Accelerated Publications

Identification and Properties of the Catalytic Domain of Mammalian DNA Polymerase β

Amalendra Kumar, John Abbotts, Essam M. Karawya, and Samuel H. Wilson*
Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892
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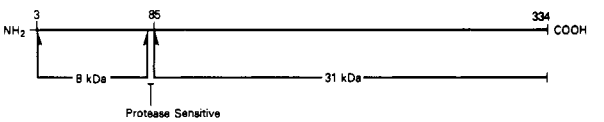
ABSTRACT: Rat DNA polymerase β (β-pol) is a 39-kDa protein organized in two tightly folded domains, 8-kDa N-terminal and 31-kDa C-terminal domains, connected by a short protease-sensitive region. The 8-kDa domain contributes template binding to the intact protein, and we now report that the 31-kDa C-terminal domain contributes catalytic activity. Our results show that this domain as a purified proteolytic fragment conducts DNA synthesis under appropriate conditions but the k_{cat} is lower and primer extension properties are different from those of the intact enzyme. A proteolytic truncation of the 31-kDa catalytic domain fragment, to remove a 60-residue segment from the NH_2-terminal end, results in nearly complete loss of activity, suggesting the importance of this segment. Overall, these results indicate that the domains of β-pol have distinct functional roles, template binding and nucleotidyltransferase, respectively; yet, the intact protein is more active for each function than the isolated individual domain fragment.

DNA polymerase β (β-pol) is a low molecular weight (39 000) eukaryotic repair DNA polymerase capable of both DNA-dependent and RNA-dependent DNA synthesis, as is the case for *Escherichia coli* DNA pol I (Travaglini & Loeb, 1974; Loeb et al., 1973; Stavrianopoulos et al., 1971). Human and rat β-polymerases are single-chain polypeptides of 335 amino acids (SenGupta et al., 1986; Zmudzka et al., 1986), and their primary structures are highly conserved during the ~80 million years generally considered as divergence time. The enzyme from human and rat sources has been overexpressed in *E. coli* (Abbotts et al., 1988a; Date et al., 1988; Kumar et al., 1990), and the sequence of the expressed rat protein matches precisely with the DNA-deduced sequence (Kumar et al., 1990), except for the absence of the N-terminal Met. The expressed enzymes are active and are found to have similar template-primer specificity and reaction properties as the corresponding natural enzymes (Abbotts et al., 1988a). In constrast to other DNA polymerases, β-pol is known to catalyze DNA synthesis in a distributive mode that requires the enzyme to dissociate from and rebind to the template during each dNMP incorporation cycle (Chang, 1975; Detera et al., 1981; Fry & Loeb, 1986; Abbotts et al., 1988a).

β-Polymerase is detected in all mammalian tissues and is generally expressed at a low level, as for a number of other so-called constitutive "housekeeping" enzymes (Zmudzka et al., 1988; Fry & Loeb, 1986; Mitchell et al., 1985). Since the β-pol expression level does not appear to be related to the proliferative state of a cell (Zmudzka et al., 1988), β-pol has long been considered to be involved in DNA repair [for reviews, see Wilson et al. (1988), Fry and Loeb (1986), and Miller and Chinault (1982a,b)], and DNA polymerase inhibitor studies of DNA repair have suggested a direct role of β-pol in certain, but not all, DNA repair processes (Hammond et al., 1990; Fry & Loeb, 1986).

Recently, circular dichroism analysis and protease domain mapping studies were carried out with rat β-pol (Kumar et al., 1990). These studies are interpreted to mean that the enzyme is a tightly folded β-sheet structure with two distinct domains separated by a short hinge-like region:



The N-terminal domain of ≃80 amino acids has single-stranded nucleic acid or template binding activity, whereas the C-terminal domain of ≃250 amino acids has negligible template binding activity. Each domain as an isolated proteolysis fragment is devoid of or has only minimal DNA polymerizing activity compared with the intact enzyme.

In this study, we further evaluated the question of DNA synthesis by the isolated 31-kDa C-terminal domain fragment. The minimal activity (≤1%) detected earlier for the C-terminal fragment could have been the result of trace contamination by the intact enzyme or an *E. coli* DNA polymerase and could not be interpreted as indicating the protein is capable of DNA synthesis. Our present data demonstrate that the 31-kDa fragment has DNA polymerase activity and that the C-terminal domain can be viewed as the catalytic domain of intact β-polymerase. This domain alone exhibits different primer elongation properties than the intact protein, indicating that the 8-kDa template binding domain plays an active role in modulating replication.

Experimental Procedures

Materials

M13mp2 DNA [viral (+)-strand] and primer 115 were as previously described (Bebenek et al., 1989) and were generous gifts from Dr. Thomas A. Kunkel (National Institutes of Environmental Health Sciences, Research Triangle Park, NC). T4 polynucleotide kinase and dNTPs were obtained from Pharmacia. [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from New England Nuclear or ICN Radiochemicals. Electrophoresis-grade acrylamide and bis(acrylamide) were obtained

* To whom correspondence should be addressed at Building 37, Room 4D-25, National Cancer Institute, National Institutes of Health.

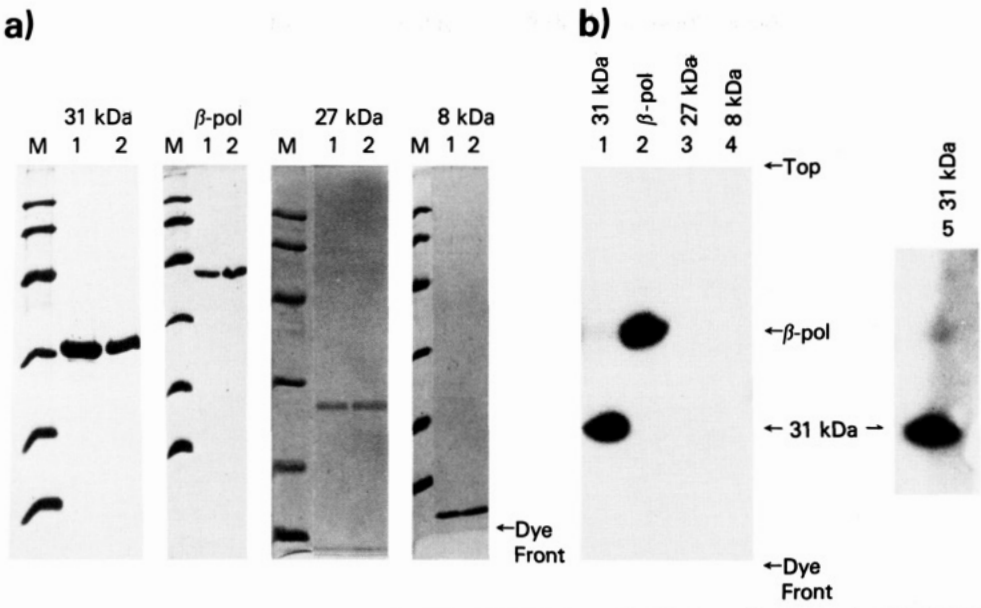


FIGURE 1: Analysis of DNA polymerase β and its fragments. (Panel a) Photograph of Coomassie blue stained 12.5% SDS–polyacrylamide gel analysis of β-polymerase and its fragments. β-Polymerase and fragments were purified as described by Kumar et al. (1990). M in each case indicates the molecular weight markers, and lanes 1 and 2 represent the two different concentrations of the protein. 31 kDa: lane 1, 10 μg of protein; lane 2, 5 μg of protein. β-pol, 27 kDa, and 8 kDa: lane 1, 2 μg of protein; lane 2, 4 μg of protein. Marker proteins (kdaltons) were phosphorylase *b* (97.4), bovine serum albumin (66.2), ovalbumin (42.7), carbonic anhydrase (31), soybean trypsin inhibitor (21.5), and lysozyme (14.4). (Panel b) Activity gel analysis of β-polymerase and its fragments. Photographs of autoradiograms are shown. The activity gel was run as described by Karawya et al. (1983) with activated DNA as template-primer and [^{32}P]dCTP as labeled dNTP. Amount of protein layered in each lane is as follows: lane 1, 1 μg; lane 2, 0.05 μg; lane 3, 2 μg; lane 4, 1 μg. Lane 5 received 1 μg of protein, but the gel was incubated with a reaction mixture containing 1 μg of 8-kDa fragment.

from Bio-Rad Laboratories. [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham.

Methods

Isolation of DNA Polymerase β and Its Fragments. The recombinant rat DNA polymerase β was purified from *E. coli* RRI (pRK248cIts, pRC-Rβ1) as described by Kumar et al. (1990). Fragments of 31 and 8 kDa were obtained after digestion of β-polymerase with trypsin, while chymotrypsin was used to generate 27-kDa fragment. These fragments were essentially purified as described by Kumar et al. (1990).

Activity Gel Analysis. Activity gel analysis was carried out as described by Karawya et al. (1983). The protein samples were electrophoresed in 10% SDS–polyacrylamide gels containing activated calf thymus DNA as template-primer and probed with a reaction mixture containing [^{32}P]dCTP as labeled dNTP.

Preparation of Labeled DNA Primers. A synthetic DNA primer, complementary to *lac Z* sequence positions 129–115 (primer 115) of the M13mp2 DNA template (Bebenek et al., 1989), with its 3′-hydroxyl group corresponding to position 115, was 5′ end labeled with ^{32}P by the procedure of Maxam and Gilbert (1980). DNA was extracted with phenol, residual phenol was removed with ether, and the DNA was precipitated with ethanol.

In Vitro DNA Synthesis on M13 DNA. Primer labeled with ^{32}P was hybridized to M13 DNA at a 1:1 molar ratio of primer-template by heating to 100 °C and slowly cooling to room temperature. DNA synthesis reactions (10 μL) contained 20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM each of dATP, dCTP, dGTP, and dTTP, 0.15 μg (63 fmol) of M13 DNA template hybridized to primer, and enzyme as indicated. Reaction mixtures were incubated at 37 °C for 1–30 min, and reactions were stopped by adding EDTA to a final concentration of 18 mM. A dye mixture in deionized formamide was then added to a total volume of 15 μL. Portions of 4 μL were loaded for gel electrophoresis.

Analysis of Products of DNA Synthesis. Products were analyzed by gel electrophoresis as previously described (Detera & Wilson, 1982). Electrophoresis was conducted on a 12% polyacrylamide–7 M urea gel (40 × 35 cm × 0.4 mm). The gel was prerun for 1 h at 40–45 W without cooling, and electrophoresis was performed at 40–45 W. After electrophoresis, the gel was transferred to Whatman 3MM paper and covered with Saran Wrap. Products were visualized by autoradiography with Kodak XAR-5 film at room temperature.

Other Methods

Protein concentration was determined by the method of Lowry et al. (1951). SDS–polyacrylamide gel electrophoresis of proteins was performed according to Laemmli (1970). Protein markers were obtained from Bio-Rad and were as follows (in kdaltons): phosphorylase *b*, 97.4; BSA, 66.2; ovalbumin, 42.7; carbonic anhydrase, 31; soybean trypsin inhibitor, 21.5; lysozyme, 14.4.

Results and Discussion

Activity Gel Analysis. By use of controlled proteolysis, we prepared the two domain fragments of rat β-pol as reported previously (Kumar et al., 1990). These are an 8-kDa N-terminal fragment and a 31-kDa C-terminal fragment, as well as a ~27-kDa fragment of the C-terminal domain, corresponding to residues 140–334 of β-pol. These purified protein preparations were analyzed by SDS–PAGE (Figure 1a) and also were examined by the activity gel assay method described by Karawya et al. (1983). *E. coli* Pol I and β-pol are particularly suited to this assay because these enzymes have similar k_{cat} in the gel and solution assays (Karawya et al., 1983), whereas other mammalian DNA polymerases, such as α, are much less active in the gel assay (Karawya & Wilson, 1982; Karawya et al., 1984). Typical results are shown in Figure 1b. It is apparent that the 31-kDa fragment has DNA synthesis activity. Yet, a much higher level of 31-kDa fragment protein is required for detection of activity than in the case of intact β-pol. The 31-kDa fragment did not produce

a detectable signal at the gel positions corresponding to either intact β-pol or *E. coli* Pol I, indicating that this preparation is not contaminated by those enzymes. When the 8-kDa and 27-kDa fragments were tested at the same protein level as the 31-kDa fragment (or at higher levels not shown), no DNA polymerase activity was detected throughout the gel lane. It is worth noting that none of the protein preparations were contaminated by *E. coli* Pol I (M_r 109 000), since this is a concern with any other DNA polymerases isolated from *E. coli*. Also shown in Figure 1b is that addition of excess purified 8-kDa fragment to the DNA polymerase incubation mixture containing the gel-embedded renatured 31-kDa fragment failed to increase activity of the 31-kDa fragment. This result is consistent with earlier results of solution assays where mixing of the 31- and 8-kDa fragments failed to restore activity similar to that of the intact protein (Kumar et al., 1990). The specific activity of the 31-kDa fragment in these assays with poly[d-(A)]·oligo[d(T)] as template-primer (Kumar et al., 1990; Abbotts et al., 1988a) is 100-fold less than that of the intact enzyme (i.e., approximately 50 μmol of dTMP incorporated per hour per milligram of protein).

Product Analysis after Synthesis on an M13 Template. We further examined the activities of DNA polymerase β and its fragments by analyzing the products of synthesis on an M13mp2 DNA template. With this method, one employs a specific primer labeled with ^{32}P on its 5′ end. The products of a DNA synthesis reaction in vitro are then separated by gel electrophoresis and visualized by autoradiography. Figure 2 displays the products formed by the proteins investigated here. We had previously shown, by conducting the analysis in the presence of template-primer excess, that β-pol is a distributive enzyme, adding a single nucleotide and then dissociating from template-product complex (Abbotts et al., 1988a). In the experiment shown in Figure 2, β-pol was present in excess, resulting in reinitiation of nascent chains during the synthesis period. After a 30-min incubation, most of the primer is extended by ~200 nucleotides or more (Figure 2, lanes 1–4). When reactions are carried out with the same concentration of 31-kDa fragment, little DNA synthesis is observed (Figure 2, lanes 5–8). Even after 30-min incubation, the primer is extended by only a few nucleotides (Figure 2, lane 8). With protein concentration increased by an order of magnitude, the 31-kDa fragment shows greater activity, but the extent of synthesis is still much less than that seen with β-pol at a lower concentration (Figure 2, lanes 9 and 10). With the 27-kDa fragment, analysis of products indicates some, very minimal, activity (Figure 2, lanes 11–13); after 60-min incubation, primer + one product is clearly seen, but we cannot rule out the possibility that the activity seen with 27-kDa fragment is due to contamination by β-pol or the 31-kDa fragment; however, it seems clear that a significant decrease in activity is observed when the 31-kDa fragment is reduced by NH_2-terminal truncation to the 27-kDa fragment.

We and others (Grosse & Krauss, 1984; LaDuca et al., 1983) have found that several different DNA-polymerizing enzymes show "pause-site" patterns during synthesis that are characteristic of the individual enzyme (Abbotts et al., 1988a,b; Bebenek et al., 1989; Becerra et al., 1990). It is noteworthy that the 31-kDa enzyme shows a pause-site pattern that is different from that of intact β-pol. For example, the 31-kDa fragment shows strong pause sites at positions 110–112, while β-pol shows modest pause sites at positions 110 and 112. Also noteworthy are the patterns seen near positions 88–97. The sequence on the M13mp2 template in this region is GGGAAAACCC, which could theoretically form a stem–loop

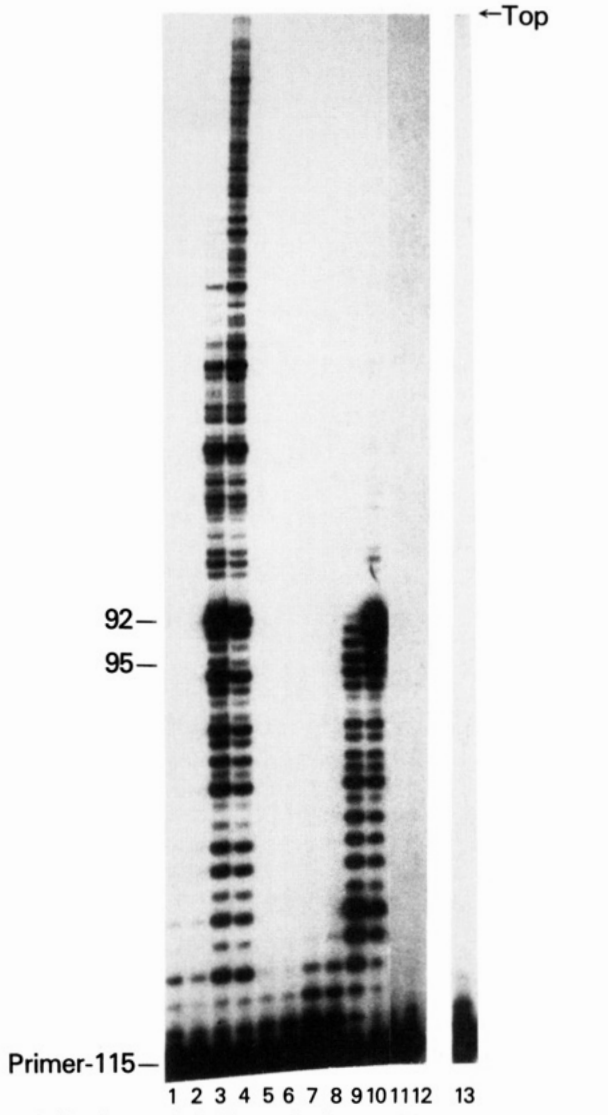


FIGURE 2: Product analysis for synthesis on an M13 DNA template. Photographs of autoradiograms are shown. DNA synthesis reactions were carried out on an M13mp2 DNA template with labeled primer 115, and the products of synthesis were examined by gel electrophoresis and autoradiography as described under Experimental Procedures. Enzyme amounts and incubation times were as follows: lane 1, 5 ng of β-polymerase, 1 min; lane 2, 5 ng of β-pol, 3 min; lane 3, 5 ng of β-pol, 10 min; lane 4, 5 ng of β-pol, 30 min; lane 5, 5 ng of 31-kDa fragment, 1 min; lane 6, 5 ng of 31-kDa fragment, 3 min; lane 7, 5 ng of 31-kDa fragment, 10 min; lane 8, 5 ng of 31-kDa fragment, 30 min; lane 9, 50 ng of 31-kDa fragment, 30 min; lane 10, 100 ng of 31-kDa fragment, 30 min; lane 11, 200 ng of 27-kDa fragment, 10 min; lane 12, 200 ng of 27-kDa fragment, 30 min; lane 13, 200 ng of 27-kDa fragment, 60 min. Lanes 1–12 were all displayed on the same polyacrylamide gel; lane 13 was displayed in a separate experiment. Markers indicate the positions of unextended primer and extension by 20 and 23 nucleotides. Position 112 corresponds to the third band above the band marked primer-115, and position 110 corresponds to the fifth band above the band marked primer-115.

structure, and we have found that several enzymes terminate processive DNA synthesis in this region [Bebenek et al. (1989) and unpublished observations]. A positive correlation between termination of synthesis by DNA polymerases and other potential stem–loop structures has been well documented (Weaver & DePamphilis, 1982). This region also shows pause sites for β-pol and 31-kDa fragment, but once again, the patterns vary with enzyme; β-pol shows strong pause sites at positions 91–93 yet can extend products from this region with time. In comparison, 31-kDa fragment shows several strong pause sites at positions 92–96 and exhibits considerable dif-

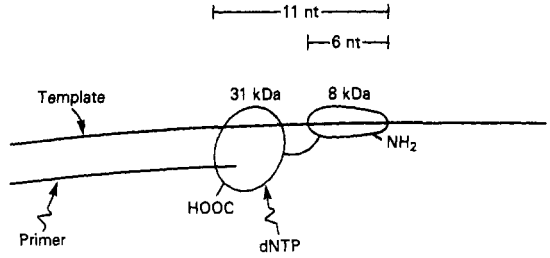


FIGURE 3: Speculative model showing proteolytic domains of β-polymerase interacting with template-primer. The hinge-like region between residues ~75 and 85 is shown as a solid line (—), whereas the 31-kDa domain and 8-kDa domain, respectively, are shown binding to a single-stranded segment of the template and the vicinity of the 3′-OH end of the primer. Binding site sizes in nucleotide residues are noted and are from Kumar et al. (1990).

ficulty extending synthesis beyond this region. The molecular basis for this difference in pause sites is unknown, although one suspects that enzyme–DNA contact phenomena are responsible.

In conclusion, we had previously shown that both the 8-kDa fragment and intact β-pol bind to single-stranded nucleic acid. Intact β-pol has a binding site size of ~11 nucleotide residues per protein monomer, while the 8-kDa fragment has a site size of ~6 nucleotides per monomer; in addition, the free energy of binding with the 8-kDa fragment is diminished by about 30% from that for intact enzyme (Kumar et al., 1990). We show here that the 31-kDa fragment retains DNA polymerase activity, but this is different in properties from the activity of the intact polymerase both quantitatively and qualitatively; the 31-kDa fragment shows a k_{cat} considerably diminished from that of the intact enzyme, as well as a different pause-site pattern during synthesis. These results, thus, indicate that the 8- and 31-kDa domains of β-pol have distinct functional roles, template binding and nucleotidyltransferase, respectively (Figure 3); yet, the intact protein is different for each function than the isolated individual domain fragment. Since the 31-kDa fragment enzyme, when used at a high level, is capable of sufficient DNA synthesis to replicate a strategic segment of M13 DNA template, a potential role of the 8-kDa template binding domain in DNA replication accuracy can be examined, and these studies are underway.

Registry No. β-Pol, 9012-90-2.

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