ACTA BIOCHIMICA POLONICA

Vol. 34 1987 No. 1

KRZYSZTOF BIELAWSKI[a], PIOTR SKOWRON[a], JÓZEF KUR[a], GRAŻYNA KONOPA[b], ANNA PODHAJSKA[a] and KAROL TAYLOR[b]

CONSTRUCTION OF A DNA-POLYMERASE I OVERPRODUCING PLASMID AND ISOLATION OF THE ENZYME*

Department of Microbiology[a] and Department of Molecular Biology[b], University of Gdańsk, Kladki 24; 80-822, Gdańsk, Poland

Received 26 August, 1986

The *polA* gene of *Escherichia coli* coding for DNA polymerase I was cloned under the control of bacteriophage λ promoter *pL* and gene *N* in a high copy number plasmid vector. The chromosomally located $\lambda cIts$ repressor gene kept the synthesis of the *polA* gene product at 28°C at a low level. Raising the temperature to 43°C resulted in inactivation of the repressor and overproduction of DNA polymerase I, which could easily be purified to homogeneity.

DNA polymerase I (deoxynucleoside triphosphate: DNA deoxynucleotidyltransferase, EC 2.7.7.7) has found many applications in the techniques of molecular biology but at present it is most often used for the labelling of DNA *in vitro* by the nick-translation method [1, 2]. Even more useful is its large proteolytic fragment (Klenow fragment), devoid of the 5'-exonuclease activity of the native enzyme [2]. The Klenov fragment is very useful both in the dideoxynucleic acid sequencing technique [3] and for the second-strand synthesis of cDNA [4]. It is also used for filling in the recessed 3'-ends [5] and finds use in rapid gel-sequencing of the RNA-primed synthesis with reverse transcriptase [6, 7]. DNA polymerase I is most often isolated from the *E. coli* lysogenic for a temperature-inducible $\lambda polA^+$ prophage NM964 [8]. The *polA* gene was cloned into the plasmid vector, giving a more efficient enzyme source [9]; however, both the DNA-polymerase overproducing strain ATL100 and the plasmid pMP5 have been patented. The DNA sequence

* This work was supported by the Polish Academy of Sciences within the Project 3.13.

coding for the Klenow fragment was cloned into the plasmid vector [10], but in this case also the plasmid pCJ55 has been patented. Therefore, we decided to construct a plasmid that would overproduce the DNA polymerase to fulfill our needs; however, we also took into consideration its possible future commercial use.

MATERIAL AND METHODS

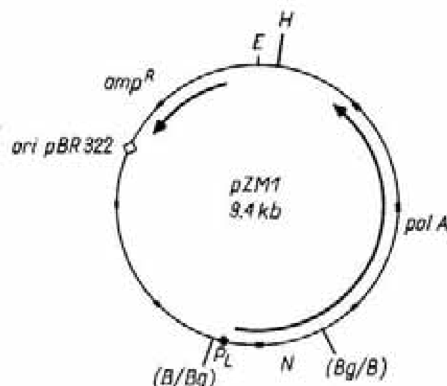
The bacteria *E. coli* N4830 were kindly given by M. Gottesman [11], the plasmid pCJ55 by C. M. Joyce [10], and the $\lambda polA^+$ phage NM964 by N. E. Murray [8]. Phage T4 lysozyme was purified according to [12]; one bacteriolytic unit corresponded to the activity of 1 µg hen egg lysozyme [13]. Restriction endonucleases and T4 polynucleotide ligase were purchased from the Department of Genetics, University of Warsaw, Polymin P (polyethyleneimine), ammonium sulphate, Coomassie Brilliant Blue R-250 and G-250, dialysis tubings and DEAE-cellulose from Serva, deoxynucleoside triphosphates, phenylmethylsulphonyl fluoride, dithiothreitol (DTT) from Sigma, NaCl (ultra pure), $MgCl_2$ (ultra pure), Amido Black 10B from Merck, Bio-Rex 70 from Bio-Rad, silicone antifoam agent from Schuchardt, [*methyl*-^{3}H]thymidine-5'-triphosphate (36 Ci/mmol) from UVVVR, Czechoslovakia.

The procedures of molecular cloning were performed according to [2]. Protein was estimated by the Coomassie [14] or Amido Black [15] method, using bovine serum albumin as a standard. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was carried out according to [16]. The activity of DNA-polymerase I was estimated at pH 9.2 since at this pH value the maximal amount of polymerase activity in impure enzyme fractions is observed [17]; the procedure of [18] with modifications recommended by Worthington was followed, but nicked T4 DNA served as the primer/template. Estimation of the activity of commercial enzymes has shown that this method gives two times higher values than the standard procedure [19], therefore the activity values of our purified preparations were appropriately corrected.

RESULTS AND DISCUSSION

Construction of the plasmid. The DNA-polymerase I-overproducing plasmid pZM1 (Scheme 1) was constructed by ligation of the *polA* gene-containing *Bgl*II-*Hin*dIII fragment of the $\lambda polA^+$ phage NM964 [8] with the *Bam*HI-*Hin*dIII fragment derived from the plasmid pλ8 [20] and present in the pCJ55 plasmid [10]. The above-mentioned fragment of pλ8 contained the origin of replication and the β-lactamase (ampicillin-resistance, Amp^r) gene from the plasmid pBR322, and the *N* gene and *pL* promoter from the

bacteriophage λ. After the separation of the appropriate restriction endonuclease digests by 0.8% agarose electrophoresis, both fragments were isolated by electroelution and ligated by T4 ligase. The ligation mixture was used to transform *E. coli* N4830 [11], and screened for ampicillin resistance.



Scheme 1. A map of the plasmid pZM1 which served as a vector for the amplification of DNA polymerase I. The plasmid is composed of three fragments derived from: (1) λ*polA* phage NM964 (*polA* gene), (2) phage λ (*N* gene and *pL* promoter), and (3) pBR322 plasmid (*Amp*r gene and origin of replication). *Bam*HI and *Bgl*II digestion both leave identical 5'-protruding sequences which, therefore, can be ligated to each other producing a hybrid junction. The construction of pZM1 is described in the text. After thermoinduction of the chromosomally-coded cI repressor, the *polA* gene is transcribed from the powerful *pL* promoter. The map is complete for the enzyme listed: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII. Ligated sticky-end junctions of former BamHI and BglII sites are denoted by B/Bg and Bg/B

The desired recombinant, pZM1, was identified by restriction analysis. The strain N4830 has the *cI857* temperature-sensitive repressor gene on a defective prophage, hence transcription of the *polA* gene starting from the *pL* promoter is repressed at 28°C. The pZM1 plasmid-carrying bacteria produce two types of colonies: small and large. As in the case of the pMP5 plasmid-carrying ATL100 [9], the bacteria that formed small colonies were the producers of DNA-polymerase I. The residual expression occurring from the *polA* own promoter, partially damaged by cutting at a *Bgl*II site, is probably responsible for the slow cell growth. The selection of normally growing (producing large colonies) non-producers of DNA-polymerase I is due to the appearance of plasmid mutants [9].

Isolation of the enzyme. DNA-polymerase I was isolated by following, in principle, the method of [9], hence only the deviations from the original procedure will be described here. One small colony of a typical morphology served for inoculating 250 ml of L broth, containing ampicillin (50 μg/ml) and vitamin B1 (20 μg/ml). After overnight incubation at 28°C, the bacteria were pelleted by centrifugation and used for the inoculation of 7 l of the same

medium in a 12 l bottle. The culture was vigorously aerated at 28°C. Air was forced through a Berkefeld G3 candle, and a silicone antifoam agent was used. At an A_{575} of about 0.45 temperature induction was initiated by pouring 3 l of the same medium preheated to 70°C. The culture was immediately transferred to a water bath at 44°C and vigorous aeration was resumed. The temperature of the culture was kept at 43°C. After 2.5 h the culture was chilled and the cells harvested by centrifugation, washed in 50 mM Tris/HCl buffer (pH 7.2), containing 20 mM EDTA, frozen in liquid nitrogen and stored at −20°C.

A crude extract was obtained by using the following modifications. The buffer for resuspending the cells contained the serine-proteases' inhibitor, phenylmethylsulphonyl fluoride at 0.02 mM. After 1 h at 4°C with occasional stirring, T4 lysozyme was added to the final activity of 1000 units/ml. After further 1.5 h, three 15-sec pulses of sonification at maximal amplitude followed.

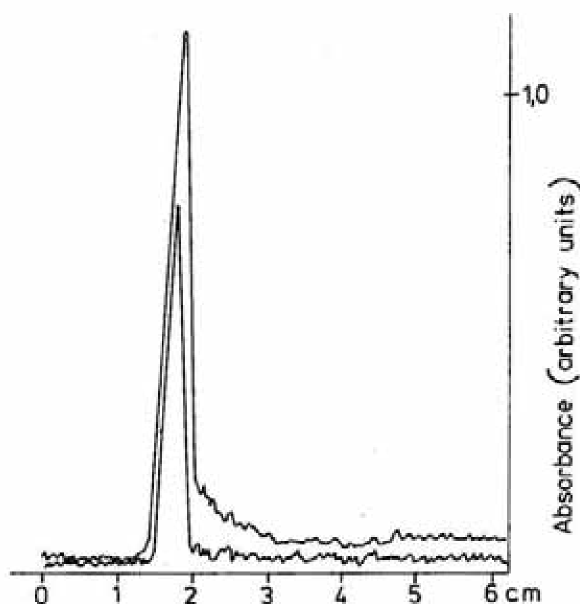


Fig. 1. Sodium dodecyl sulphate polyacrylamide 10% gel electrophoresis of 20 µg and 50 µg (overload) of DNA-polymerase I purified from the pTM1 plasmid-carrying *E. coli* N4830

The modifications of the purification procedure were as follows. The polymerase-containing extracts obtained from Polymin precipitates were brought to 0.2 M K/PO_4 buffer (pH 6.5), containing 1 mM DTT, by overnight dialysis and run through the DEAE-cellulose column (2.5 × 5 cm). The breakthrough eluate was fractionated by ammonium sulphate precipita-

tion at pH 7.0. The 0.4 - 0.85 sat. fraction was loaded onto a Bio-Rex 70 column (1.2 × 15 cm). The polymerase-containing eluate was placed in the dialysis tubing, concentrated by covering the tubing with polyethylene glycol 20 000, and dialysed against 100 mM K/PO_4 buffer (pH 7.0), containing 10 mM DTT. After addition of an equal volume of glycerol the enzyme solutions were stored at −20°C.

The yield of DNA-polymerase I was at least a hundredfold higher than that obtained from the wild type *E. coli*, and the specific activity of the final preparations amounted to about 30 000 units/mg. Figure 1 gives an indication of the purity of the final material. The conversion of the supercoiled into the relaxed form of pBR322 plasmid DNA served for tracing endonuclease contamination. Only prolonged (16 h) incubation of 10 polymerase units at high (10 mM) Mg^{2+} concentration revealed the presence of endonucleases. We use our DNA-polymerase I preparations for ^{32}P-labelling of isolated DNA by the nick-translation method, and as a starting material for the isolation of the Klenow fragment, which does not reveal any contamination with endonucleases and is used with success in the Sanger's [3] DNA sequencing.

We acknowledge the expert technical assistance of Ewa Brzychcy.

REFERENCES

1. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA-polymerase I. *J. Mol. Biol.*, **113**, 237 - 251.
2. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular cloning: a laboratory manual.* Cold Spring Harbor, New York. Cold Spring Harbor Press.
3. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 5463 - 5467.
4. Rougeon, F., Kourilsky, P. & Mach, B. (1975) Insertion of a rabbit β-globin gene sequence into an *E. coli* plasmid. *Nucl. Acids Res.*, **2**, 2365 - 2378.
5. Telford, J. L., Kressman, A., Koski, R. A., Grosschedl, R., Muller, F., Clarkson, S. G. & Brinstiel, M. L. (1979) Delimitation of a promoter for RNA polymerase III by means of a functional test. *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2590 - 2594.
6. Brownlee, G. E. & Cartwright, E. M. (1977) Rapid gel sequencing of RNA by primed synthesis with reverse transcriptase. *J. Mol. Biol.*, **114**, 93 - 117.
7. Smith, A. J. H. (1980) DNA sequence analysis by primed synthesis. *Methods Enzymol.*, **65**, 560 - 580.
8. Murray, N. E. & Kelley, W. S. (1979) Characterization of *λpolA* transducing phages; effective expression of the *E. coli polA* gene. *Mol. Gen. Genet.*, **175**, 77 - 87.
9. Minkley, Jr., E. G., Leney, A. T., Bodner, J. B., Panicker, M. M. & Brown, W. E. (1984) *Escherichia coli* DNA polymerase I. Construction of a *polA* plasmid for amplification and an improved purification scheme. *J. Biol. Chem.*, **259**, 10386 - 10392.

10. Joyce, C. M. & Grindley, N. D. F. (1983) Construction of a plasmid that overproduces the large proteolytic fragment (Klenow fragment) of DNA polymerase I of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 1830 - 1834.
11. Gottesman, M. E., Adhya, S. & Das, A. (1980) Transcription antitermination by bacteriophage lambda *N* gene product. *J. Mol. Biol.*, **140**, 57 - 75.
12. Tsugita, A., Inouye, M., Terzaghi, E. & Streisinger, G. (1968) Purification of bacteriophage T4 lysozyme. *J. Biol. Chem.*, **243**, 391 - 397.
13. Bieńkowska, K. & Taylor, A. (1979) Low molecular weight substrate for the lysozyme of T4 bacteriophage. *Eur. J. Biochem.*, **96**, 581 - 584.
14. Bradford, A. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248 - 254.
15. Schaffner, W. & Weismann, C. (1973) A rapid and specific method for the determination of proteins in dilute solution. *Anal. Biochem.*, **56**, 502 - 514.
16. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)*, **227**, 680 - 685.
17. Kelley, W. S. & Stump, K. H. (1974) A rapid procedure for isolation of large quantities of *Escherichia coli* DNA polymerase I utilizing a *λpolA* transducing phage. *J. Biol. Chem.*, **254**, 3206 - 3210.
18. Setlow, P. (1974) DNA polymerase I from *Escherichia coli*. *Methods Enzymol.*, **29**, 3 - 12.
19. Richardson, C. C., Schildkraut, L. C., Aposhian, W. H. & Kornberg, A. (1964) Enzymatic synthesis of deoxyribonucleic acid. Further purification and properties of deoxyribonucleic acid polymerase of *Escherichia coli*. *J. Biol. Chem.*, **239**, 222 - 232.
20. Reed, R. R. (1981) Transposon-mediated site-specific recombination: a defined *in vitro* system. *Cell*, **25**, 713 - 719.