OLIGORIBONUCLEOTIDES AS PRIMER FOR TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE

G. FEIX

Institut für Biologie III, Universität Freiburg, 78 Freiburg i. Br., German Federal Republic

Received 16 August 1971

1. Introduction

Terminal deoxynucleotidyl transferase from calf thymus gland was isolated and extensively purified by F.J. Bollum and coworkers [1, 2]. This enzyme catalyzes the template independent sequential addition of nucleotides from nucleoside triphosphates to the 3'-end of an oligodeoxynucleotide or DNA. This reaction has been used for the synthesis of many polydeoxynucleotides [3–8]. This communication shows that under certain conditions terminal deoxynucleotidyl transferase will also accept an oligoribonucleotide as primer, giving rise to the possibility of synthesizing covalently linked polyribodeoxynucleotides. Under the conditions used working with $(A-A-A-A-A-A)_r$ as primer, and dCTP as substrate, $(A-A-A-A-A-A)_r-(C_d)_n$ could be synthesized.

2. Materials and methods

$(A-A-A-A-A-A)_r$ was obtained from Miles Laboratory (USA), unlabelled dCTP and [^{3}H]-dCTP (specific activity 35 Ci/μmole) from Schwarz Bioresearch (USA), [α-^{32}P]-dCTP (specific act. 1.6 Ci/μmole) from Internat. Chem. and Nuclear Corp. (USA). Salts were reagent grade from Merck (Germany). $(pT-T-T-T)_d$ was kindly given by Dr. H. Kössel of this department. Terminal deoxynucleotidyl transferase was prepared according to L. Chang and F. J. Bollum and had a specific activity of 9,000 units per mg protein as measured with dCTP and $CoCl_2$ [2]. The rather low specific activity resulted from partial inactivation during the last step of the enzyme purification because of too dilute enzyme solution. Protein was measured by the method of Lowry [9].

Concentrations of nucleotides and oligonucleotides were determined spectrophotometrically using a Zeiss PMQ spectrophotometer.

All radioactive determinations were made in a Beckman liquid scintillation spectrophotometer using a toluene based scintillator for counting the samples on either membrane filters (Sartorius, Germany) or cut-up chromatography paper.

Paper chromatography was carried out with Schleicher and Schüll paper No. 2043 b in descending fashion in the following systems: (1) Ethanol – 0.5 M NH_4 acetate (7:3 v/v), pH 3.8. (2) N-propanol – conc. NH_4OH – H_2O (55:10:35 v/v).

High voltage paper electrophoresis was carried out on the same paper in 0.15 N ammonium acetate (adjusted to pH 3.45 with acetic acid) at 2,000 V in the Savant-Instr. apparatus, Model No. LT 20 A.

3. Results

The results of a typical [^{3}H]-dCTP incorporation experiment of the enzyme preparation in response to different primers are summarized in table 1. With the oligodeoxynucleotide $(pT-T-T-T)_d$ as primer the dCTP substrate was almost completely exhasuted after 40 min of incubation. The use of $(A-A-A-A-A-A)_r$ as primer at the same concentration gave rise to only little incorporation of dCTP. But with higher concentrations of $(A-A-A-A-A-A)_r$ the yield of the enzymatic reaction is considerably increased. An alkali digested $(A-A-A-A-A-A)_r$ sample (18 hr, 0.3 N KOH, 37°, neutralized with

North Holland Publishing Company – Amsterdam

Table 1
Incorporation of [^{3}H]-dCTP into 6% TCA insoluble material.

Primer [conc.]	Incorporation [cpm]		
	20 min	30 min	40 min
no primer	350	406	526
r(Ap)$_5$A[10 μM]	486	776	1630
r(Ap)$_5$A[500 μM]	2482	4050	6818
r(Ap)$_5$A[250 μM] after alkaline digestion	410	505	622
d(pT)$_4$[10 μM]	121,360	159,142	185,750

The enzyme assays (100 μl) containing 20 μmoles K-cacodylate (pH 7.0), 100 nmoles $CoCl_2$, 1.8 μmoles KCl, 100 nmoles mercaptoethanol, 100 nmoles [^{3}H]-dCTP (Specific activity 11000 cpm/nmole), 21 μg enzyme and primers in the amounts listed in the table were incubated at 35°. At the times indicated 20 μl aliquots were taken, diluted with a mixture of 0.05 ml 8% $Na_4P_2O_7$ (pH 7.0) and 0.1% bovine serum albumin, and acid precipitated with 6% TCA. The acid precipitates were collected on membrane filters and after washing with 6% TCA and drying, the radioactivity was measured as described in "Materials and methods".

Dowex-50 X 8 resin) has lost its priming capacity indicating that the oligoribonucleotide, and not a contaminating oligodeoxynucleotide, is responsible for the priming of the reaction.

Since under the conditions used the enzyme reaction displays also some primer independent synthesis, a further experiment was carried out to show conclusively that the observed synthesis of acid precipitable polymers is the result of a covalently linked addition of αCPM to the 3′-end of the primer.

A covalent linkage between the primer and the product would follow the reaction:

$$(A{-}A{-}A{-}A{-}A{-}A)_r + (pppC_d)_n \longrightarrow$$
$$(A{-}A{-}A{-}A{-}A{-}A)_r{-}(pC_d)_n + nPPi$$

The use of [α-^{32}P]-dCTP as the substrate followed by alkaline hydrolysis of the product would permit a nearest neighbour analysis according to the reaction:

$$(A{-}A{-}A{-}A{-}A{-}A)_r{-}\overset{*}{p}C_d{-}(\overset{*}{p}C_d)_{n-1} \xrightarrow{\text{alkali}}$$
$$5\,Ap + A\overset{*}{p} + C_d - (\overset{*}{p}C_d)_{n-1}$$

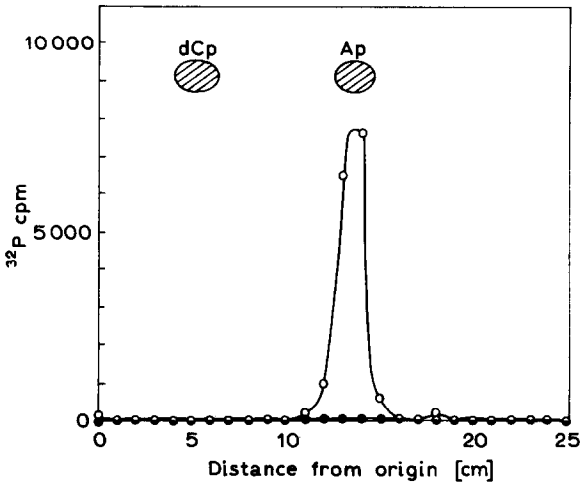

Fig. 1. An assay mixture (120 μl) containing 24 μmoles K-cacodylate (pH 7.0) 120 nmoles $CoCl_2$, 2.2 μmoles KCl, 120 nmoles mercaptoethanol, 120 nmoles [^{32}P]-dCTP (specific activity 16,000 cpm/nmole), 100 μg enzyme and 60 nmoles $(A{-}A{-}A{-}A{-}A{-}A)_r$ was incubated for 30 min at 20° and afterwards for 3 hr at 31°. After the incubation 20 μl were taken to determine the acid precipitable radioactivity (for procedure, see table). The remaining 100 μl of the incubation mixture were filtered through a Sephadex G-25 column (10 ml volume, equilibrated with 0.05 M triethylammoniumbicarbonate (pH 7.5) to remove the salts. The fractions containing radioactivity as detected by Cerenkov radiation were pooled, evaporated to dryness and taken up in 0.6 ml of 0.3 N KOH. After an incubation for 18 hr at 37° the mixture was neutralized with pyridinium Dowex-50 X 8 resin. The resin was washed with 30% pyridine and the combined solutions were taken to dryness in a Evapo-Mix (Buchler instruments). 50 μl H_2O were added and the solution was spotted on chromatography paper. The paper was run for 15 hr in system 1. The Ap spot (identified by marker Ap) was localized by UV-light quenching and eluted from the paper with 0.2% NH_3. After drying the sample and resolving it in 50 μl of H_2O the solution was run on a chromatogram in system 2 for 21 hr. The Ap spot was eluted again in the same way and then run for 3½ hr on high voltage paper electrophoresis in the system indicated in "Materials and methods". The Ap spot was eluted and run for a second time. Afterwards the paper was cut serially in 1 cm strips and counted in the liquid scintillation counter (in the figure curve with open circles). Another assay mixture without addition of any primer was run and worked up in an identical manner. The final radioactivity profile of this enzyme incubation is represented in the figure by the line with the closed circles.

The ^{32}P counts are corrected for the radioactive decay.

The [^{32}P]-label from the first addition residue pC_d will thus be transferred to its nearest neighbour, liberating a free Ap and provide a conclusive evidence for a covalent linkage.

The Ap generated by alkaline digestion was purified from the other radioactive products by various chromatographic procedures. Two different paper chromatography systems were used to separate mononucleotides from oligonucleotides and polynucleotides. High voltage paper electrophoresis at pH 3.45 was used to differentiate between the different nucleotides.

Fig. 1 represents the final electrophoretic distribution of radioactivity which coincides with Ap (open circles). The other line in the figure (closed circles) is the result of an identical enzyme assay without the addition of $(A-A-A-A-A-A)_r$. In this case no radioactivity can be detected. During the chromatographies and the electrophoreses the Ap spot was always identified by the position of an Ap marker prepared by alkaline digestion of the $(A-A-A-A-A-A)_r$ used as primer.

The radioactive Ap spot of the final electrophoresis was eluted and further identified as Ap by its A_{280}/A_{260} ratio of 0.17 at pH 7. This absorbance measurement indicated further that 50% of the input $(A-A-A-A-A-A)_r$ primer was recovered after the processing of the sample. Considering this 50% yield of Ap as well as the incorporation of 1.12 nmoles of the primer $(A-A-A-A-A-A)_r$ out of a total of 60 nmoles of primer supplied, it is concluded that under the conditions of the experiment every 27th primer molecule was used. In this particular reaction 20 nmoles of dCTP out of a total of 120 nmoles in the assay mixture became acid insoluble as tested with a sample of the incubation mixture.

4. Discussion

The above experiments demonstrate that terminal deoxynucleotidyl transferase from calf thymus glands is able to accept oligoribonucleotides as primers. Compared to oligodeoxyribonucleotides, the priming capacity is low and in order to achieve some appreciable synthesis rather high primer and enzyme concentrations have to be used. Since those oligoribonucleotide primer molecules to which a deoxynucleotide has been added will be more efficiently used by the enzyme than the unreacted ones it is difficult to synchronize the reaction.

Investigations aiming at overcoming these limitations are being carried out in this laboratory. The reaction of adding deoxynucleotides to the 3′-end of oligoribonucleotides will be helpful for the *in vitro* synthesis of various polynucleotides, the use of which may be of interest towards an understanding of the biological function of nucleic acids. So far only the synthesis of statistical mixed polymers and the limited addition of one or two deoxynucleotides to the 3′-end of an oligoribonucleotide has been achieved [10–12]. The possible biological relevance of this type of reaction is unclear especially since even the biological role of the oligodeoxynucleotide primed reaction of this enzyme needs clarification [13].

Acknowledgements

I wish to thank Dr. R. Roychoudhury of this department for valuable advice with the enzyme purification. The excellent technical assistance of Miss A. Malchert is gratefully acknowledged. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 46).

References

[1] M. Yoneda and F.J. Bollum, J. Biol. Chem. 240 (1965) 3385.
[2] L.M.S. Chang and F.J. Bollum, J. Biol. Chem. 246 (1971) 909.
[3] E. Hansbury et al., Biochim. Biophys. Acta 199 (1970) 322.
[4] R.H. Jensen et al., Biochem. Biophys. Res. Commun. 43 (1971) 384.
[5] F.N. Hayes et al., European J. Biochem. 6 (1968) 485.
[6] C.F. Lefler and F.J. Bollum, J. Biol. Chem. 244 (1969) 594.
[7] L.M.S. Chang and F.J. Bollum, Biochemistry 10 (1971) 536.
[8] F.N. Hayes et al., J. Biol. Chem. 246 (1971) 3631.
[9] D.H. Lowry et al., J. Biol. Chem. 193 (1951) 265.
[10] J.Y. Chon and M.F. Singer, Biochem. Biophys. Res. Commun. 42 (1971) 306.
[11] G. Kaufmann and U.Z. Littauer, FEBS Letters 4 (1969) 79.
[12] S. Bon, Th. Godefroy and M. Grunberg-Manago, European J. Biochem. 16 (1970) 363.
[13] L.M.S. Chang, Biochem. Biophys. Res. Commun. 44 (1971) 124.