Cloning and overproduction of the large proteolytic fragment (Klenow) of *Escherichia coli* DNA polymerase I

N. A. Kaushik, R. P. Sanzgiri, M. S. Patil and V. N. Pandey

Radiation Biology and Biochemistry Division, Bhabha Atomic Research Centre, Bombay 400 085, India

Using polymerase chain reaction (PCR) technique we have selectively amplified two-thirds of *E. coli* DNA polymerase I gene from the genomic DNA corresponding to the coding region of proteolytically derived 'Klenow fragment'. The PCR product was cloned in the *E. coli* expression vector pET-3a. Transcription of Klenow gene fragment in this clone was initiated from T7 RNA polymerase promoter, whereas translation was driven by the Shine–Dalgarno sequence and the initiator AUG codon of the T7 gene 10 message. The clone was introduced into an appropriate *E. coli* strain in which T7 RNA polymerase was expressed under the control of the lac promoter. Under optimal conditions of induction with isopropylthiogalactopyranoside (IPTG), Klenow polypeptide made in this bacterial strain constituted 10–15% of total cellular protein. Klenow polypeptide has been purified to homogeneity in a single step by immunoaffinity column chromatography. The purified Klenow fragment showed identical specific activity as the commercially available product. The availability of such a clone will be greatly helpful in carrying out site-directed mutagenesis to examine the regions on the enzyme molecule which are implicated in the DNA synthesis.

DNA polymerase I of *E. coli* (pol I) is a prototype multifunctional enzyme of approximately 103 kDa molecular mass[1]. It contains three distinct catalytic domains, viz. DNA polymerizing activity, 3′–5′ and 5′–3′ exonuclease activities[2]. Limited proteolysis of the pol I generates the enzymatically active fragments of 68 kDa and 35 kDa molecular mass[3,4]. The larger fragment contains 3′–5′ exonuclease and polymerase activities and the smaller fragment, 5′–3′ exonuclease activity[3,4]. The Klenow fragment of this enzyme is an excellent model to study structure–function relationship of DNA polymerases and for understanding the molecular details of template-directed DNA synthesis. Thus, site-specific reagents have been used to identify the important regions in the enzyme that are involved in the binding of dNTP substrate and template-primer[5–9]. The Klenow fragment is now cloned and a plasmid that overproduces this fragment has been constructed using conventional gene fusion and molecular cloning techniques[10]. Since this clone is patented, it is not easily available for studies based on site-directed mutagenesis. To achieve this objective a simple protocol was designed to construct a superproducing clone of Klenow, the Klenow coding region of pol I gene was amplified by polymerase chain reaction (PCR) on the *E. coli* genomic DNA and the amplified PCR product was cloned in an expression vector that overproduces the Klenow fragment in *E. coli*.

Materials and methods

Taq DNA polymerase, deoxyribonucleoside triphosphates (dNTPs), restriction enzymes and isopropyl-β-D-thiogalacto pyranoside (IPTG) were obtained from M/s Boehringer while T7 DNA sequencing kit was from US Biochemicals. Synthetic template-primers were obtained from Pharmacia, Sweden and ^{32}P labelled dNTPs and ATP were the products of BRIT, Bhabha Atomic Research Centre, Bombay. Expression vector pET-3a and expression strain *E. coli* BL-21 (DE3) were purchased from Novagen, Madison, USA. All other chemicals were of high purity grade obtained from SISCO Research Lab, Bombay.

Preparation of genomic DNA from E. coli

Five ml of Luria broth (LB) medium was inoculated with *E. coli* HB 101 and grown in a shaker-incubator at 37°C until the culture was saturated. The genomic DNA from the saturated bacterial culture was isolated following the standard protocol[11].

Preparation of Klenow–Sepharose 4B column

About 15 mg of Klenow polypeptide was covalently linked to Cyanogen bromide (CNBr) activated Sepharose-4B column as described before[12] and used as the affinity column for purification of antibodies specific to Klenow protein.

Preparation of monospecific anti-Klenow antibody

Polyclonal antibodies were raised against purified preparation of Klenow fragment. About 500 μg of Klenow protein in saline was emulsified with equal volume of Freund's complete adjuvant (Difco) and injected subcutaneously into a rabbit at multiple sites. Four booster doses were also given at two weeks intervals and serum was obtained after two weeks of the last booster. Whole serum thus obtained was loaded onto the column of Klenow–Sepharose-4B (5 ml) at a very slow flow rate. The flowthrough was collected and reloaded on the column 3–4 times and then washed extensively with phosphate buffer-saline (PBS: 10 mM phosphate buffer, pH 7.3 and 0.16 M saline) until its OD_{280} was negligible. The monospecific anti-Klenow antibodies were eluted with 0.5 M acetic acid. Two ml fractions were collected and monitored at OD_{280}. The peak fractions were pooled, neutralized with solid Tris and extensively dialysed against PBS at 4°C. The antibodies were finally concentrated by centriprep ultracentrifugation device and used for the preparation of immunoaffinity column.

Preparation of anti-Klenow antibody (immunoaffinity) column

Monospecific antibodies obtained as above were covalently linked to protein A-Sepharose as described before[13]. Briefly, the rabbit polyclonal monospecific anti-Klenow antibodies were mixed with protein A-Sepharose (2 mg IgG/ml protein A-Sepharose). After shaking for one hour at room temperature in 0.2 M triethylamine -HCl, pH 8.7, solid dimethyl suberimidate (3 mg/ml) was added and mixing continued for further four hours at room temperature. The gel suspension was poured in a column and washed successively with 10 column volumes each of PBS, 0.2 M NaCl, 3.5 M $MgCl_2$, 0.2 M NaCl and finally with PBS containing 0.05% sodium azide and stored in the same buffer at 4°C.

Amplification of Klenow gene fragment

The Klenow gene fragment of *E. coli* DNA polymerase I was amplified by PCR with the reagents from Boehringer Mannheim and DNA thermal cycler from Hybaid, Hook and Tucker Institute, UK. PCR mixtures contained 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, gelatin (1 mg/ml), 200 μM each of four dNTPs, 2.5 units of Taq DNA polymerase, 100 ng of *E. coli* genomic DNA and 0.5 μg of upstream and downstream primers each per 100 μl reaction mixture. Thermal cycle parameters were 94°C, 2 min (denaturation); 42°C, 2 min (annealing); 72°C, 3 min (elongation) and a total of 30 cycles. A time delay of 7 min at 94°C (for initial denaturation of genomic DNA) and 5 min at 72°C (final extension) was introduced at the beginning and at the end, respectively. The following oligonucleotides were synthesized in an Applied Biosystem DNA synthesizer model 380B with phosphoramidite chemistry. For the Klenow gene fragment the upstream primer was TATGGGGCCATATGATTTCTTATGAC and downstream primer was TATAGGGCAAGCTTTT-AGTGGGCCTGATCC. The upstream primer contains *Nde*I site (CATATG) and downstream primer contains *Hind*III site (AAGCTT). The 12 base sequence 3′ to *Nde*I site of upstream primer and 18 base sequence 3′ to *Hind*III site of downstream primer are homologous to 5′ ends of sense and antisense strands of Klenow coding gene, respectively[14]. All primers contain additional hexanucleotides (clamping sequences) at the 5′ end for optimal restriction enzyme recognition.

Cloning and expression

The PCR product was gel purified, restricted with *Nde*I and *Hind*III and ligated with pET-3a restricted with these two enzymes. Initial transformation and screening were done in *E. coli* HB 101. Prospective clones were then introduced into *E. coli* BL-21 (DE3) for expression. For the production of Klenow fragment, *E. coli* BL-21 (DE3) transformed with pET-3a-K was grown at 37°C in LB containing 100 μg/ml ampicillin. When the culture reached an A_{590} of 0.3, IPTG was added to 100 μM and growth was continued for another 2 h. The culture was quick chilled and cells were harvested by centrifugation.

Purification of Klenow polypeptide

The cells grown and harvested as above were resuspended in the lysis buffer (1 ml/100 mg cells) containing 0.2% lysozyme. The lysis buffer contained 50 mM Tris-HCl pH 8.0, 1 mM diothiothreitol (DTT) and 2 mM EDTA. After incubating the cell suspension on ice for 45 min, 5 M NaCl and 20% Triton X-100 were added to a final concentration of 100 mM and 0.2%, respectively. The suspension was homogenized, sonicated briefly to decrease the viscosity and centrifuged at 12,000 *g* for 30 min to obtain the crude extract. The Klenow polypeptide from the crude extract was purified either following the protocol of Joyce and Grindley[10] or by immunoaffinity column chromatography as described below.

The anti-Klenow antibody column was activated by successive washings with 10 column volumes each of 50 mM Tris-HCl pH 8.0, 3.5 M $MgCl_2$ and 100 mM NaCl in the same buffer. The crude extract was applied

on the antibody column at a very slow flow rate. The flow through was collected and reapplied on the column 3-4 times. The column was washed thoroughly with 100 mM NaCl in 50 mM Tris-HCl, pH 8.0 (Tris saline buffer). The Klenow polypeptide bound to the antibody column was eluted with 2 column volumes of 3.5 M $MgCl_2$ solution. The eluates were diluted five times with 50 mM Tris-HCl pH 8.0 and concentrated by centriprep ultrafiltration device. The concentrated enzyme sample was then extensively dialysed against several changes of 50 mM Tris-HCl pH 8.0 and finally against 50% glycerol containing 10 mM phosphate buffer pH 7.5 containing 1 mM DTT. To obtain a sequencing grade Klenow, a gel filtration step was also included to remove any contaminating DNA pol I arising from the host cell.

Enzyme assay

Polymerase activity of the Klenow fragment was assayed as described before[5-7] using activated calf thymus DNA or synthetic template-primers and ^{3}HdNTP or α^{32} PdNTP as the radiolabelled substrate.

Results

Amplification and cloning of Klenow coding region of pol I gene

The genomic DNA of *E. coli* HB 101 was used as the template and synthetic oligonucleotides with overhanging *Nde*I and *Hind*III sequences were used as upstream and downstream primers to selectively amplify two-thirds of the 2.7 kb pol I gene corresponding to the Klenow coding region by PCR. The 1.8 kb PCR product was gel purified and restriction digested with *Nde*I and *Hind*III and then inserted into an expression vector pET-3a (Figure 1). Initial transformation and screening of the recombinant pET-3a-K containing the Klenow coding insert was done in *E. coli* HB 101. Figure 1 (inset) shows the restriction analysis of several recombinant clones (pET-3a-K) containing the 1.8 kb insert. In order to further confirm that the insert in pET-3a corresponded with the Klenow-coding region, several pET-3a-K clones were subjected to DNA sequencing by dideoxy sequencing method[15]. The results of the sequence analysis are illustrated in Figure 2 which clearly read through Klenow coding region starting from ATG codon.

Expression of pET-3a-K clone in E. coli BL-21 (DE3) strain

Several prospective recombinant clones were used to check the expression of Klenow polypeptide in *E. coli*

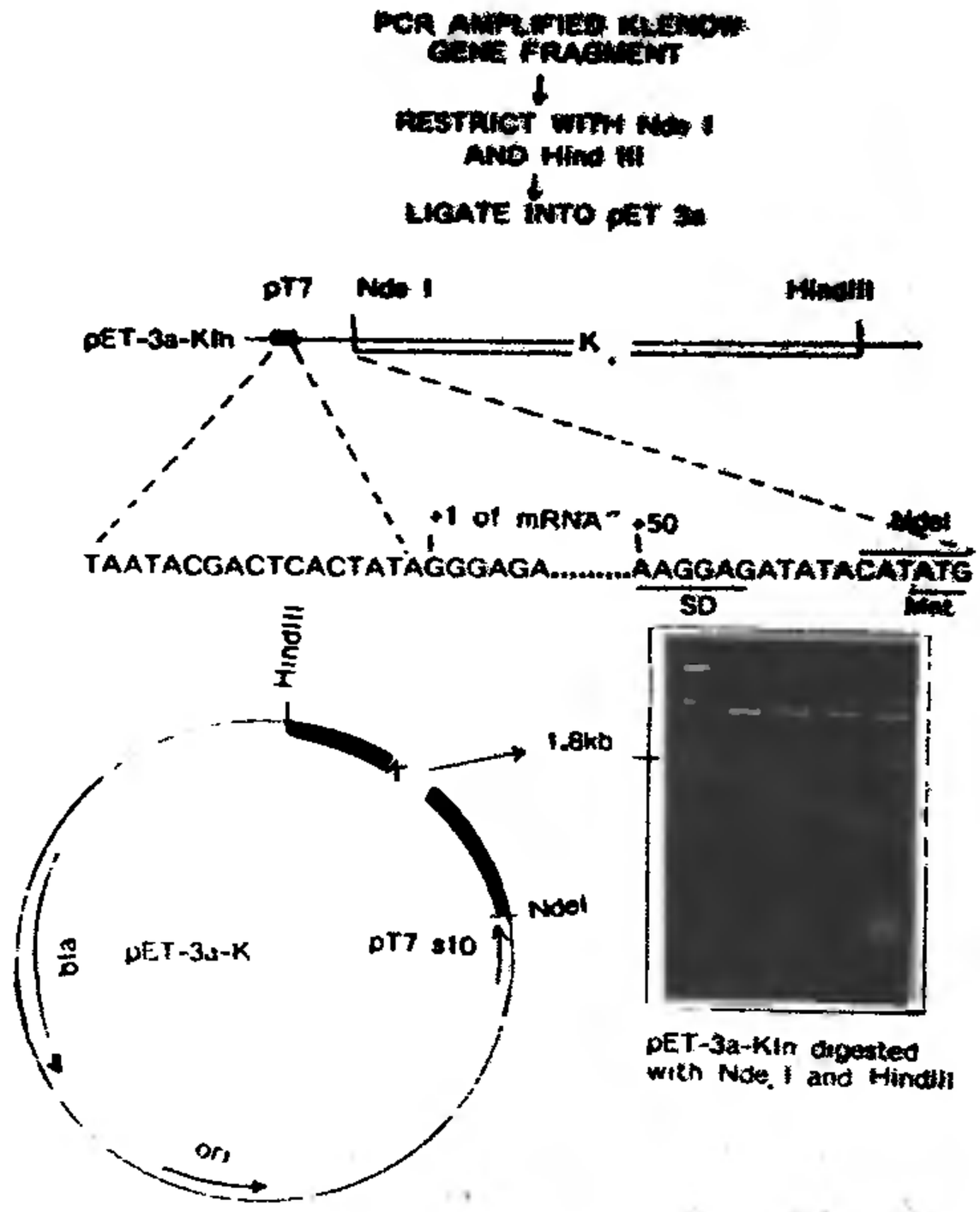

Figure 1. Cloning of PCR amplified (Klenow coding) pol I gene fragment into pET-3a. The 4.5-kb vector pET-3a with relevant restriction sites is shown at the top[16]. The T7 terminator (T7) and the transcriptional and translational start regions (pT7-s10) are indicated. Unique restriction sites for *Nde*I and *Hind*III were used for cloning of the Klenow (K) gene. PCR amplification of the K gene with *Nde*I and *Hind*III primers and subsequent insertion into pET-3a are described in the text. Sequences of transcriptional and translational control region are shown at the bottom. SD, Shine–Dalgarno sequence. The inset shows restriction analysis of several clones (pET-3a-K) containing the 1.8 kb insert.

BL-21 (DE3). The clones were introduced in this strain and induced with IPTG as described in Materials and Methods. This strain contains T7 RNA polymerase gene under the control of lac uv5 promoter[16]. Under the condition of induction, T7 RNA polymerase is induced which recognizes its own promoter on the recombinant pET-3a-K clone and transcribes massively the insert downstream to T7 promoter. This, in turn, results in the accumulation of large amount of translated product. Figure 3 shows Coomassie blue-stained protein gel profile of cell lysate from several induced and uninduced clones. As seen in the figure, all the clones expressed a major polypeptide of 68 kDa which migrated along with the standard Klenow fragment.

To ascertain that the overproduced polypeptide represented the authentic Klenow fragment, whole cell extracts of IPTG induced and uninduced strain were

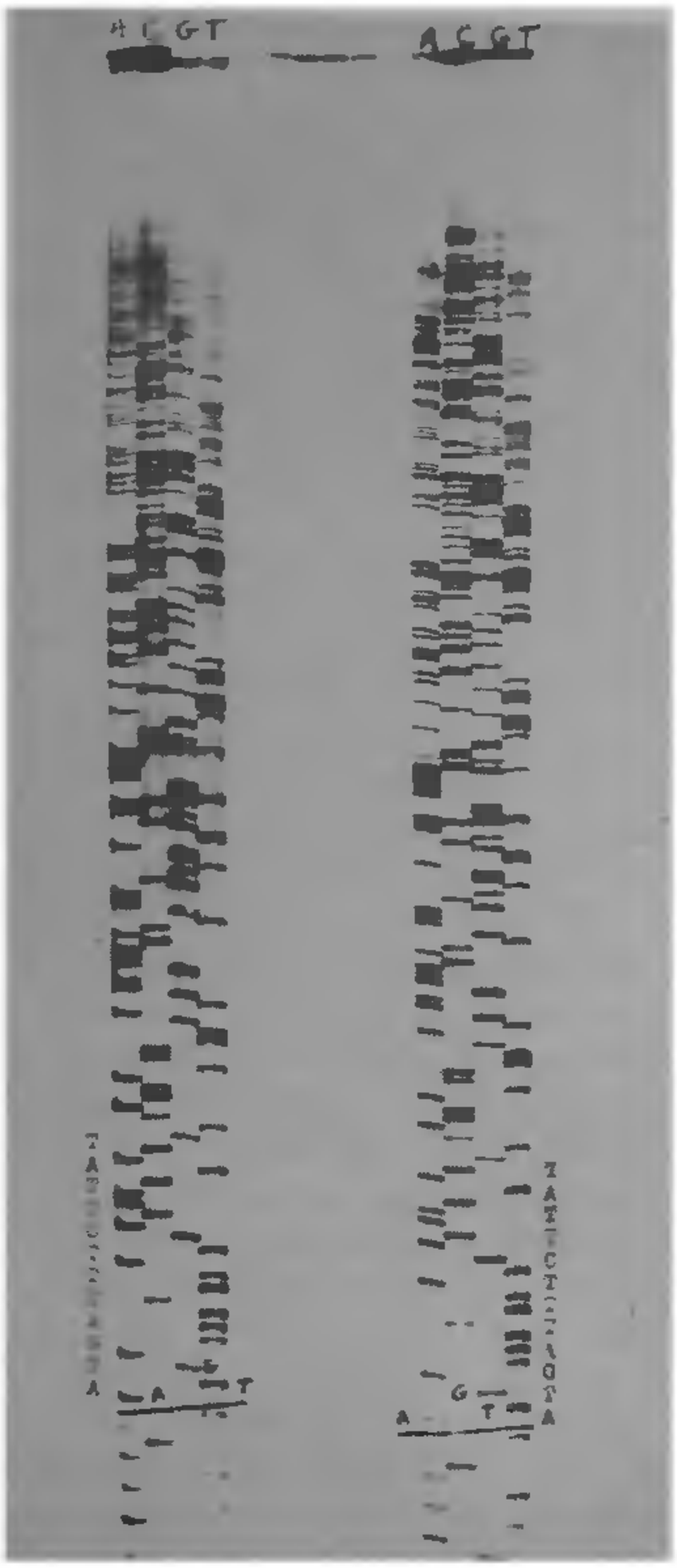

Figure 2. DNA sequencing of 1.8 kb insert in pET-3a-K. Nucleotide sequences were determined by the dideoxy chain termination method[15] using pET-3a-K plasmid as the template and T7 promoter as the sequencing primer.

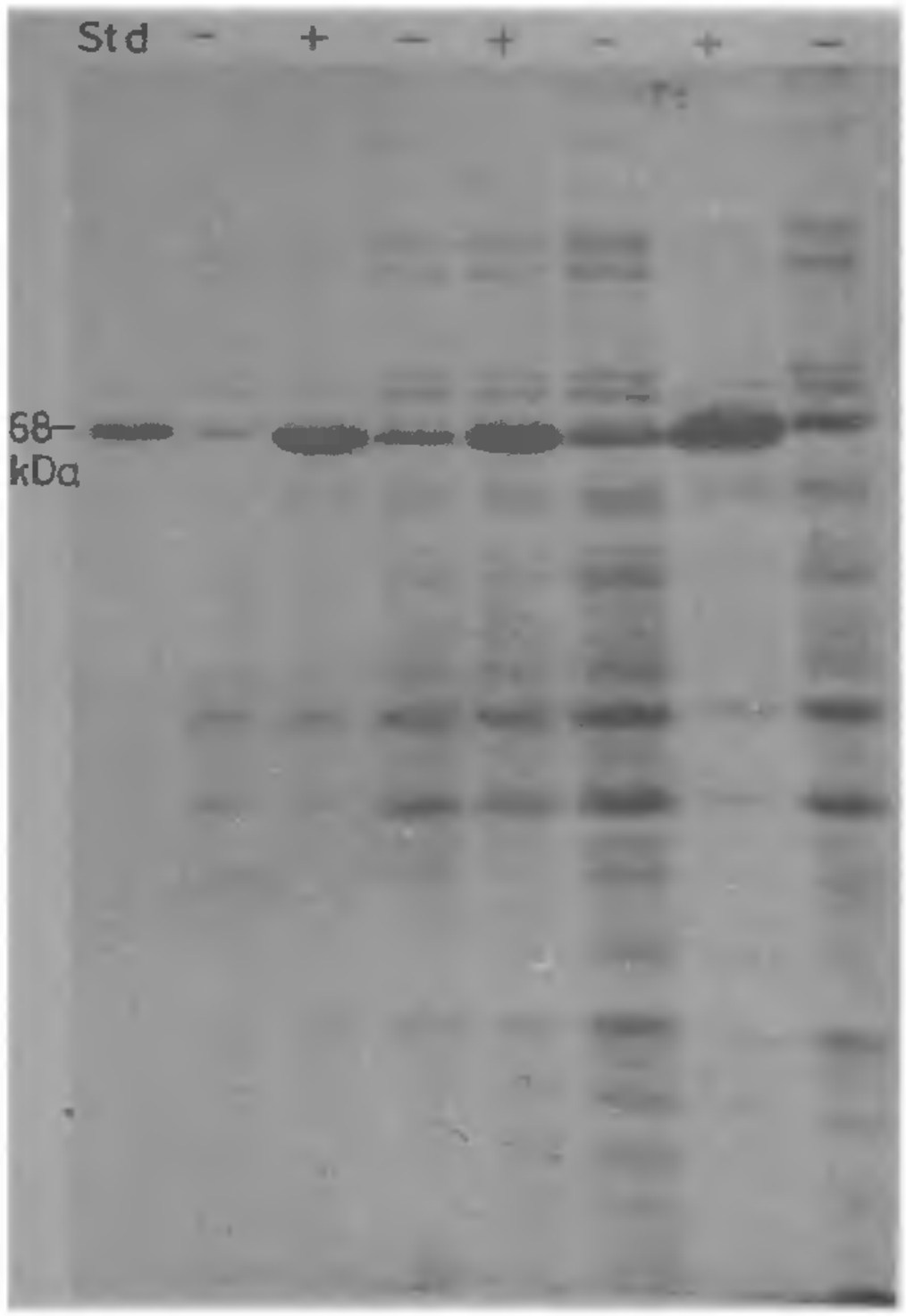

Figure 3. Overproduction of recombinant Klenow polypeptide in *E. coli*. IPTG induced ('+' lanes) and uninduced ('−' lanes) *E. coli* BL-21 (DE3) containing the pET-3a-K clones were lysed in Laemmli sample buffer[17]. Total cellular proteins (50 μg) were electrophoresed in polyacrylamide gels containing SDS by the method of Laemmli[17]. Standard Klenow polypeptide is shown in the extreme right lane.

electrophoresed on SDS polyacrylamide gel and the protein was probed by Western blotting using polyclonal rabbit anti-Klenow antibody. The results presentéd in Figure 4 show that the antibody reacted specifically with the overproduced 68 kDa polypeptide. Cell lysate from induced *E. coli* BL-21 (DE3) containing the vector without the Klenow coding insert did not show any reaction with anti-Klenow antibody (Figure 4, lane 3).

Purification of Klenow polypeptide

The induced Klenow polypeptide was purified either by conventional procedure as described by Joyce and Grindley[10] or by a single step immunoaffinity column directly from the crude extract. The clear cell lysate was applied on a pre-equilibrated affinity column prepared by covalently linking the anti-Klenow rabbit antibody to protein-A-Sepharose. The unbound proteins were removed by extensive washing of the column with the buffer. The elution of the bound Klenow polypeptide was achieved by 3.5 M $MgCl_2$. The high Mg^{2+} salt was rapidly removed by using the centriprep ultrafiltration device and finally by extensive dialysis against the phosphate buffer containing 50% glycerol. Using either of the above procedures, approximately 10 mg of Klenow polypeptide could be isolated per g of induced cells. The purified Klenow was found to be as active as the commercially available Klenow. The specific activity of the enzyme was determined using various template primers. The results shown in Table 1 indicate that the highest specific activity obtained was with activated DNA whereas the lowest was with synthetic template primer poly d(AT). The Klenow purified by either of the procedures was found to be free from pol I contamination arising from the host cell and hence excellent for DNA sequencing.

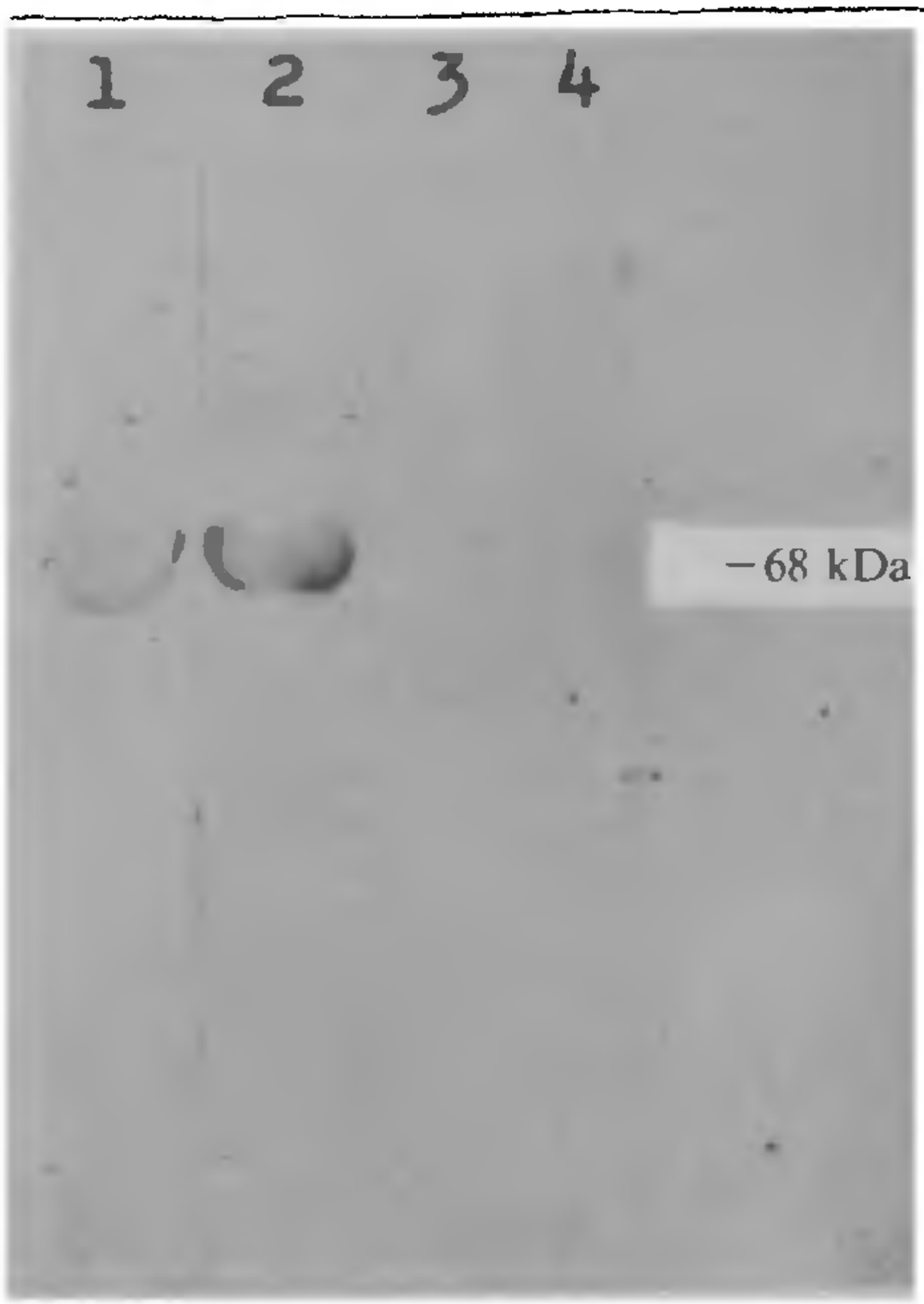

Figure 4. Western blot of overproduced Klenow protein. Following SDS-PAGE separation of total cellular proteins of *E. coli* BL-21 (DE3) containing various plasmids, Western blotting was carried out as described by Burnette[18] using polyclonal anti-Klenow rabbit antibody. Lane 1, standard Klenow polypeptide; lane 2, pET-3a-K; lane 3, pET-3a; lane 4, no plasmid.

Table 1. Polymerase activity of purified Klenow

Template primer	Specific activity (units/mg protein)
Poly (dAT)	1.25×10^4
Poly (dT)·$(dA)_{15}$	1.00×10^4
Poly (dA)·$(dT)_{15}$	2.70×10^4
Poly (dG)·$(dC)_{15}$	2.40×10^4
Poly (dC)·$(dG)_{15}$	1.50×10^4
Activated DNA	2.00×10^5

Polymerase assays with the indicated template primers (TP) were carried out as described in materials and methods. A typical reaction mixture contained: 50 mM Tris-HCl, pH 7.8; BSA, 100 μg/ml, DTT, 1 mM; 3 μg of activated calf thymus DNA or 1 μg of synthetic template primer, each dNTP, 20 μm; *[3H]dNTP or* α *[P-*^{32}*]dNTP*, 1.5 μCi; and enzyme, 4.4 nM in a final volume of 100 μl. Reactions were initiated by adding 10 mM $MgCl_2$ when TP was activated DNA, poly (dG) $(dC)_{15}$ or poly (dC) $(dG)_{15}$ and by 1 mM $MnCl_2$ when TP was poly (rA) $(dT)_{15}$ or poly (dT)·$(dA)_{15}$. Following incubation at 37°C for 15 min, reactions were terminated by the addition of ice-cold 5% TCA. The acid-precipitable radioactivity was collected on Whatman GF-B filter and counted.

Discussion

The polymerase chain reaction technique was used as a tool to selectively amplify two-thirds of the *E. coli* DNA polymerase I gene from the genomic DNA of *E. coli* corresponding to the coding region of the Klenow fragment. This was done with the objective of constructing a superproducing clone of this enzyme, which would make available large quantities of Klenow fragment for carrying out structural and functional studies involving site-directed mutagenesis. The over-producer clone constructed by Joyce and Grindley[10] using conventional gene fusion and molecular cloning techniques is patented and, therefore, not easily available for carrying out such studies.

Synthetic oligonucleotides with overhanging *Nde*I and *Hind*III sequences were used as upstream and downstream primers to amplify the Klenow coding region. The choice of introducing *Nde*I and *Hind*III sites in the respective upstream and downstream primers was because sites for these enzymes are absent in the Klenow coding region and that the expression vector pET-3a has unique sites for these restriction enzymes. The 1.8 kb PCR product was inserted into an expression vector pET-3a which is a derivative of pBR322 and a member of the pET (expression T7) series of vectors, all of which contain the strong T7 RNA polymerase promoter (Figure 2) preceding gene 10 in the T7 genome[16]. In mRNA form, pET-3a also contains the highly efficient translation initiation signals—Shine Dalgarno sequence and initiator AUG codon of the gene 10 along with a portion of its N-terminal coding sequences. We decided to use the unique *Nde*I and *Hind*III sites of this vector to clone the Klenow coding pol I gene fragment as this would remove the gene 10 coding sequences, thereby producing a Klenow protein devoid of any extraneous amino-acid residues. The pET-3a containing 1.8 kb Klenow coding insert was screened in *E. coli* HB 101 and the size of the DNA insert and its sequence were confirmed by restriction analysis (Figure 1) and dideoxy sequencing (Figure 2).

The clone was introduced into an expression strain *E. coli* BL-21 (DE3), a lysogen containing lambda derivative prophage (DE3), in which T7 RNA polymerase gene is under the control of the lac uv5 promoter[16]. Under conditions of induction with 1 mM (IPTG), Klenow polypeptide made in this bacterial strain was so overwhelmingly greater in amount that most of the induced polypeptide remained stacked with the membrane and hence was difficult to solubilize. However, at lower concentration of IPTG (50–100 μM), most of the polypeptide from the cell lysate remained in soluble form in the cell extracts while only a fraction of it was associated with the membrane which could be

solubilized by using a neutral detergent and sonication treatment. The Klenow polypeptide in the cell extract was purified to homogeneity in a single step immunoaffinity column chromatography. The purified enzyme showed specific activity identical to that of the commercially available product.

The availability of such a clone would be of immense help in designing strategies for site-directed mutagenesis which is undeniably the most effective approach for identifying the regions in the enzyme molecule that take part in different steps in DNA synthesis.

1. Jovin, T. M., Englund, P. T. and Bertsch, L. L., *J. Biol. Chem.*, 1969, **244**, 2996–3008.
2. Kornberg, A., *DNA Replication*, W. H. Freeman, San Francisco, 1980.
3. Klenow, H. and Henningson, I., *Proc. Natl. Acad. Sci. USA*, 1970, **65**, 168–175.
4. Brutlag, D., Atkinson, M. R., Setlow, P. and Kornberg, A., *Biochem. Biophys. Res. Commun.*, 1969, **37**, 982–989.
5. Pandey, V. N., Williams, K. R., Stone, K. L. and Modak, M. J., *Biochemistry*, 1987, **26**, 7744–7748.
6. Pandey, V. N. and Modak, M. J., *J. Biol. Chem.*, 1988, **263**, 6068–6073.
7. Pandey, V. N., Kaushik, N. A., Pradhan, D. S. and Modak, M. J., *J. Biol. Chem.*, 1990, **265**, 3879–3884.
8. Mohan, P. M., Basu, A., Basu, S., Abraham, K. I. and Modak, M. J., *Biochemistry*, 1988, **27**, 226–233.
9. Basu, S., Basu, A. and Modak, M. J., *Biochemistry*, 1988, **27**, 6710–6716.
10. Joyce, C. M. and Grindley, N. D. F., *Proc. Natl. Acad. Sci. USA*, 1982, **80**, 1830–1834.
11. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., *Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Inter Sciences, John Wiley and Sons, New York, 1987.
12. Kohli, J. K., Patil, M. S., Pandey, V. N. and Pradhan, D. S., *J. Gen. Appl. Microbiol.*, 1989, **35**, 429–439.
13. Pandey, V. N., Dave, V. P., Patil, M. S., Pradhan, D. S., Amrute, S. B. and Modak, M. J., *Biochemistry*, 1990, **29**, 4037–4041.
14. Joyce, C. M., Kelly, W. S. and Grindley, N. D. F., *J. Biol. Chem.*, 1982, **257**, 1958–1964.
15. Sanger, F., Nicklen, S. and Coulson, A. R., *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 5463–5467.
16. Rosenberg, A. H., Lade, B. N., Chui, D., Lin, S. W., Dunn, J. J. and Studier, F. W., *Gene*, 1987, **56**, 125–135.
17. Laemmli, V. K., *Nature*, 1970, **227**, 680–685.
18. Burnette, W. N., *Anal. Biochem.*, 1981, **112**, 195–203.

ACKNOWLEDGEMENTS. One of the authors (R. P. S.) thanks CSIR, New Delhi for a fellowship.

Received 29 May 1992; revised accepted 3 October 1992

Enigma of the negative $\delta^{18}O$ pulse at the last glacial maximum in the Arabian Sea

S. K. Gupta and P. Sharma

Physical Research Laboratory, Ahmedabad 380 009, India

Sarkar *et al.*[1] had reported a negative excursion of up to 1‰ in the $\delta^{18}O$ of planktonic foraminiferal species at the time of last glacial maximum (LGM) about 18 kyr ago from an east Arabian sea core SK-20-185. They attributed this spike to increased influx of low salinity water from Bay of Bengal through intensified westward coastal circulation caused by stronger NE winter monsoon at LGM. Krishnamurthy[2], however, opined that this negative excursion in $\delta^{18}O$ could be accounted for by the increase in sea surface temperature (SST) resulting from reduced upwelling due to weakening of the summer SW monsoon at the time of LGM. Here we use the limited available data from the east Arabian sea to show that the seasonal abundance pattern of the planktonic species is inconsistent with the mechanism invoked by Krishnamurthy[2]. In view of problems with the mechanisms proposed both by Sarkar *et al.*[1] and Krishnamurthy[2] for the observed $\delta^{18}O$ excursion, we put forward a new mechanism which invokes increased discharge of melt-water from the Tibet and Himalayas into the Bay of Bengal in response to a warming event around the time of LGM.

SARKAR *et al.*[1] have reported variations in the oxygen isotope content of four planktonic foraminiferal species (*G. menardii, G. ruber, O. universa and G. sacculifer*) from an east Arabian sea core SK-20-185 showing a negative $\delta^{18}O$ spike of up to 1‰ at the time of last glacial maximum (LGM) about 18 kyr ago (Figure 1). Alongside of Figure 1 is also shown the measured ^{14}C dates on their core which suggest a time interval of 22–18 kyr BP for the negative $\delta^{18}O$ spike. However, if a uniform sedimentation rate (2.2 cm kyr^{-1}) obtained by considering all the radiocarbon dated levels is assumed, as was done by Sarkar *et al.*[1], the period of $\delta^{18}O$ spike would correspond to around 18 kyr. They[1] cited this spike as an evidence for increased influx of low salinity water from the Bay of Bengal to the core location (10 N, 71°50′ E) through the intensified westward coastal circulation due to a stronger NE winter monsoon at LGM. This interpretation has already provoked a comment by Krishnamurthy[2] who has opined that the negative pulse in the $\delta^{18}O$ of up to 1‰ could be accounted for by the weakening of the summer monsoon and the