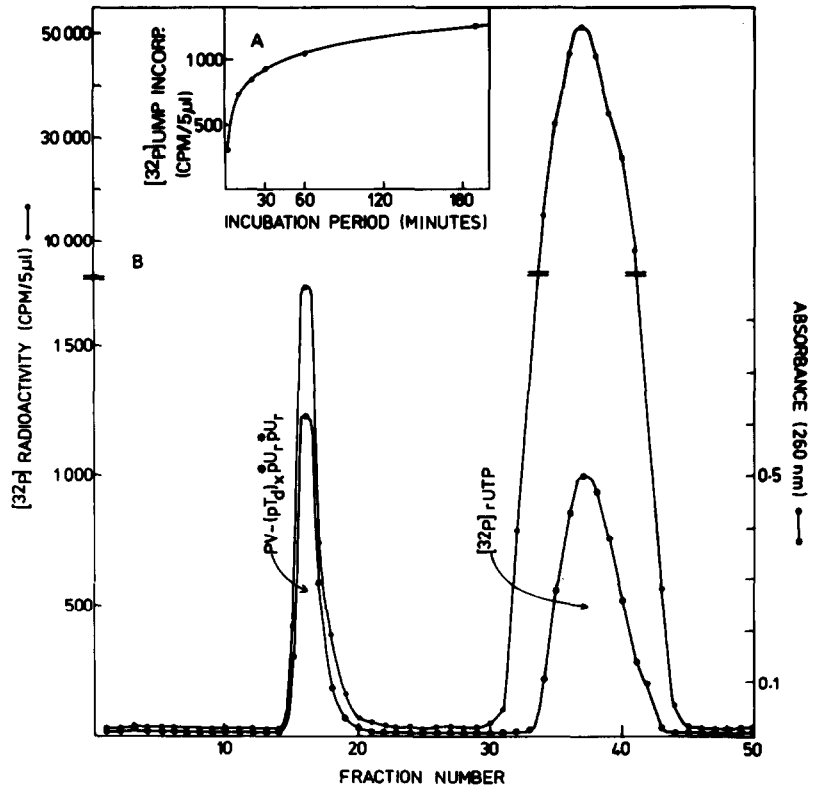

Fig. 1. Formation and isolation of PV-$(pT_d)_x p^*U_r p^*U_r$. The incubation mixture (200 µl) contained 40 mM potassium cacodylate (pH 6.8), 8 mM $MgCl_2$, 1 mM $CoCl_2$, 200 µM dithiothreitol, 1 mM [α-^{32}P] rUTP (25 000 cpm/nmole), 8 A_{260} units/ml of PV-$(pT_d)_x$ and 160 µg/ml of terminal deoxynucleotidyl transferase. At the time intervals indicated in A, 5 µl aliquots were tested for acid precipitable radioactivity. After the plateau had been reached (180 min) the remaining 170 µl were chromatographed (B) on th combination column as described under Materials and methods. The upper curve represents the radioactivity profile. Fractions 15–17 were pooled, evaporated to dryness and redissolved in 500 µl of water (A_{260} = 2.13; A_{280} = 1.78; 3435 cpm per 50 µl).

[6] onto PV-$(pT_d)_x$ catalyzed by terminal deoxynucleotidyl transferase was studied first. As evident from fig. 1A, after an initial rapid phase the addition rate gradually decreases to a lower level which continues for several hours. It seems that the rapid synthesis during the initial phase is caused by the more accessible primer chains, while during the later phase primers of shorter chain length or primers more buried in the polyvinyl alcohol matrix are utilized. The covalent linkage of the radioactive ribouridylic acid residues is evident from the chromatography of the product mixture on a combination column (fig. 1B) in which the product PV-$(pT_d)_x p^*U_r p^*U_r$ appears in the void volume well separated from the excess of unreacted [α^{32}P] rUTP. For the characterization of the product, material of the product peak of fig. 1B was hydrolyzed with alkali (0.3 M KOH, 18 hr 37°C). After neutralization and treatment with phosphatase an aliquot of the product mixture was subjected to paper chromatography in the *n*-propanol–conc. NH_3–H_2O system (55:10:35, v/v/v). As expected for a diaddition product about equal amounts of radioactivity were observed at the origin (corresponding to the monoaddition product PV-$(pT_d)_x p^*U_r$ and in the region of inorganic phosphate (fig. 2). The major portion of the monoaddition product thus obtained was purified by chromatography on the combination column similar to fig. 1B.

Next the various compounds indicated in fig. 3A were tested as primers for the polymerization of

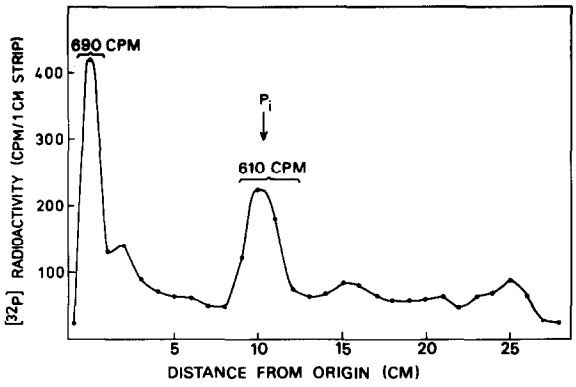

Fig. 2. Characterization of PV-(pT_d) $p^*U_rp^*U_r$ by alkali and phosphatase treatment. Isolated material of fractions 15–17 of fig. 1B was treated with 0.5 N KOH at 37°C for 20 hr (final volume 600 μl. After the pH was brought to 8 by addition of HCl, 10 μl of bacterial alkaline phophatase (26 units/ml) were added and the incubation continued at 37°C for 12 hr. A 25 μl aliquot was then analyzed by paper chromatography alongside with a ^{32}P-inorganic phosphate marker.

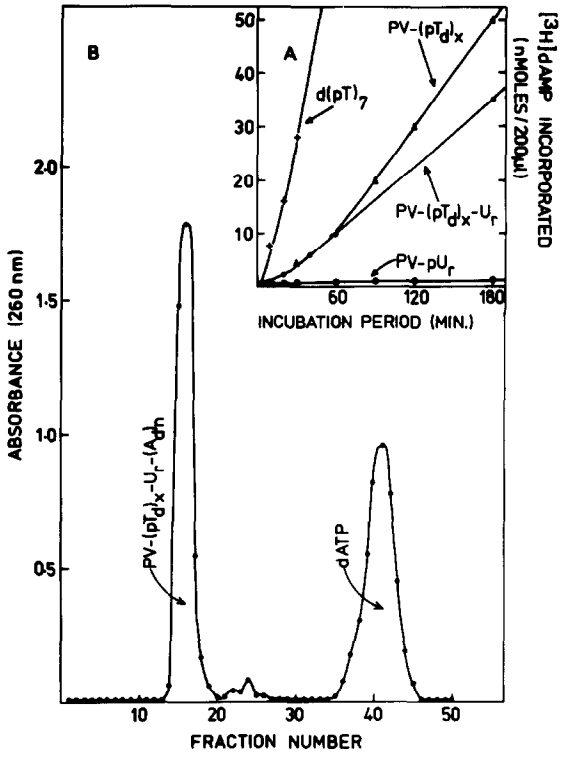

Fig. 3. Formation and isolation of PV-$(pT_d)_x$-U_r-$(A_d)_n$. The kinetics of incorporation of [^{3}H]dAMP residues onto the various primers in the presence of terminal deoxynucleotidyl transferase is shown in the insert (A). The reaction mixtures (200 μl) contained 40 mM potassium cacodylate (pH 6.8), 10 mM $MgCl_2$, 1 mM $CoCl_2$, 200 μM dithiothreitol, 1.6 mM [^{3}H]dATP (7500 cpm per nmole) and 80 μg/ml of enzyme. The respective primer concentrations were 2.5 A_{260} units/ml of PV-pU_r, 3.0 A_{260} units/ml of PV-$(pT_d)_x$, 2.5 A_{260} units/ml of PV-$(pT_d)_xp^*U_r$ and 3.0 A_{260} units/ml of $(pT)_7$. At the time intervals indicated 10 μl aliquots were assayed for acid precipitable radioactivity. The remaining material from the PV-$(pT_d)_xpU_r$ primed reaction (which was carried out on 500 μl scale) after 20 hr of incubation was analyzed by chromatography on a combination column (B) as described under Materials and methods.

[^{3}H]dATP catalyzed by terminal deoxynucleotidyl transferase. While as expected no polymerization was primed by PV-pU_r (showing only a slight background probably consisting of unspecifically absorbed dATP), comparatively high priming efficiency was observed with PV-$(pT_d)_x$ and PV-$(pT_d)_x$-U_r. For comparison a kinetics is also shown for a $(pT)_7$ primed reaction which is faster by more than a factor of 3. The lower efficiency of the PV linked primers as compared to $(pT)_7$ probably is due to the presence of shorter chains and/or to steric influences of the PV matrix. The column chromatographic separation of a product mixture from a PV-$(pT_d)_x$-U_r primed reaction is shown in fig. 3B, indicating that about half of the dATP was converted to the PV linked polymer product PV-$(pT_d)_x$-U_r-$(A_d)_n$. Similar results were obtained when a PV-$(pT_d)_x$-U_r primer was used in which the 3′-terminal U_r had been connected chemically. As evident, however, from nearest neighbour data (products obtained from PV-$(pT_d)_x$-U_r and [α^{32}P]dATP, digested with spleen and micrococcus nuclease after alkaline hydrolysis) a considerable portion of the primers utilized, had lacked the 3′-terminal U_r; thus, in a typical experiment 65% T_dp^* [representative for PV-$(pT_d)_x(p^*A_d)_n$] and 35% U_rp^* [representative for PV-$(pT_d)_xpU_r(p^*A_d)_n$] were obtained. Therefore, the product peak of fig. 3B has to be regarded as a mixture of PV-$(pT_d)_x$-$(A_d)_n$ and PV-$(pT_d)_x$-U_r-$(A_d)_n$, and further work will be necessary in order to achieve ribointernucleotide linkages exclusively between the primer and the product chains.

Finally, it was tested whether the poly dA chains of PV-$(pT_d)_x(A_d)_n$ and PV-$(pT_d)_x$-U_r-$(A_d)_n$ can serve as templates for the polymerization of dTTP in the presence of oligo dT primers and DNA polymerase I.

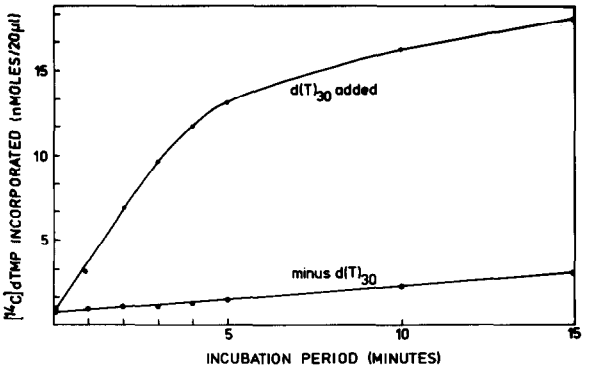

Fig. 4. Kinetics of incorporation of [^{14}C]dTMP residues onto $d(T)_{30}$ in the presence of DNA polymerase I and a mixture of PV-$(pT_d)_x$-$(A_d)_n$ and PV-$(PT_d)_x$-U_r-$(A_d)_n$ as template. The incubation mixtures (200 µl) contained 100 mM glycylglycine–KOH buffer (pH 7.5), 10 mM $MgCl_2$, 1 mM [^{14}C]dTTP (6000 cpm per nmole), 100 µM of template, 10 µM of $d(T)_{30}$ as primer (or no primer) and 5 units/ml of *E. coli* DNA polymerase I. At the time intervals indicated 20 µl aliquots were tested for acid precipitable radioactivity.

The data of fig. 4 indicate that polymerization reaches nearly one template equivalent and that the reaction is dependent on the addition of the $d(T)_{30}$ primer.

Polynucleotide celluloses have been reported as solid state primers and templates for polymerizing enzymes [7]. Similar to the water soluble Ficoll linked primers, reported more recently [8], the polyvinyl alcohol linked oligonucleotides investigated in this paper combine the advantages of polymer support synthesis and of synthesis in homogeneous aqueous phase.

References

[1] Schott, H. (1973) Angew. Chem. 85, 263–264; Angew. Chem. Internat. Edit. 12, 246.
[2] Brandstetter, F., Schott, H. and Bayer, E. (1973) Tetrahedron Letters 32, 2997–3000.
[3] Roychoudhury, R. and Kössel, H. (1971) Eur. J. Biochem. 22, 310–320.
[4] Kössel, H. and Roychoudhury, R. (1974) J. Biol. Chem. 249, 4094–4099.
[5] Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R. and Kössel, H. (1973) Biochemistry 12, 932–938.
[6] Roychoudhury, R. (1972) J. Biol. Chem. 247, 3910–3919
[7] Jovin, T. M. and Kornberg, A. (1968) J. Biol. Chem. 243, 250–259.
[8] Scheffler, I. E. and Richardson, C. C. (1972) J. Biol. Chem. 247, 5736–5745.