Mammalian DNA Polymerase β: Characterization of a 16-kDa Transdomain Fragment Containing the Nucleic Acid-Binding Activities of the Native Enzyme†

José R. Casas-Finet,‡ Amalendra Kumar,§ Richard L. Karpel,‡ and Samuel H. Wilson*,§

Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228, and Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: The 39-kDa DNA polymerase β (β-Pol) molecule can be readily converted into two constituent domains by mild proteolysis; these domains are represented in an 8-kDa N-terminal fragment and a 31-kDa C-terminal fragment [Kumar et al. (1990a) *J. Biol. Chem. 265*, 2124–2131]. Intact β-Pol is a sequence-nonspecific nucleic acid-interactive protein that binds both double-stranded (ds) and single-stranded (ss) polynucleotides. These two activities appear to be contributed by separate portions of the enzyme, since the 31-kDa domain binds ds DNA but not ss DNA, and conversely, the 8-kDa domain binds ss DNA but not ds DNA [Casas-Finet et al. (1991) *J. Biol. Chem. 266*, 19618–19625]. Truncation of the 31-kDa domain at the N-terminus with chymotrypsin, to produce a 27-kDa fragment (residues 140–334), eliminated all DNA-binding activity. This suggested that the ds DNA-binding capacity of the 31-kDa domain may be carried in the N-terminal segment of the 31-kDa domain. We used CNBr to prepare a 16-kDa fragment (residues 18–154) that spans the ss DNA-binding region of the 8-kDa domain along with the N-terminal portion of the 31-kDa domain. The purified 16-kDa fragment was found to have both ss and ds polynucleotide-binding capacity. Thermodynamic binding properties for these activities are similar to those of the intact enzyme. The results indicate that all of the nucleic acid-binding capacity of β-Pol is carried in the first 154 residues and permit localization of the ss- and ds-binding capacities, respectively, to contiguous segments within this region of the protein.

Mammalian DNA polymerase β (β-Pol), a 39-kDa monomeric DNA-polymerizing enzyme without associated nuclease activity [for review, see Wilson (1990)], has been overexpressed in *Escherichia coli* (Abbotts et al., 1988; Date et al., 1991; Kumar et al., 1990a); the recombinant proteins from human and rat appear fully active in DNA synthesis and possess template–primer specificity and catalytic properties similar to those of the natural enzymes (Abbotts et al., 1988). Detailed studies on recombinant β-polymerase domains have been carried out by proteolytic and chemical cleavage of the enzyme under controlled conditions (Kumar et al., 1990a). These studies indicate that β-Pol is organized in two tightly folded, functionally distinct domains of 8- and 31-kDa, respectively. The 8-kDa domain contributes single-stranded (ss)[1] nucleic acid binding, and the 31-kDa domain contributes catalytic activity and has double-stranded (ds) nucleic acid-binding capacity (Kumar et al., 1990a; Casas-Finet et al., 1991). In this study, we have extended our earlier work on nucleic acid-binding properties of intact β-pol and its purified 8- and 31-kDa domains (Casas-Finet et al., 1991) to the chymotryptic-prepared 27-kDa fragment and a CNBr-prepared 16-kDa fragment (Kumar et al., 1990a). The latter fragment spans the protease-sensitive segment linking the 8- and 31-kDa domains and contains the region previously shown to be responsible for the ss nucleic acid-binding function of β-polymerase. Another region present in the 16-kDa fragment could possibly contribute the ds nucleic acid-binding function of intact β polymerase. Therefore, we characterized thermodynamic properties of nucleic acid binding by the 16-kDa fragment (occluded binding site size, intrinsic affinity, cooperativity, salt dependence) and compared these properties with those of intact β-polymerase and the individually purified 8- and 31-kDa domains. Our results indicate that the segment from residues 18–154 of β-polymerase contributes the enzyme's nucleic acid-binding sites. Thus, we found that the 16-kDa fragment is able to bind both ss and ds nucleic acid lattices, and the binding parameters are similar to those of intact β-Pol. Boundaries of these various fragment peptides of the enzyme are summarized in Figure 1. These results pointing to the contiguous location of the ss and ds nucleic acid-binding sites within the structure of the protein could have important implications for the enzyme's function.

MATERIALS AND METHODS

Isolation of DNA Polymerase β and the 27-kDa Fragment. Recombinant rat DNA polymerase β was purified from *E. coli* RRI (pRK248clts, pRC-Rβ1) as previously described (Kumar et al., 1990a). The 27-kDa fragment was prepared by controlled proteolysis of β-polymerase with chymotrypsin (25:1, w/w) for 30 min, in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, and 1 mM phenylmethanesulfonyl fluoride at 25 °C. The 27-kDa fragment was purified by phosphocellulose column chromatography as previously described (Kumar et al., 1990a).

Preparation of the 16-kDa Fragment. A 3.4-mg sample of homogeneous β-polymerase in 20 mM Tris–HCl, pH 7.5, containing 0.5 M NaCl, 1 mM Na_2EDTA, and 1 mM phenylmethanesulfonyl fluoride was made to 70% formic acid

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* To whom correspondence should be addressed: Sealy Center for Molecular Science, H-51, University of Texas Medical Branch, Galveston, TX 77550.

‡ University of Maryland.

§ National Cancer Institute.

[1] Abbreviations: bp, base pair; ds, double-stranded; Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; fwhm, line-width (full width at half-maximum); poly(A), poly(riboadenylic acid); poly(dA), poly(deoxyadenylic acid); poly(εA), poly(1,N^6-ethenoadenylic acid); poly(dT), poly(deoxythymidylic acid); poly(U), poly(uridylic acid); SDS, sodium dodecyl sulfate; ss, single-stranded.

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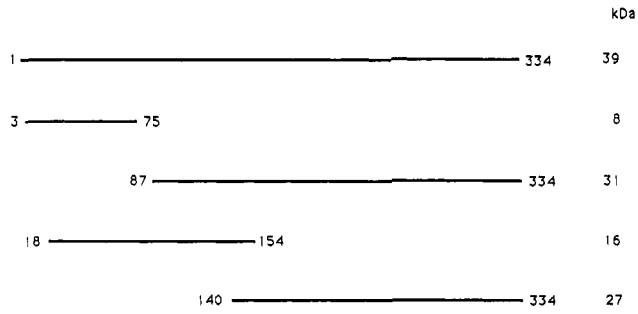

FIGURE 1: Summary of fragments of DNA polymerase β. The N-terminal sequences of the four fragments were measured, but the C-terminal sequences were assumed on the basis of size measurements. The 31-kDa fragment was a mixture of two peptides, one starting at residue 83 and the other at residue 87 as shown (Kumar et al., 1990a).

and treated in the dark at 25 °C with a freshly prepared solution of CNBr in 90% formic acid at a protein to CNBr ratio of 1:3 for 48 h. Upon digestion, the sample was diluted to 30 mL and lyophilized. This was repeated once again after resuspension in H_2O. The lyophilized powder was resuspended in 3 mL of H_2O and dialyzed for 24 h against 25 mM Tris–HCl, pH 7.5, containing 250 mM NaCl, 1 mM Na_2EDTA, and 1 mM DTT.

Purification of the 16-kDa Fragment. The dialyzed sample was layered on a phosphocellulose column (1 × 4 cm) equilibrated with 25 mM Tris–HCl, pH 7.5, containing 250 mM NaCl, 1 mM Na_2EDTA, and 1 mM DTT. The column was washed stepwise with 3-mL aliquots of the equilibration buffer containing either 250 mM, 500 mM, 600 mM, 800 mM, or 1 M NaCl. The column was run at room temperature while 1.5-mL fractions were collected on ice. The 500 mM NaCl fractions, containing the 16-kDa fragment at a concentration of ~0.2 mg mL^{-1}, were pooled and stored at −70 °C. Overall yield of 16-kDa fragment per milligram of treated β-polymerase was 150 μg. The 16-kDa fragment was found to be highly purified by SDS–PAGE analysis; a sample containing 20 μg of the 16-kDa fragment peptide also contained about 0.1 and 0.4 μg of contaminating peptides at 21 and 10 kDa, respectively.

The concentrations of stock solutions of β-polymerase and 16- and 27-kDa fragments were calculated from their ultraviolet absorbance, using the following molar absorption coefficients estimated from the amino acid composition: $\epsilon_{280} = 21.2 \times 10^3$ M^{-1} cm^{-1} for β-polymerase, $\epsilon_{280} = 17.6 \times 10^3$ M^{-1} cm^{-1} for the 27-kDa fragment, and $\epsilon_{274} = 7.2 \times 10^3$ M^{-1} cm^{-1} for the 16-kDa fragment. Absorption spectra were acquired at 25 °C with a Gilford Response spectrophotometer using matched 1-cm path-length Suprasil quartz cells. The concentration of the 16-kDa fragment was determined also from Bradford assays (Bradford, 1976) performed in triplicate. Molecular weight markers were obtained from Bio-Rad. Markers and their respective molecular masses were as follows: phosphorylase *b*, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 42.7 kDa; carbonic anhydrase, 31.0 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa; bovine trypsin inhibitor, 6.2 kDa; and insulin B chain, 3.4 kDa.

Partial Proteolysis of the 16-kDa Fragment with Trypsin. Proteolytic cleavage of the 16-kDa fragment was carried out at 25 °C by incubating the 16-kDa fragment (0.13 mg mL^{-1}) with trypsin (substrate to enzyme ratio, 1000:1, w/w) in 25 mM Tris–HCl, pH 7.5, containing 300 mM NaCl, 1 mM Na_2EDTA, and 1 mM DTT for different times. The reaction was terminated by boiling the samples in SDS–PAGE sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.001% bromophenol blue).

Protein Microsequencing of the 16-kDa Fragment Subdomains. The 16-kDa fragment was cleaved by trypsin as described above, and the resulting fragments were separated by electrophoresis on a 15% SDS–polyacrylamide gel and electrotransferred to a 0.45-μm poly(vinylidene difluoride) (PVDF) membrane (Immobilon P; Millipore) in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), pH 11.0, containing 10% (v/v) methanol. The PVDF membrane was stained with Coomassie Brilliant Blue, the bands were isolated, and the fragments were subjected to microsequence analysis using an Applied Biosystems Inc. 477A sequencer.

Polynucleotides. Poly(dA), poly(A), poly(U), poly(dT), poly(εA), $p(dT)_8$, and $p(dT)_{16}$ were obtained from Pharmacia-PL Biochemicals; calf thymus DNA was from Sigma. Polynucleotide concentration was determined spectrophotometrically using a molar absorption coefficient (per mole of phosphate) $\epsilon_{257} = 3.7 \times 10^3$ M^{-1} cm^{-1} for poly(εA), $\epsilon_{257} = 10.0 \times 10^3$ M^{-1} cm^{-1} for poly(dA), $\epsilon_{260} = 10.3 \times 10^3$ M^{-1} cm^{-1} for poly(A), $\epsilon_{260} = 9.2 \times 10^3$ M^{-1} cm^{-1} for poly(U), $\epsilon_{264} = 8.52 \times 10^3$ M^{-1} cm^{-1} for $p(dT)_8$, $p(dT)_{16}$, and poly(dT), and $\epsilon_{260} = 6.5 \times 10^3$ M^{-1} cm^{-1} for calf thymus DNA. Heat-denatured calf thymus DNA was prepared by boiling, followed by rapid quenching in ice.

Circular Dichroism Spectroscopy. Spectra were collected at 25.0 ± 0.1 °C on an Aviv 60DS CD spectrometer over the wavelength range 200–260 nm, using strain-free Suprasil quartz cells with 0.2-cm path length, as described previously (Casas-Finet et al., 1991).

Fluorometric Titrations. The model polynucleotide probe poly(εA) is a single-stranded polyribonucleotide which contains the fluorescent base ethenoadenine. In this polynucleotide, the fluorescence of the base is significantly quenched, presumably as a result of stacking interactions. Binding of proteins generally brings about an enhancement of fluorescence emission due to perturbation of the polynucleotide's structure. Titrations were performed in either 1 or 10 mM sodium phosphate buffer, pH 7.0, and 0.1 mM EDTA at 25 °C in 0.2 × 1.0 cm path-length Suprasil quartz cells as described earlier (Kumar et al., 1990a), unless otherwise indicated.

Gel Mobility Shift Assays. The 180-bp promoter fragment (−114 to +62) of the human β-polymerase gene (Widen et al., 1988) was labeled at its 5′-end with ^{32}P by the procedure of Maxam and Gilbert (1980) and used as probe in the assays. Unincorporated [^{32}P]ATP was removed by passing the reaction mixture over a NICK column (Pharmacia) following the manufacturer's suggested protocol. ss DNA was prepared by boiling the ds DNA for 5 min and then cooling the mixture immediately with vigorous shaking in an ice bath.

Samples for the binding reaction (10-μL aliquots) contained either intact β-polymerase or a purified fragment and 1.5 fmol (1.3×10^4 cpm) of ds DNA or 3 fmol (1.3×10^4 cpm) of ss DNA. The buffer for the binding reaction was either 10 mM Tris–HCl, pH 7.5, containing 1 mM Na_2EDTA and 40 mM NaCl, or 10 mM potassium phosphate, pH 7.5, containing 1 mM Na_2EDTA and 80 mM NaCl. The binding mixture was incubated for 10 min at 25 °C; then, 2.5 μL of sample dye (73.8% sucrose, 0.038% bromophenol blue) was added, and the samples immediately were applied on a nondenaturing 6% polyacrylamide gel. The gel was run in a 0.5× TBE (50 mM Tris–borate, pH 8.0, and 1 mM EDTA) buffer at 18 mA for 2 h. After electrophoresis, the gel was transferred to Whatman 3MM paper and covered with sheets

of polyethylene film (Saran). Products were visualized by autoradiography with Kodak XAR-5 film at −70 °C.

UV Photochemical Cross-Linking of the 16-kDa Fragment to ^{32}P-Labeled p(dT)$_{16}$. p(dT)$_{16}$ was phosphorylated at the 5′-terminus with T4 polynucleotide kinase by the procedure of Maxam and Gilbert (1980). [^{32}P]5′-p(dT)$_{16}$ was separated from unreacted ATP by passing the mixture over a DuPont NEN Sorb 20 column, following the manufacturer's suggested protocol.

The final binding mixture (volume, 15 μL) contained 16-kDa fragment (8.3 μM) and [^{32}P]p(dT)$_{16}$ (6.6 μM) in 25 mM Tris–HCl, pH 8.0, 33 mM NaCl, and 1 mM Na_2EDTA. Following a 10-min incubation at 25 °C, the reaction mixture was spotted on Parafilm and immediately irradiated (254 nm) in a UV Stratalinker apparatus (Stratagene) for various times. The cross-linked products were analyzed by electrophoresis on a SDS–polyacrylamide (15%) gel, according to Laemmli (1970). The dried gels were subject to autoradiography at −70 °C with X-Omat film (Kodak). Bands for the 16-kDa fragment/probe complex were quantified by scanning densitometry and integration. The integrated values were plotted vs ionic strength (I) and fitted by a third-degree polynomial. The extrapolated value at zero ionic strength is assumed to represent quantitative (>99%) binding of the 16-kDa fragment by the radioactive probe. This value was then used to assess [16 kDa] bound at various salt concentrations

$$[\text{16-kDa fragment}]_{\text{bound}} = (\text{integrated value at } I \text{ ionic strength}/\text{integrated value at zero ionic strength})\,[\text{16-kDa fragment}]_{\text{total}}$$

$$[\text{16-kDa fragment}]_{\text{free}} = [\text{16-kDa fragment}]_{\text{total}} - [\text{16-kDa fragment}]_{\text{bound}}$$

Since the binding site size is known, apparent affinities

$$K_{\text{app}} = \frac{[\text{16-kDa fragment}]_{\text{bound}}}{[\text{16-kDa fragment}]_{\text{free}}[\text{probe}]_{\text{free}}}$$

were calculated and plotted as log $K\omega$ vs log [Na^+].

RESULTS

Purification and Structural Properties of the 16-kDa Fragment of β-Polymerase: Large-scale expression of rat β-polymerase in *E. coli* has allowed production of this protein in amounts sufficient for detailed physicochemical study of individual domain fragments (Kumar et al., 1990a). β-polymerase has six methionine residues and yields, upon prolonged CNBr cleavage, a 16-kDa fragment along with a number of fragments in the 2–6-kDa range. Characterization of the 16-kDa fragment, an end product of CNBr digestion spanning residues 18–154 (Figure 1), is the subject of the current study. After CNBr digestion, the fragment mixture was renatured by prolonged dialysis against a buffer containing 250 mM NaCl and 1 mM DTT. The 16-kDa fragment has high affinity for phosphocellulose or FPLC mono S columns, and we made use of this property to purify the fragment. Thus, upon chromatography of the dialyzed CNBr digest on a phosphocellulose column in buffer containing 250 mM NaCl, most of the smaller fragments appeared in the flow-through, whereas the 16-kDa fragment bound tightly to the column and subsequently eluted in highly purified form with a 500 mM NaCl wash (Figure 2a). This fraction was used for all experiments described in this work; it contained at least two minor contaminating peptides, as can be seen in Figure 2a.

As the 16-kDa fragment was generated under denaturing conditions and then renatured prior to purification, it was essential to establish its refolding into a native conformation. The circular dichroism (CD) spectrum of the 16-kDa fragment (not shown) displayed a prominent band of negative ellipticity at ~220 nm, with a shoulder of 208 nm, characteristic of α-helical structure. These results indicated that the purified 16-kDa fragment had secondary structure. A computer-run Garnier–Osguthorpe–Robson algorithm (Garnier et al., 1978), which predicts secondary structure based on primary sequence, suggested that the 16-kDa fragment is 58% α-helix, 16% β-sheet, 13% β-turn, and 13% random coil. This prediction was in good agreement with the results of CD analysis. Refolding into native structure next was examined by limited proteolysis with trypsin (Figure 2b). The 16-kDa fragment is a transdomain fragment spanning portions of the 8- and 31-kDa domains; in the intact enzyme, these domains are connected by a region that is highly susceptible to several proteases (Kumar et al., 1990a), including trypsin. Trypsin digestion of the purified 16-kDa fragment at 25 °C generated two fragments of ~5.8 and ~7.5 kDa within the first 5 min of incubation; these fragments remained relatively resistant to further proteolysis for 60 min under the conditions used (Figure 2b). This pattern of rapid cleavage and then resistance of two fragments to further degradation is very similar to the picture observed with intact β-Pol digested with trypsin under the same conditions (Kumar et al., 1990a).

The amino-terminal residues of the 5.8- and 7.5-kDa fragments were investigated by sequencing (Figure 2c). The results indicated that the smaller fragment starts at Leu^{18}, which is the N-terminal residue of the 16-kDa fragment. The 7.5-kDa fragment begins at residue Lys^{83}, which corresponds to the N-terminal residue of one of the two peptides in the 31-kDa domain sample obtained by trypsin cleavage of intact β-Pol; the other peptide begins with Lys^{87} (Kumar et al., 1990a). Thus, trypsin cleavage of the 16-kDa fragment occurs in the same region as trypsin cleavage of intact β-Pol, and the cleavage products of the 16-kDa fragment digestion were similarly resistant to trypsin as those of intact β-Pol. The presence of a protease-hypersensitive site and the relative proteolytic resistance of the subdomains of the 16-kDa fragment suggest that the refolded structure of the 16-kDa fragment resembles that of the corresponding portion of the native enzyme. Thus, these results suggested proper refolding of the 16-kDa fragment.

Analysis of Double-Stranded Polynucleotide Binding by the 27-kDa Fragment of β-Polymerase. The 27-kDa fragment of β-Pol failed to exhibit binding to ss polynucleotides, as indicated by the absence of a fluorescence enhancement in the poly(εA) assay and by the lack of poly(dA)-induced quenching of intrinsic protein fluorescence (data not shown). This result was expected since the 27-kDa fragment is an N-terminal truncation product of the 31-kDa domain, which itself is devoid of ss polynucleotide binding activity (Figure 1). Interestingly, the 27-kDa fragment also failed to exhibit binding to ds calf thymus DNA under conditions where the 31-kDa domain binds to this lattice (Casas-Finet et al., 1991). Thus, the 27-kDa fragment differed from the 31-kDa domain in that the smaller fragment (1) failed to exhibit any intrinsic fluorescence change in the presence of ds calf thymus DNA, (2) was not retarded on a column of ds calf thymus DNA–cellulose in the presence of 50 mM KCl, and (3) did not produce a gel mobility shift with ds DNA as a probe. These results suggested to us that the region spanning residues 87–139 of β-pol (i.e., the difference between the 27- and 31-kDa

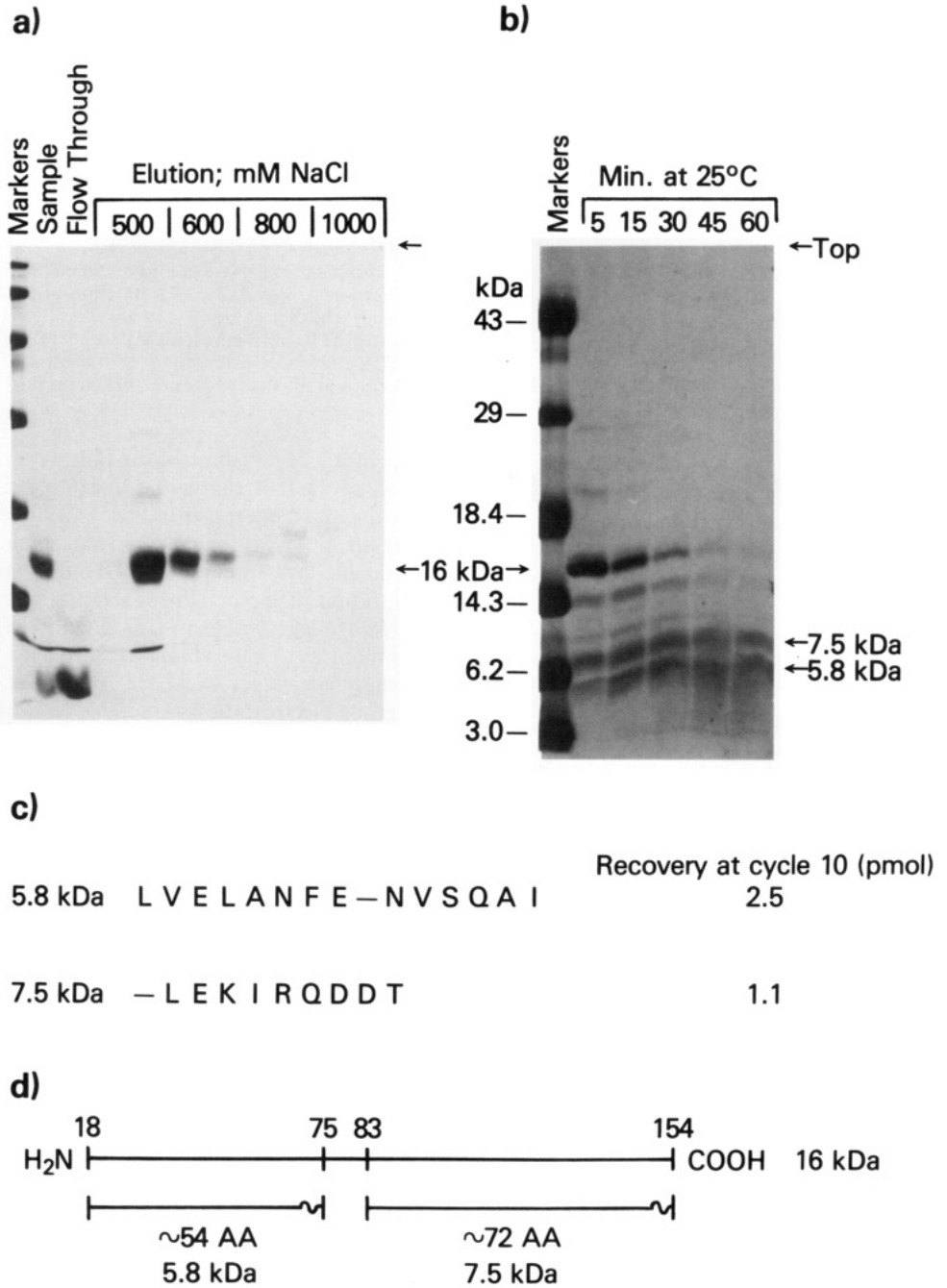

FIGURE 2: Purification and domain characterization of the 16-kDa fragment. (a) A photograph of a Coomassie Brilliant Blue-stained 15% SDS–polyacrylamide gel is shown. The 16-kDa fragment was purified by chromatography on a phosphocellulose column; 20 μL of successive column fractions was mixed with sample buffer and layered on the gel. The flow-through fraction was obtained after the column was washed with 25 mM Tris–HCl, pH 7.5, containing 250 mM NaCl; bound protein was eluted with increasing NaCl concentration as indicated. Markers (2 μg each), as described in Materials and Methods, and an aliquot of the CNBr digest (5 μg) are also shown. (b) Photograph of a 15% SDS–polyacrylamide gel illustrating the controlled proteolysis of the purified 16-kDa fragment with trypsin. Markers and time course of digestion are indicated at the top of the panel. (c) Amino-terminal sequence analysis of the 5.5- and 7.5-kDa fragments. The 16-kDa fragment was cleaved with trypsin; fragments were separated by SDS–PAGE and transferred to PVDF membrane as described in the Materials and Methods. The amino acid identified in each cycle for the 5.8- and 7.5-kDa fragments is shown; (–) indicates that a residue could not be identified. The picomole values refer to the amounts detected in the tenth cycle of sequence analysis; the last cycle shown for the 5.8-kDa fragment corresponds to detection of ≤1 pmol. (d) Schematic representation of the two subdomains obtained from tryptic digestion of the 16-kDa fragment. The 5.8-kDa fragment begins at Leu^{18} and spans residues 18–~75. The 7.5-kDa fragment starts at residue Lys^{83}; although Lys was detected in the first cycle, this residue is not assigned (– in the figure) because other amino acids also were detected; assignment in the subsequent cycles shown was not complicated by the presence of other residues. Wavy lines indicate the C-terminal sequence has not been determined.

fragments; see Figure 1) may contain a domain necessary for the double-stranded nucleic acid-binding capacity of the 31-kDa domain. An alternative explanation of these results could be that the structure of a ds nucleic acid-binding site remaining in the 27-kDa fragment had been altered or destroyed by the proteolytic digestion. The availability of the purified 16-kDa fragment afforded the possibility of testing these ideas, since the 16-kDa fragment spans the portion of the 31-kDa domain that had been removed in the production of the 27-kDa fragment (see Figure 1). Thus, if this N-terminal region of the 31-kDa domain is a modular ds nucleic acid-binding domain, the 16-kDa fragment would be expected to exhibit

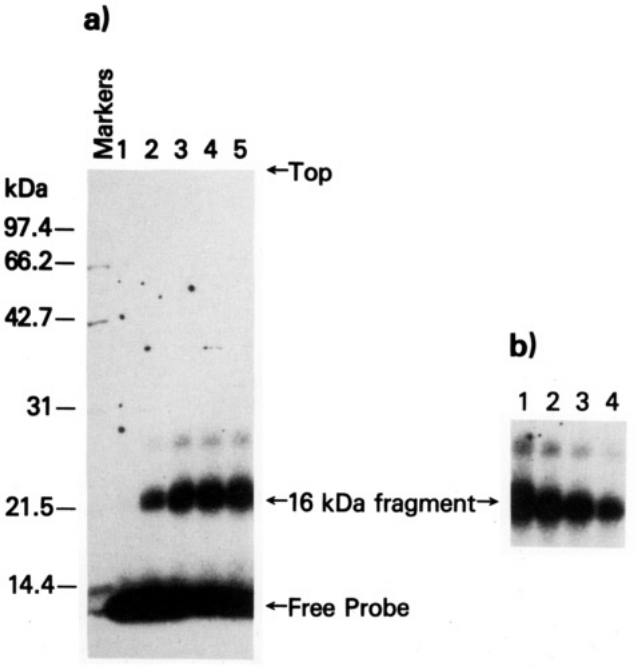

FIGURE 3: Photographs of autoradiograms illustrating results of two experiments of UV cross-linking of the 16-kDa fragment to [^{32}P]p(dT)$_{16}$, as a function of irradiation time (a) and ionic strength (b). In panel a, the cross-linking was carried out for various times and samples were applied on a 15% SDS–polyacrylamide gel: lane 1, un-cross-linked fragment; lane 2, 0.4 min; lane 3, 0.8 min; lane 4, 1.2 min; lane 5, 2.0 min. In panel b, cross-linking was carried out for 2 min in sample buffer containing 25 (lane 1), 50 (lane 2), 75 (lane 3), or 100 mM NaCl (lane 4). The arrows labeled 16-kDa fragment indicate the 16-kDa fragment/probe complex.

double-stranded nucleic acid binding, as well as single-stranded nucleic acid-binding contributed by the segment corresponding to the 8-kDa domain.

16-kDa Fragment/[^{32}P]p(dT)$_{16}$ Binding Revealed by UV Cross-Linking. We first conducted UV cross-linking of mixtures of [^{32}P]p(dT)$_{16}$ and the 16-kDa fragment to confirm interaction between the fragment and this single-stranded nucleic acid probe. Covalent UV-induced cross-linking of complexes between oligodeoxythymidylate and proteins can provide information on amino acids at the interface of the oligonucleotide/protein complex (Merrill et al., 1984, 1988), and in addition, some properties of the binding interaction can be examined [e.g., see Sobol et al. (1991)]. Intact β-pol and the 8-kDa fragment are ss nucleic acid-binding proteins, and we had found earlier that both can be cross-linked to oligo(dT) by exposure to UV light.[2]

Our results here indicated that the 16-kDa fragment can be cross-linked to [^{32}P]p(dT)$_{16}$ (Figure 3a). Most of the cross-linked complex migrated in an SDS–polyacrylamide gel as an apparent 22-kDa species; this was consistent with one molecule of 16-kDa fragment cross-linked to one molecule of [^{32}P]-oligonucleotide, since the 5–6-kDa retardation in migration of the protein corresponds to the size of one molecule of p(dT)$_{16}$. We note that a minor amount of cross-linked product also was observed at approximately 27 kDa.

Formation of the protein/oligodeoxythymidylate complex appeared to be salt-dependent, as cross-linking decreased when the salt concentration in the binding mixture was increased from 25 to 100 mM NaCl (Figure 3b). Densitometry of the autoradiogram allowed quantitative analysis of the effect of salt on cross-linking. The results are summarized in Figure

[2] A. Kumar and S. Wilson, unpublished observations on the 8-kDa domain.

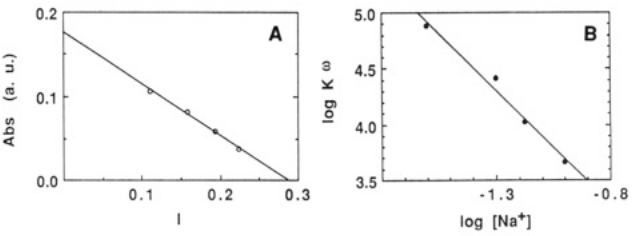

FIGURE 4: Analysis of the effect of NaCl on binding between the 16-kDa fragment and p(dT)$_{16}$. (a) Amount of 16-kDa fragment cross-linked to [^{32}P]p(dT)$_{16}$ as a function of square root of ionic strength (I); (b) plot of log (K_ω) vs log [Na$^+$] for 16-kDa fragment binding to p(dT)$_{16}$ in 10 mM sodium phosphate, pH 7.0, and 0.1 mM Na$_2$EDTA at 25 °C. Values for overall binding affinity ($K\omega$) were calculated from the data in Figure 3b, as described in Materials and Methods.

4a in the form of amount of cross-linked species plotted as a function of ionic strength; the linear plot is indicative of proportionality between these two variables. The overall binding affinity ($K\omega$), calculated from these data, was used for a double-logarithmic plot of binding affinity vs Na$^+$ concentration. The plot is linear and characterized by a slope, δ log ($K\omega$)/δ log [Na$^+$], of –2.0 (Figure 4B); this suggests that as many as three Na$^+$ ions are displaced from the oligo(dT) phosphate–sugar backbone (Record et al., 1976) and replaced by electrostatic interactions with basic residues in the protein. The contribution of nonelectrostatic interactions to the free energy of binding was calculated from the overall binding affinity at the reference state of 1 M Na$^+$, which was equivalent to free energy of –2.4 kcal mol^{-1}. These results indicate that electrostatic interactions contribute significantly to the binding of the 16-kDa fragment to oligo p(dT), accounting for about two-thirds of the free energy of binding at 0.025 M Na$^+$ (–6.5 kcal mol^{-1}).

Quantitative Aspects of ss and ds Polynucleotide Binding by the 16-kDa Fragment. The 16-kDa fragment is capable of binding the ss polynucleotide probe poly(ϵA), as indicated by an enhancement effect of the protein on the polynucleotide's fluorescence. Binding at saturation corresponded to an average binding site size (nucleotides residues occluded) of 6 ± 1, and binding at saturation induced an enhancement of poly(ϵA) fluorescence by a factor of 2.7-fold (not shown). Alternatively, we found that there was quenching of the 16-kDa fragment's intrinsic Tyr fluorescence in the presence of the ss polynucleotide, poly(dT) (Figure 5A), and we could make use of this property to measure binding affinity and site size, which was also equal to about six nucleotides per protein molecule (Figure 5A).

Poly(ϵA) competition experiments indicated that the affinity of the 16-kDa fragment for poly(ϵA) was ~15-fold higher than that for poly(dT), the affinity of the 16-kDa fragment for p(dT)$_8$ was about one-half that for poly(dT), and there was intermediate affinity for p(dT)$_{16}$ (Figure 5B). The affinity for other polynucleotides assayed was within a factor of 4 of that for poly(dT). Affinity for various ss polynucleotides ranked in the following order: poly(ϵA) > poly(dT) ≈ poly(U) > ss DNA > poly(dA) ≈ poly(A).

The 16-kDa fragment also was able to bind double-stranded polynucleotides, as shown both from competition titrations of calf thymus ds DNA with poly(ϵA) as probe and by the ability of ds DNA to quench the fragment's intrinsic Tyr fluorescence. Poly(ϵA) competition assays indicated that the 16-kDa fragment exhibited a modest binding preference for ss DNA over ds DNA; the fragment bound these lattices with affinities 26- and 41-fold lower, respectively, than the affinity for poly(ϵA). Intact β-Pol also exhibits a modest preference for ss

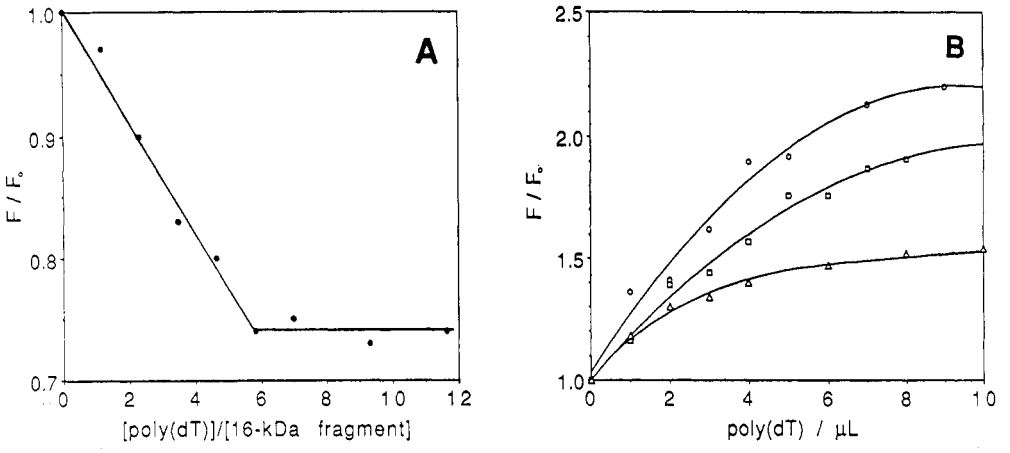

FIGURE 5: (A) Fluorometric equilibrium binding isotherms of the 16-kDa fragment obtained by intrinsic protein Tyr fluorescence (A) and by poly(εA) competition (B). In panel A, 9 μM 16-kDa fragment with poly(dT) as indicated in nucleotides, in 10 mM sodium phosphate buffer, pH 7.0, containing 1.0×10^{-4} M Na_2EDTA. The ordinate is the ratio of Tyr fluorescence in the presence of poly(dT) (F) and absence of poly(dT) (F_0). The abscissa is the ratio of concentrations of poly(dT) in nucleotides and the 16-kDa fragment. In panel B, competition of $p(dT)_8$ (circles), $p(dT)_{16}$ (squares), or poly(dT) (triangles) for 16-kDa fragment binding to the probe poly(εA) at 1 mM. Experiments were conducted as described in Materials and Methods. The abscissa represents increasing amounts of the respective deoxythymidylate polymer in the final binding mixture, and the ordinate represents poly(εA) fluorescence.

Table I: Summary of Nucleic Acid-Binding Properties of β-Polymerase and Its Fragments in 13 mM Na^+

protein	ss nucleic acid binding by direct binding to poly(εA) ($K\omega$) (M^{-1})	ds DNA binding by competition for poly(εA) ($K\omega$) (M^{-1})
8 kDa[a]	4×10^6	ND[b]
16 kDa	6×10^7	2×10^6
31 kDa	no detectable binding	ND[b]
β-Pol[a]	8×10^7	5×10^6

[a] Data taken from Casas-Finet et al. (1991). [b] Could not be determined by competition since there was no detectable binding to poly(εA).

DNA over ds DNA (Casas-Finet et al., 1991). Some comparisons of binding parameters for the 16-kDa fragment and intact β-polymerase are shown in Table I. In summary, the binding affinity of the 16-kDa fragment is similar to that of intact β-pol for both single-stranded and double-stranded DNA and also is similar for the single-stranded polynucleotide probe, poly(εA).

Single- and Double-Stranded Nucleic Acid-Binding Properties of the 16-kDa Fragment Revealed by Gel Mobility Shift Assay. Binding of the 16-kDa fragment to ss and ds natural DNA was further examined by gel mobility shift assays. The results of this method illustrate qualitative aspects of binding between the 16-kDa fragment and DNA; an excess of protein was used to drive the binding reaction. For ds DNA binding to a 180-bp probe (Figure 6), one relatively fast-migrating band-shift signal was seen at lower protein concentrations; this binding signal, for example, was observed at 2.5 pmol of 16-kDa fragment. However, at levels of 6–12.5 pmol of 16-kDa fragment, saturation of the lattice appeared to be complete (Figure 6a and b). This sharp onset of complete binding, to produce slower migrating complexes over a narrow concentration range of protein level, suggests that the 16-kDa fragment binds ds DNA in a cooperative manner. The overall affinity of binding appeared to be ionic strength-dependent, as a slightly higher protein concentration was required to saturate ds DNA at 80 mM NaCl as compared to 40 mM NaCl (Figure 6). It is interesting to note that a number of bands were observed of mobility intermediate between that of the fastest-migrating signal (seen at lower protein levels) and that of the slowest-migrating signal, which represented saturation of the probe. These intermediate bands probably represent complexes with successively increasing amounts of 16-kDa fragment complexed to a single probe molecule. Well over 15 intermediate bands could be visualized by use of different exposure times for the gels depicted in Figure 6.

Binding of the 16-kDa fragment to a heat-denatured 80-bp ss DNA probe also was monitored by a gel mobility shift assay (Figure 7). Band-shifting was observed, although the binding process did not appear to be cooperative.

Double-Stranded DNA Binding Properties of β-Polymerase and the 31-kDa Fragments. We had found previously that neither β-pol nor the 31-kDa fragment has affinity for ds DNA lattices when the binding assay is conducted in the presence of 150 mM NaCl (Kumar et al., 1990a). In the present work, we found that intact β-pol is able to bind ds DNA in the presence of 40 or 80 mM NaCl (Figure 8a and b). The binding, as revealed by the gel mobility shift assay, was similar to that observed for the 16-kDa fragment, and the mobility shift pattern, suggesting cooperativity and multiple intermediate species, also was similar. The ds DNA-binding capacity of the 31-kDa fragment was examined also (Figure 8c–e). The 31-kDa fragment bound ds DNA, but binding was observed only in the presence of a relatively large amount of protein (Figure 8c). There was a noticeable decrease in binding at 80 mM NaCl (Figure 8d) compared with 40 mM NaCl; no binding was observed when the salt concentration in the binding mixture was increased to 150 mM (Figure 8e), as expected from previous studies of the 31-kDa fragment. ds DNA binding by the 31-kDa fragment appeared to be substantially weaker than that of β-polymerase and also was much weaker than that of the 16-kDa fragment. This suggests that proteolytic cleavage of β-Pol to produce the 8- and 31-kDa domains impairs the ds DNA-binding function of the intact enzyme.

DISCUSSION

The use of purified fragments of mammalian β-polymerase has led to significant insights into the relationship between particular structural elements of the enzyme and its various activities, such as single-stranded and double-stranded nucleic acid binding and nucleotidyltransferase catalysis (Kumar et al., 1990a,b). The association of these properties with defined portions of the protein is fully consistent with the existence of tightly folded subdomains linked by short solvent-exposed regions. The action of several proteases on rat β-polymerase

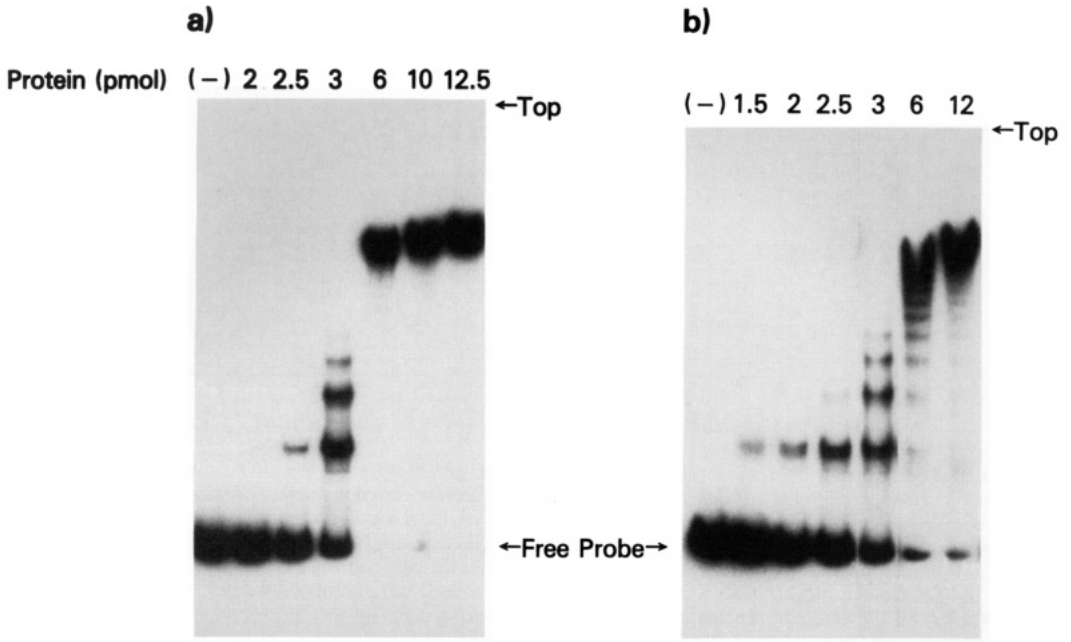

FIGURE 6: Photographs of autoradiograms illustrating ds DNA binding by the 16-kDa fragment, as detected by gel mobility assays. ^{32}P-Labeled human ds DNA probe was incubated with increasing concentrations of 16-kDa fragment (indicated at the top of the panel); the probe was resolved on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography. In panel a, binding mixtures contained 40 mM NaCl, whereas in panel b, binding mixtures contained 80 mM NaCl. The lane designated (−) indicates the minus-protein control. Gel mobility shift assay was conducted as described in Materials and Methods.

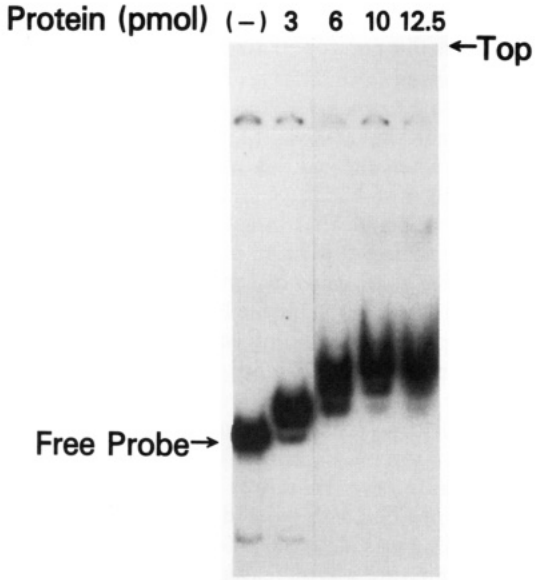

FIGURE 7: ss DNA binding by the 16-kDa fragment of β-polymerase evaluated by gel mobility shift assay. An autoradiogram of the gel is shown. Increasing concentrations of 16-kDa fragment (indicated at the top of the panel) were mixed with ^{32}P-labeled ss DNA probe, and complexes were resolved on a nondenaturing 6% polyacrylamide gel. The lanes designated (−) indicate the minus-protein control. Gel mobility shift assay was conducted as described in Materials and Methods.

is characterized by the rapid release of these subdomains, which are relatively resistant to further digestion (Kumar et al., 1990a).

The full-length enzyme appears to have a mostly α-helical N-terminal domain of ~75 amino acids, connected by a protease-sensitive segment to a C-terminal domain of ~250 amino acid residues, which has high content of β-structure (Casas-Finet et al., 1991). As noted above, nucleic acid-binding studies have shown that the purified N-terminal domain (8 kDa) is capable of single-stranded nucleic acid-binding activity with affinity approximately $^2/_3$ that of the intact enzyme, but unlike the full-length protein, the 8-kDa domain does not also have double-stranded nucleic acid-binding activity. In contrast, the C-terminal domain (31 kDa) has little or no affinity for single-stranded lattices, but does bind double-stranded nucleic acid. The 31-kDa domain also carries nucleotidyltransferase activity, whereas the 8-kDa domain does not (Kumar et al., 1990b). The segment spanning residues 87–139 in the N-terminal region of the 31-kDa domain appears to be a folded subdomain, since this segment can be removed from the 31-kDa domain by chymotryptic or tryptic proteolysis. Removal of this segment is associated with loss of the ds DNA-binding activity of the 31-kDa domain, suggesting that this subdomain contributes ds DNA binding to β-Pol. In view of the finding that the ds DNA-binding activity of the 31-kDa domain is substantially less than that of β-Pol, we decided to evaluate the question of ds DNA-binding activity of the putative residue 87–139 subdomain by characterizing a CNBr fragment carrying this portion of the 31-kDa domain along with most of the N-terminal-contiguous ss nucleic acid-binding domain.

Since the 16-kDa fragment, residues 18–154, described here binds to single-stranded lattices, about as well as intact β-Pol and somewhat better than the 8-kDa domain, the single-stranded nucleic acid-binding site clearly does not reside in the first 17 residues of β-Pol and is enhanced by the C-terminal contiguous residues. Partial homology between residues 35–44 of β-polymerase and the "RNP-1" consensus sequence of many other eukaryotic single-stranded nucleic acid-binding proteins (Kumar et al., 1990a) suggests that the single-stranded nucleic acid-binding site includes this sequence. We found earlier that binding of double-stranded DNA to β-polymerase is able to displace poly(εA) from the single-stranded nucleic acid-binding site (Casas-Finet et al., 1991), and this led us to speculate that the latter site may share common structural elements with the double-stranded nucleic acid-binding site in the 31-kDa domain. The observation that the 16-kDa fragment binds ss nucleic acid with greater affinity than the purified 8-kDa domain is consistent with this idea. The

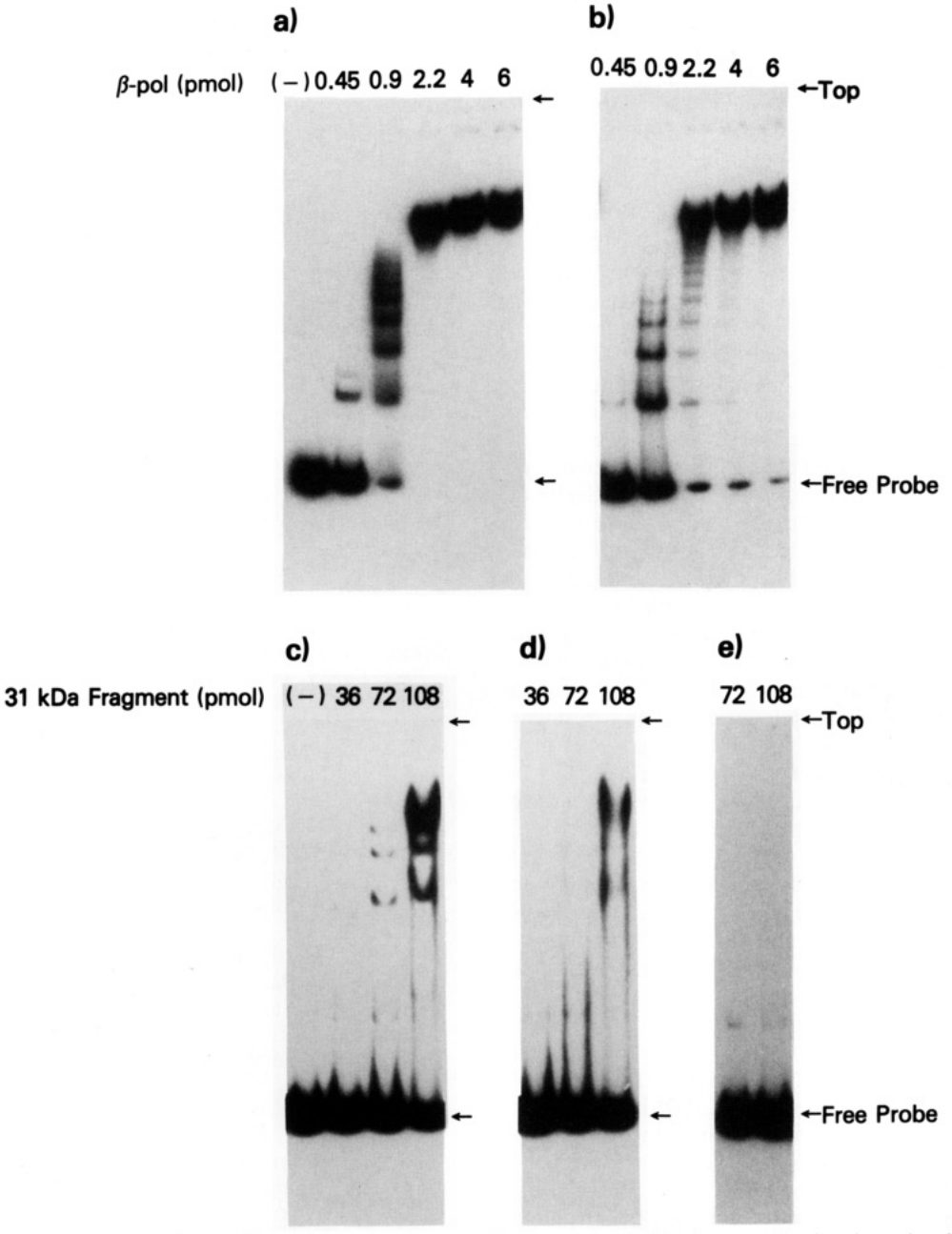

FIGURE 8: Screening of ds DNA-binding activity of intact β-polymerase (a, b) and the 31-kDa fragment (c–e), using gel mobility shift assays. ^{32}P-Labeled ds DNA probe was incubated with increasing concentrations of β-polymerase at (a) 40 and (b) 80 mM NaCl. Complexes were separated on a nondenaturing 6% polyacrylamide gel and visualized by autoradiography. Similarly, the 31-kDa fragment was assayed at (c) 40, (d) 80, and (e) 150 mM NaCl.

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 60 RRILILGKLNNLVKEWIREIRESKNLPQSVIENVGGKIFTFGSYRLGVHT 109   PAP
    :|    :.||. :.: : |:        ... .||:. | .:..||
  2 KRKAPQETLNGGITDMLVEL.......ANFEKNVSQAIHKYNAYR..... 39   β Pol

110 KGADIDALCVAPRHVDRSDFFTSFYDKLKLQEEVKDLRAVEEAFVPVIKL 159   PAP
    |:|.: |               .: .|:| ..|.|.|.:|:. :
 40 KAASVIA..............KYPHKIKSGAEAKKLPGVGTKI...... 68   β Pol

160 CFDGIEIDILFA...RLALQT 177   PAP
       : .|| ::|   || |:.
 69 ...AEKIDEFLATGKRLKLEK 86   β Pol
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FIGURE 9: Computer-derived alignment of primary sequence of the single-stranded DNA-binding domains of poly(A) polymerase (residues 60–177) and β-polymerase (residues 2–86).

observations that the 8-kDa fragment does not bind ds DNA, whereas the 16-kDa fragment binds ds DNA with about the same affinity as intact β-Pol allows us to propose that the segment spanning from the protease-hypersensitive site (near residue 83) to reside 154 (the end of the 16-kDa fragment) contains a subdomain functionally active in binding ds nucleic acids. This segment of the 31-kDa fragment does not appear to be the complete double-stranded DNA-binding site, however, since additional residues appear to be required for full binding to double-stranded lattices.

β-polymerase function may depend on the interplay of separate domains that contribute distinct functional roles. The ss nucleic acid-binding and ds nucleic acid-binding activities of β-Pol may correspond to template- and template·primer-binding functions, respectively. Alternatively, the ds-binding activity could be involved in the enzyme's ability to recognize a short gap (Wang & Korn, 1980) by virtue of binding to the ds component downstream of the primer. The observation that these two nucleic acid-binding activities of β-Pol can be separated by proteolytic cleavage indicates that they are contributed, at least in part, by different regions of the enzyme. Nevertheless, their close approximation appears to be required for full activity of each, since as noted above, the ds DNA-binding activity of the ds DNA-binding subdomain appears to be enhanced by the presence of the 8-kDa domain. Thus, it is possible that although each subdomain has a defined primary function, a subdomain may enhance the activities of other subdomains. For instance, residues Ser^{44} and Ser^{55} can be phosphorylated by protein kinase C, and this results in inactivation of β-polymerase activity (Tokui et al., 1991). The phosphorylated enzyme retains binding to ss DNA–cellulose (Tokui et al., 1991); therefore, it seems that phosphorylation of these residues in the 8-kDa domain blocks some required function other than ss DNA binding. In view of the fact that divalent metal binding has been proposed to be involved in dNTP binding and catalysis, it is interesting that an Asp residue located in the 8- and 31-kDa domains, respectively, may coordinate with the divalent metal ion (Delarue et al., 1990). In the sequence of β-polymerases, two closely spaced Asp residues in the 31-kDa domain have been proposed to participate in enzyme function (Argos, 1988). Mutation of these two Asp residues in β-polymerase suggests that they are located in the active site (Date et al., 1991); one residue is likely to be involved in a functional role in catalysis, while the second may bind Mg^{2+} in concert with the single Asp residue in the 8-kDa domain (Delarue et al., 1990).

An interesting feature of the modular character of β-Pol is provided by a comparison of its primary sequence with that of poly(A) polymerase (Raabe et al., 1991). The region of poly(A) polymerase spanning residues 62–138 is predicted to contain the RNA-binding domain of this enzyme, and we found extensive homology with the 8-kDa single-stranded DNA-binding domain of β-polymerase (Figure 9); according to our alignment, 30% of the amino acids are identical and there is an overall similarity of 52%, including conservative replacements. On the other hand, no significant match of the ds DNA-binding region of β-polymerase could be found within the poly(A) polymerase (not shown). We note that the alignment in Figure 9 spans the entire sequence of the 8-kDa domain and that aromatic and basic residues, which may be involved in the nucleic acid-binding process, are particularly conserved.

[3] J. Kraut and S. Wilson, unpublished observations.

In summary, the results presented in this study suggest that DNA polymerase β is a multidomain protein in which the subdomains contribute distinct functions. Solution of the structure of β-Pol at atomic resolution is now within reach, as intact β-polymerase has been crystallized as an apoenzyme and the crystals are found to diffract to high resolution.[3] However, as a degree of conformational flexibility is expected from structural considerations and may, in fact, be required for enzyme activity, solution studies of β-polymerase and its fragments such as the 16-kDa one can provide a dynamic picture of the multiple interactions of β-polymerase with various ligands.

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