Structure–Function Analysis of DNA Polymerase-β Using Monoclonal Antibodies: Identification of a Putative Nucleotide Binding Domain

Anthony J. Recupero,‡ Diane C. Rein, and Ralph R. Meyer*

Department of Biological Sciences, University of Cincinnati, Cincinnati, Ohio 45221-0006

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ABSTRACT: DNA polymerase-β was purified from Novikoff hepatoma and used as an antigen in an in vitro immunization system to produce monoclonal antibodies. These reagents surprisingly showed cross-reactivity to a number of proteins, including several DNA polymerases. Nearly all of these proteins possess nucleotide binding sites, which suggested the potential value of using the monoclonals to elucidate structure–function relationships within polymerase-β. Furthermore, these antibodies were able to partially neutralize (40–50%) polymerase-β activity, and this effect could be blocked by dNTP[1] but not by dNMP or rNTP. The limited neutralization phenomenon is at least partially explained by the weak binding affinity of these antibodies. Scatchard analysis of immunoprecipitation data predicted a K_d of 1.8×10^{-8} M. Epitope mapping studies showed that the region of polymerase-β recognized by one of the monoclonal antibodies is within residues 235–335, and sequence homology studies indicated that the epitope is probably located in the region of amino acids 283–320. At least a portion of this area, namely residues 301–308 and 311–315, appears to be part of a nucleotide binding domain which has sequence homology with a portion of the highly conserved ATP binding site in adenylate kinase.

Polymerase-β-like enzymes are present throughout the animal kingdom (Chang, 1976), and tryptic peptide mapping and immunological studies indicate that the primary structure of polymerase-β in vertebrates is highly conserved (Tanabe et al., 1981, 1984; Chang et al., 1982a). Mammalian β-polymerases share several characteristics: low molecular weight (32–40 kDa), alkaline pI and pH optimum, stimulation by 50–100 mM salt, inhibition by phosphate, general resistance to *N*-ethylmaleimide (NEM), resistance to aphidicolin, and sensitivity to dideoxythymidine triphosphate (ddTTP). Purified β-polymerases are unable to conduct processive DNA synthesis, lack intrinsic nuclease activity, and do not catalyze detectable levels of pyrophosphate exchange, pyrophosphorolysis, or dNMP turnover. Attempts to kinetically distinguish reaction intermediates have been unsuccessful. The β-polymerase activity is relatively constant throughout the cell cycle under a variety of conditions. The exact cellular role of this enzyme remains uncertain; however, a number of studies suggest that the enzyme is involved in DNA repair [for reviews, see Fry and Loeb (1986) and Rein et al. (1990)]. Nonetheless, the apparent simplicity of polymerase-β makes it an attractive choice for structure–function studies. Rat and human β-polymerases are single-chain polypeptides of 335 amino acids, and both enzymes have been overexpressed in *Escherichia coli* (Date et al., 1988; Abbotts et al., 1988; Kumar et al., 1990a). These recombinant enzymes have similar template-primer specificity and reaction properties to their naturally derived counterparts (Abbots et al., 1988). Kumar et al. (1990a) utilized limited proteolysis of recombinant rat polymerase-β to study the structural domains. They reported a protease-sensitive region between residues 82 and 86 which, when cleaved, divides the protein into 8-kDa and 31-kDa fragments. The 8-kDa fragment, but not the 31-kDa fragment, was able to bind to ssDNA;[1] however, it showed a reduced affinity for DNA compared to the intact protein. Subsequently, Kumar et al. (1990b) reported that the C-terminal 31-kDa domain does in fact possess DNA polymerase activity when assayed by activity gels and on an M13 DNA substrate, but at reduced levels when compared to the intact protein.

The present investigation was designed to explore structure–function relationships of DNA polymerase-β. Novikoff hepatoma DNA polymerase-β was utilized as an antigen to produce several monoclonal antibodies using an in vitro immunization protocol. These antibodies were alike in their cross-reactivity with other proteins as well as in their ability to inhibit polymerase-β activity in vitro. Epitope mapping studies along with sequence homology comparisons suggest that the epitope is at or near a putative nucleotide binding site in the C-terminal region of the protein.

EXPERIMENTAL PROCEDURES

Materials

Enzymes/Proteins. E. coli polymerase I (EC 2.7.7.7) and SDS–polyacrylamide gel electrophoresis (SDS–PAGE) prestained low M_r (3–45 kDa) protein standards were from Bethesda Research Labs (Gaithersburg, MD). Calf thymus terminal deoxynucleotidyl transferase (EC 2.7.7.31) was obtained from New England Nuclear or Boehringer-Mann-

* Address correspondence to this author.

‡ Present address: Department of Molecular Biology, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.

[1] Abbreviations: BSA, bovine serum albumin; ddH_2O, double-distilled H_2O; DTT, dithiothreitol; dNMP, deoxyribonucleoside monophosphate; dNTP, deoxyribonucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody(-ies); NTCB, 2-nitro-5-thiocyanobenzoic acid; PBS, phosphate- (10 mM $NaPO_4$, pH 7.2) buffered saline (150 mM NaCl); PC buffer, 50 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol; pol-α, DNA polymerase-α; pol-β, DNA polymerase-β; PVDF, poly(vinylidene difluoride); rNTP, ribonucleoside triphosphate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SSB, single-stranded DNA-binding protein of *Escherichia coli*; SSB-48, 48-kDa single-stranded DNA-binding protein of Novikoff hepatoma; ssDNA, single-stranded DNA; TBS, Tris- (50 mM Tris-HCl, pH 7.4) buffered saline (200 mM NaCl); Tris, tris(hydroxymethyl)aminomethane.

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heim (Indianapolis, IN). SDS–PAGE M_r (14–205 kDa) protein standards and biotinylated M_r protein standards were from Sigma Chemical Co. Endoproteinase Lys-C (EC 3.4.99.30) was purchased from Boehringer-Mannheim (Indianapolis, IN). Calf DNA polymerase-α–primase complex (Perrino & Loeb, 1989) was the generous gift of Dr. Fred Perrino (Bowman Gray School of Medicine, Winston–Salem, NC). The *E. coli* SSB protein was purified in this laboratory by Perrino (1986). DNA polymerase III holoenzyme was purified according to McHenry and Kornberg (1977). *E. coli* RecA protein, bovine serum albumin, ovalbumin, trypsin inhibitor, α-lactalbumin, β-galactosidase, and carbonic anhydrase were purchased from Sigma. Factor II (D. C. Rein and R. R. Meyer, unpublished) and SSB-48 (Koerner & Meyer, 1983) were purified in this laboratory to greater than 90% homogeneity.

Methods

DNA Polymerase-β Assays. DNA polymerase-β assays in 62.5-μL volumes, consisting of 25 mM Tris-HCl, pH 8.4, 5 mM β-mercaptoethanol, 15% (w/v) glycerol, 10 mM magnesium acetate, 0.5 mM EDTA, 15 μM of each dNTP, [^{3}H]-dTTP, or [^{3}H]dATP (specific activity 325 mCi/mmol), 270 μg/mL of activated DNA, and 0.01–0.30 unit of DNA polymerase-β, were incubated for 30 or 60 min at 37 °C. Polymerase-β was also assayed on poly(dA)·poly(dT) substrate in 62.5-μL reactions consisting of 20 mM Tris-HCl, pH 8.4, 5 mM β-mercaptoethanol, 15% (w/v) glycerol, 1 mM $MnCl_2$, 0.5 mM EDTA, 50 mM NaCl, 15 μM of each complementary dNTP, [^{3}H]dTTP, or [^{3}H]dATP (325 mCi/mmol), 50 μM (nucleotide) poly(dA)·poly(dT), and varying amounts of polymerase-β as noted above. The reactions were terminated and processed according to Stalker et al. (1976).

Purification of Novikoff and Recombinant DNA Polymerase-β. The purification protocol for Novikoff pol-β was essentially the same as that of Stalker et al. (1976) and Mosbaugh and Meyer (1980). When used as an antigen, Novikoff pol-β was purified in the absence of BSA. The recombinant clone, JMpβ5, was the kind gift of Dr. Akio Matsukage (Aichi Cancer Center Research Institute, Nagoya, Japan), and this β-polymerase was purified according to the method of Date et al. (1988).

Production of Monoclonal Antibodies to Polymerase-β. The following procedure was a modification of that of Boss (1986). Spleens were recovered from four female (age 8–12 weeks) CB6F1/J mice (F1 of a Balb/c female and C57BL/6 male cross) and made into a single-cell suspension using a cellector (Bellco). The cells were grown at 1×10^7 cells/mL in Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS), 5×10^{-5} M β-mercaptoethanol, 4 mM glutamine, 1 mM sodium pyruvate, 100 units penicillin/mL, and 100 μg streptomycin/mL. For immunization, 10–20 μg of Novikoff fraction VI polymerase-β (without BSA) was added to the spleen cell culture along with 20 μg/mL of the peptide adjuvant, MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine) (Calbiochem). The culture was incubated, undisturbed for 4–5 days at 37 °C in a 5% CO_2 incubator. Spleen cells were then mixed with the established P3-X63/Ag8.653 myeloma cell line (Kearney et al., 1979) in a 3:1 spleen to myeloma cell ratio. To this cell pellet a solution of 45% polyethylene glycol (Merck PEG 4000) was added to promote fusion. The cells were resuspended in Gibco Opti-MEM medium containing 15% FBS, 5.5×10^{-5} M β-mercaptoethanol, 4 mM glutamine, 2× HAT (1× HAT = 680 μg/mL hypoxanthine, 9.5 μg/mL aminopterin, and 194 μg/mL thymidine), 100 units of penicillin/mL, and 100 μg of streptomycin/mL. The cell suspension was then plated at 8×10^4 original myelomas per well in 96-well culture plates already containing feeder cells (mouse peritoneal macrophages in DMEM + 10% FBS) at 6×10^3 cells per well. These plates were incubated at 37 °C in a 10% CO_2 incubator and the supernatants tested for the presence of polymerase-β-specific antibodies by ELISA or by immunoblot analysis. Positive cultures were cloned twice by limiting dilution. Stable cell lines were injected into pristane-primed CB6F1/J mice for ascites immunoglobulin production.

Western Blot Analysis. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membrane or PVDF [poly(vinylidene difluoride)] membrane (Immobilon-P) for immunoblotting was performed essentially as described by Towbin et al. (1979). After transfer, the membrane was blocked in a solution of TBS + 1% BLOTTO (nonfat dry milk) for 30 min at room temperature followed by incubation with purified monoclonal antibody, diluted in PBS + 0.5% BLOTTO to 10–20 μg/mL, for 2 h at room temperature with gentle agitation. This was followed by subsequent incubation with alkaline phosphatase-conjugated secondary antibody and then reacted with a NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate) substrate.

Purification of IgM Monoclonal Antibodies. IgM antibodies were purified from mouse ascites fluid or from expansion cultures in dialysis tubing (Jwo & LoVerde, 1988) by euglobulin precipitation according to García-González et al. (1988).

Immunoprecipitation of Polymerase-β. Novikoff polymerase-β (fraction VI, containing BSA) was precipitated by using one of the anti-polymerase-β monoclonal antibodies along with anti-mouse IgM linked to agarose (Calbiochem). Reactions were carried out in 0.1 M Tris-HCl, pH 8.0, in a total of 30 μL in 1.5-mL Eppendorf tubes at 1–2 °C. Polymerase-β and monoclonal antibody were incubated together for 1 h, followed by the addition of 10 μL of agarose-linked anti-IgM and incubation for an additional 30 min. The immune complexes were sedimented at 30000*g* for 20 min at 4 °C. Finally, the supernatants were assayed for polymerase-β activity on activated DNA and poly(dA)·poly(dT) substrates. The percent polymerase-β activity remaining in the supernatant was compared to control reactions devoid of the monoclonal antibody as well as reactions containing nonimmune IgM and reactions lacking the secondary antibody.

Neutralization of Novikoff Polymerase-β Activity by Monoclonal Antibody. Each of the monoclonal antibodies raised against Novikoff polymerase-β was tested for its ability to inhibit ("neutralize") enzymatic activity. Incubations were carried out in 0.1 M Tris-HCl, pH 8.0, in a final volume of 26 μL in 0.5-mL Eppendorf tubes at 1–2 °C. Polymerase-β and monoclonal antibody were incubated together for 1 h. An aliquot of this mixture was then assayed for polymerase activity on activated DNA and poly(dA)·poly(dT) as described above, with the exception of a lower dNTP concentration (3.75 or 7.5 μM) in the reaction mixture. Percent polymerase activity was compared to control reactions containing nonimmune IgM and those devoid of IgM for each dNTP concentration tested.

Tryptic Digestion at Lysines and Arginines. In a typical experiment, trypsin, in 1 mM HCl, was added to 60-μL (10-μg) aliquots of recombinant polymerase-β (dialyzed into PC buffer + 0.15 M NaCl) in substrate to trypsin ratios of 1500:1, 1000:1, 500:1, 100:1, 50:1, 10:1, and 2:1 (w/w), and the mixture was incubated for 15, 30, or 90 min at room temperature. To stop the reaction, an aliquot (3–4 μg of polymerase-β) was removed and put into 3× SDS sample buffer and subsequently loaded onto a 15% SDS–PAGE minigel. The gel was blotted to PVDF, and the peptides were tested

against monoclonal antibodies to polymerase-β in a Western blot analysis. The gel, as well as an identical sister gel, was then silver stained (Wray et al., 1981) and photographed. The molecular weights of any immunoreactive peptides were determined relative to molecular weight standards.

Cyanogen Bromide (CNBr) Cleavage at Methionines. Recombinant polymerase-β (60 μg) in PC + 0.15 M NaCl was denatured with formic acid (70% final concentration) and treated (in the dark at room temperature) with a freshly prepared solution of CNBr in 88% formic acid at a protein to reagent ratio of 1:3 (Kumar et al., 1990a). At various time points (2, 4, 12, 16, and 24 h), 5-μg samples were removed and diluted in ddH_2O. The excess formic acid was removed by drying the samples in a Speed-Vac. The process of dilution in ddH_2O and drying was repeated at least two more times. The cleavage products were analyzed on SDS–PAGE (5 μg per lane) and by Western blot analysis as described above.

Cleavage at Cysteines with 2-Nitro-5-thiocyanobenzoic Acid (NTCB). Cleavage was performed essentially as described by Jacobson et al. (1973) and Stark (1977). Recombinant polymerase-β was dried in a Speed Vac, resuspended at 0.2 mg/mL in 0.2 M Tris-HCl, pH 9.0, 8 M urea, and 5 mM DTT, and incubated at 37 °C for 1 h. Varying amounts (80 μM–16 mM) of NTCB (in 0.2 M Tris-HCl, pH 9.0, 8 M urea) were added to aliquots of protein, and the pH was adjusted to 9.0 by the addition of 1 N NaOH. Incubation was at room temperature from 24 to 48 h. Aliquots of 4 μg were subsequently removed, added to SDS sample buffer, and directly loaded onto 15% SDS–PAGE gels to be analyzed as described above. It is critical that the pH be adjusted to 9.0 after the addition of NTCB for optimal cleavage to occur.

Digestion at Lysines with Endoproteinase Lys-C. Recombinant polymerase-β (in PC + 0.15 M NaCl) was digested with endoproteinase Lys-C (0.1 mg/mL in 50 mM Tricine, pH 8.0, 10 mM EDTA) at 100:1, 50:1, and 20:1 substrate to enzyme ratios (w/w) at room temperature for 12–48 h. Aliquots (5 μg) were removed and added to SDS sample buffer to stop the reaction. Digested products were analyzed by SDS–PAGE and Western blot analysis.

Amino Acid Composition and N-Terminal Sequencing of an Immunoreactive Peptide. Recombinant polymerase-β was digested with endoproteinase Lys-C at a 50:1, pol-β to enzyme ratio for 12 h at room temperature. The products were run on a 15% SDS–PAGE minigel (10 μg per lane) which was blotted to PVDF in 10 mM CAPS (3-cyclohexylamino-1-propanesulfonic acid) and 20% methanol, pH 11.0 (Matsudaira, 1987) for 90 min at 100 mA. A small portion of the blot was immunostained as for a typical Western blot, and the remainder was stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol for 5 min followed by destaining in 50% methanol and 10% acetic acid in order to visualize the proteins. The blot was rinsed 5–10 min in ddH_2O and air dried, and the appropriate band was cut out with a razor blade. PTH-amino acid analysis was conducted, and amino acid sequencing was performed in an Applied Biosystems, Inc., gas-phase sequencer at the protein-sequencing core facility in the Department of Pharmacology and Cell Biophysics, University of Cincinnati [supported in part by NIH Grant PO1 HL 22619 (Core 2) under the supervision of Dr. Terence L. Kirley].

RESULTS

Purification of Polymerase-β from Novikoff Hepatoma. Fraction VI (without BSA) polymerase-β was utilized in the in vitro immunizations as well as ELISA, Western blot, and immunoblot assays. Fraction VI (containing BSA) was used in the neutralization and immunoprecipitation assays. Due to the limited quantities of Novikoff hepatoma polymerase-β obtainable, recombinant pol-β, which is easily recovered in both high quantity and high concentration, was isolated to be used for the epitope mapping studies.

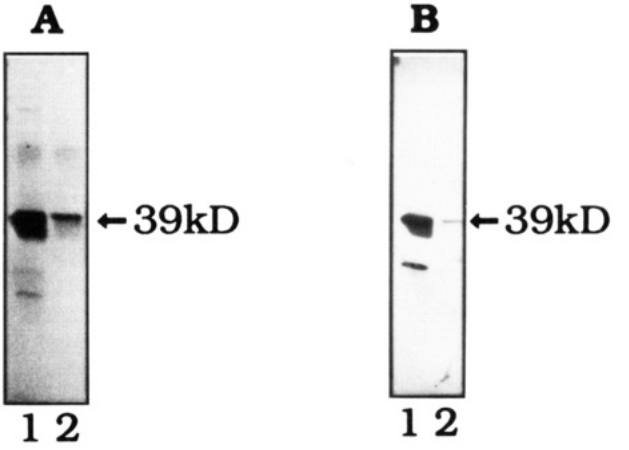

FIGURE 1: SDS–PAGE and Western blot analysis of DNA polymerase-β. (A) Silver-stained 11% SDS–polyacrylamide gel; lane 1, 2.0 μg recombinant (JMpβ5) pol-β; lane 2, 0.5 μg Novikoff hepatoma pol-β (after concentration). (B) Western blot analysis immunostained with IgM mAb AJR5D10-β-6. Arrow denotes location of pol-β.

Purification of Recombinant Polymerase-β. From 6 g (wet weight) of cells, approximately 2.5 mg of nearly homogeneous recombinant polymerase-β was isolated. When visualized on SDS–polyacrylamide gels, this preparation appears as major band of 39 kDa and a minor band of 31 kDa, which represents a pol-β degradation product since it reacts to pol-β mAb (Figure 1B). When visualizing this preparation after repeated freeze–thaw cycles, generally 3–5 additional lower M_r bands are revealed (see Figures 4 and 5, lane 0). These bands also appear to represent pol-β degradation products since their intensity increases with each subsequent freeze–thaw cycle.

Production and Characterization of Monoclonal Antibodies to DNA Polymerase-β. From two separate immunization/fusion procedures, a total of six stable monoclonal hybridoma cell lines producing antibody to Novikoff hepatoma polymerase-β were isolated. These cell lines are designated as follows: AJR3B5-β-1, AJR2B9-β-2, AJR2E7-β-3, AJR6B8-β-4, AJR3F3-β-5, and AJR5D10-β-6. Supernatants from each cell line were tested for immunoglobulin class in ELISA and immunoblot assays by using secondary antibodies specific for IgG (γ-chain) and IgM (μ-chain). All six cell lines were shown to produce only IgM monoclonal antibodies to polymerase-β. Large-scale production of mAb was accomplished by expansion cultures in dialysis tubing or by ascites production in mice. Typically, the dialysis tubing culture method would yield mAb in the culture supernatant at 0.2–0.4 mg/mL, and the ascites method would yield 4–10 mg of IgM per 1 mL of ascites supernatant. The IgM purified from these supernatants by euglobulin precipitation as described above was homogeneous as judged by Coomassie staining of the samples on SDS–polyacrylamide gels. These IgM fractions were then tested for the ability to recognize both Novikoff and recombinant pol-β in immunoblot analysis and Western blot analysis. Figure 1B shows a Western blot analysis of recombinant and Novikoff pol-β with mAb AJR5D10-β-6. All six mAb gave the same pattern of reactivity. A degradation product of the recombinant pol-β also reacts with these antibodies. Neither the recombinant pol-β nor the Novikoff pol-β react with a control nonimmune mouse IgM (data not shown).

Testing Monoclonal Antibodies for Cross-Reactivity. All six mAb were subsequently tested for cross-reactivity to several

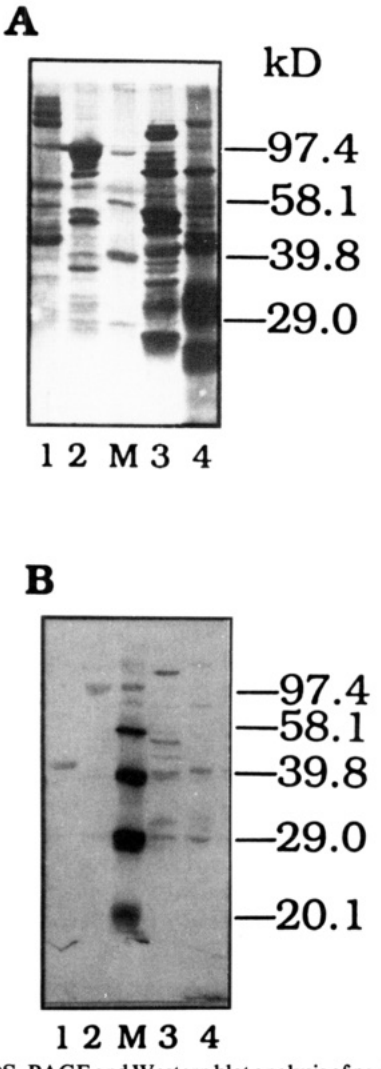

FIGURE 2: SDS–PAGE and Western blot analysis of partially purified proteins which react with mAb to polymerase-β. (A) 11% silver-stained SDS–gel; lane 1, calf DNA polymerase-α–primase complex (140–180, 68, 56, and 46 kDa subunits); lane 2, DNA polymerase I (109 kDa); lane 3, DNA polymerase III holoenzyme (major subunits of 130, 71, 52, 41, 32, 28, and 10 kDa); lane 4, terminal transferase (60-, 44-, 33-, and 11-kDa polypeptides); lane M, biotinylated M_r standards, M_r in kDa denoted to right of gel and blot. (B) Western blot analysis immunostained with mAb AJR5D10-β-6.

proteins known to contain similar functional domains (e.g., other polymerases) as well as to several proteins which should have little primary sequence homology with pol-β. All six antibodies were tested against DNA polymerase I, DNA polymerase III holoenzyme, DNA polymerase-α, terminal deoxynucleotidyl transferase, Novikoff hepatoma factor II, SSB, BSA, and ovalbumin. Each of the six mAb reacted with the above proteins except for BSA and ovalbumin. Western blot analysis (Figure 2) of partially purified preparations of DNA polymerase-α, DNA polymerase I, DNA polymerase III holoenzyme, and terminal transferase showed that the 46-kDa subunit of calf polymerase-α is recognized by the mAb, while intact DNA polymerase I (109 kDa) is the only reactive protein in a highly contaminated commercial polymerase I preparation. Several subunits of DNA polymerase III holoenzyme are recognized by the mAb: 130 kDa (α-subunit), 71 kDa (τ), 52 kDa (γ), 41 kDa (β), 32 kDa (δ), and 28 kDa (ϵ). Terminal transferase is synthesized as a catalytically active single polypeptide of 60 kDa; however, lower M_r forms can be found in crude extracts and during enzyme purification (Chang et al., 1982b; Deibel et al., 1983). The 60-kDa protein is specifically cleaved to produce a 44-kDa or a two-polypeptide enzyme consisting of 11-kDa (α) and 33-kDa (β) polypeptides. In the Western blot analysis, the predominant reactive bands are at approximately 44 kDa (presumably the α–β polypeptide) and 29 kDa (most likely the β polypeptide).

Table I: Neutralization Assay[a]–Titer of [dNTP] in Reaction Mixture

substrate	dNTPs in rxn mix	[dNTP], μM	% control activity	100% activity (pmol incorp)
poly(dA)·poly(dT)	A, T	3.75	66	3.6
		7.5	49	4.8
		15.0	67	5.3
		30.0	81	5.7
		45.0	83	6.5
		75.0	87	7.6
activated DNA	G, C, A, T	1.875	78	4.8
		3.75	59	8.2
		7.5	64	11.8
		15.0	100	13.2
		37.5	100	15.7
		75.0	100	15.8

[a] Neutralization assays were performed as described in the Methods section. Approximately 0.1 unit of fraction VI Novikoff hepatoma polymerase-β was incubated with 0.1 μg of IgM monoclonal antibody, AJR5D10-β-6. Controls for each dNTP concentration were incubated with antibody buffer only or with a nonimmune mouse IgM. Aliquots were assayed on the indicated substrates with varying concentrations of dNTPs in the reaction mixture as indicated (the concentration listed is that for each dNTP present). Results from a typical experiment are shown in which each data point was assayed in triplicate and averaged. The 100% control activity is reported as the number of pmol of total nucleotide incorporated into the substrate.

At least two of the six mAb (including AJR5D10-β-6) were tested by Western blot analysis and shown to react with carbonic anhydrase, but not with Novikoff hepatoma SSB-48, *E. coli* RecA protein, trypsin, endoproteinase Lys-C, trypsin inhibitor, α-lactalbumin, and β-galactosidase (data not shown).

Neutralization of Polymerase-β Activity by mAb. Monoclonal antibodies were tested for their ability to neutralize pol-β activity in order to ascertain if any of the mAb recognize an epitope at or near an active site of the enzyme. Polymerase-β neutralization assays were carried out using purified IgM mAb as described under Experimental Procedures. Each of the six mAb (10^{-1}–10^{-3} μg) were able to inhibit pol-β activity from 40–50% on an activated DNA substrate or a poly(dA)·poly(dT) substrate with a dNTP concentration of 3.75 or 7.5 μM, respectively, in the reaction mixture. Nonimmune mouse IgM is not able to neutralize pol-β activity, and mAb to pol-β are unable to affect the polymerase activity of *E. coli* DNA polymerase I. The ability to detect neutralization activity of the mAb is strictly dependent on the dNTP concentration in the reaction mixture (Table I). When the total concentration of dNTP is 15 μM [7.5 μM each nucleotide on poly(dA)·poly(dT) or 3.75 μM each nucleotide on activated DNA], neutralization activity of the mAb is maximal. At the lowest dNTP concentration listed for each substrate, the percent control activity is unexpectedly higher than that for the next highest dNTP concentration tested. These reproducible results suggest that at a very low dNTP concentration pol-β may have an increased dNTP affinity. Under the conditions of a standard polymerase-β assay (15 μM of each complementary dNTP on activated DNA or 60 μM total dNTP), however, mAb neutralization activity is undetectable. To investigate this phenomenon further, a modified neutralization assay was designed, and the results are shown in Table II. Polymerase-β was preincubated with the indicated concentration of dNTP prior to incubation with mAb. The neutralization assay was then performed as usual with the dNTP concentration in the reaction mixture held constant at 7.5 μM for each dNTP in assays on poly(dA)·poly(dT) and at 3.75 μM for each dNTP in assays on activated

Table II: Preincubation[a] with dNTP Reduces Monoclonal Antibody Neutralization Activity

substrate	dNTPs preincubation	[dNTP], μM	% control activity
poly(dA)·poly(dT)	none	0	57
poly(dA)·poly(dT)	A, T	15	95
	G, C	15	95
	G, C, A, T	15	100
poly(dA)·poly(dT)	A, T	150	100
	G, C	150	100
	G, C, A, T	150	100
activated DNA	none	0	64
activated DNA	A, T	15	85
	G, C	15	87
	G, C, A, T	15	100
activated DNA	A, T	150	100
	G, C	150	100
	G, C, A, T	150	100

[a] Neutralization assays were carried out essentially as described in the Methods section and Table I; however, polymerase-β was first preincubated with the indicated concentration of each listed dNTP for 1 h at 1–2 °C. An aliquot was then assayed for pol-β activity. The concentration of complementary dNTPs in the reaction mixture was held constant at 7.5 μM for the poly(dA)·poly(dT) assay and at 3.75 μM for assays on activated DNA. In the control assays for each entry, antibody buffer was substituted for the IgM mAb. Each data point was assayed in triplicate and averaged.

DNA. The different combinations and concentrations of nucleotides used demonstrate that inhibition of mAb activity is dependent only on total dNTP concentration and is not related to the binding of substrate-specific nucleotides [preincubation with dGTP and dCTP before assaying on poly(dA)·poly(dT)]. In other words, these data indicate that pol-β can bind dNTPs in the absence of a DNA substrate. It was previously thought that pol-β must first bind template-primer before binding dNTP [for review, see Rein et al. (1990)]. From the above results, it is clear that the dNTP concentration affects the ability of mAb to neutralize or to inhibit pol-β enzymatic activity. Addition of rNTPs to the reaction mix, as well as preincubation of pol-β with rNTPs, had no significant effect on mAb neutralization activity, as would be expected if the polymerase did not bind ribonucleotide triphosphates (data not shown). In addition, preincubation with dNMPs has no effect on the mAb activity (data not shown).

The above experiments strongly suggest that the mAb recognize at least a portion of the dNTP binding site on pol-β. The inability of the mAb to completely neutralize pol-β activity could be due to the possibility that the dNTP binding domain is made up of a number of discontinuous residues or possibly that the mAb recognize a site adjacent to the nucleotide binding domain. On the other hand, this observation could simply be due to the mAb having a weak binding affinity.

Immunoprecipitation of Polymerase-β. Since polymerase activity was only partially inhibited by the mAb and reversed by high dNTP concentrations, experiments were carried out to determine if the polymerase could be immunoprecipitated. Monoclonal antibodies AJR2E7-β-3 and AJR5D10-β-6 were tested in this assay and shown to bind 60–70% of the pol-β activity present in control reactions which did not contain mAb. Maximal binding activity was observed at 10^{-2}–10^{-1} μg of IgM per assay. Although mAb and pol-β were repeatedly titered against each other in this assay, it was not possible to find conditions where greater than 70% of the enzyme was bound. Similar results were obtained when a mixture of all six mAb was utilized. Quantitative analyses of the binding of purified monoclonal IgM to Novikoff pol-β (Figure 3) generated linear Scatchard plots (Scatchard, 1949). From this analysis, the calculated dissociation constant (K_d) of 1.8 × 10^{-8} M predicted a weak binding affinity. This explains

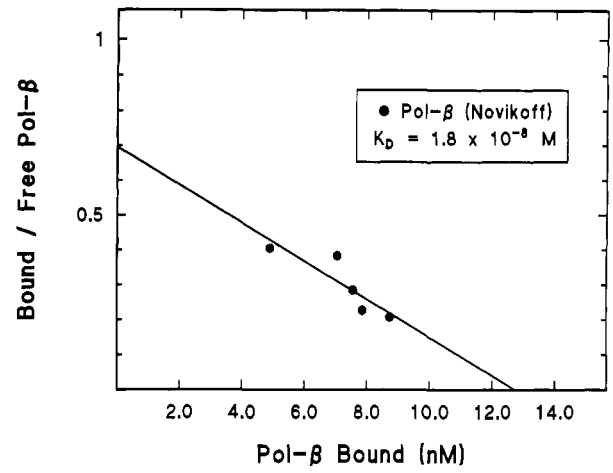

FIGURE 3: Scatchard analysis of the interaction of mAb AJR5D10-β-6 with Novikoff polymerase-β. Polymerase-β immunoprecipitations were performed as described under Experimental Procedures. The straight line analyses of the kinetics data were computer-generated by the method of least squares. Fraction VI Novikoff hepatoma polymerase-β (0.07–0.2 units) and 0.01 μg AJR5D10-β-6 IgM were incubated together for 1 h followed by the addition of 10 μL of agarose-linked anti-IgM and incubation for an additional 30 min. The immune complexes were sedimented at 30000*g* for 20 min at 4 °C. The supernatants were assayed for polymerase-β activity on an activated DNA substrate. Total enzyme activity in each reaction was measured in a control reaction containing antibody buffer without IgM. Free enzyme was determined as the polymerase activity that remained in the supernatant fraction after immunoprecipitation; bound enzyme was determined as the difference between the enzyme activity in the control supernatant and that remaining in the immune supernatant. The molarity of polymerase-β was based on a molecular weight of 39 kDa and a theoretical specific activity under standard assay conditions of 3500 units/mg.

the inability of the mAb to completely immunoprecipitate the pol-β activity at various antibody concentrations. In contrast, it has been shown that monoclonal antibodies prepared to DNA polymerase-α had significantly greater binding affinities, with K_d values in the range of 1.0 × 10^{-9} to 3.4 × 10^{-10} M (Tanaka et al., 1982). These differences can be attributed to the high degree of evolutionary conservation of pol-β as well as the fact that the mAb raised to pol-α were of the IgG class rather than the IgM class as those mAb raised against pol-β.

Mapping of the mAb Epitope on Polymerase-β. Since the previous experiments suggest the recognition of a nucleotide binding domain by the mAb, it was of great interest to localize this site and obtain amino acid sequence information. These data could then be compared to those for other known nucleotide binding sites. Several methods were utilized in an effort to isolate the smallest possible immunoreactive fragment of pol-β. Before use in the epitope mapping studies, the purified fraction IV recombinant polymerase-β (in PC buffer + 0.6 M KCl) was dialyzed into PC buffer + 0.15 M NaCl to avoid possible protease inhibition by high salt and also to avoid the insoluble precipitate formed when high concentrations of potassium are in the presence of SDS.

(A) Tryptic Digestion at Lysines and Arginines. At pol-β to trypsin ratios of 500:1 (15, 30, 90 min) and 100:1 (15 and 30 min), pol-β is digested to yield major products at 31, 27, and 8 kDa. The 31- and 27-kDa fragments are highly immunoreactive, while the 8-kDa band shows no reaction with the mAb (data not shown). With increasing trypsin concentration, an immunoreactive fragment of approximately 12 kDa was generated. Subsequent attempts to generate smaller immunoreactive fragments by altering trypsin concentration and digestion time proved to be unsuccessful. By analysis of the pol-β primary sequence for trypsin cleavage sites, it was determined that the 12-kDa immunoreactive fragment cor-

Table III: Amino Acid Sequence Analysis of Polymerase-β Fragments

fragment size,[a] kDa	amino acids included	reaction with mAb[b]
31 (trypsin)	87–335[c]	yes
27 (trypsin)	141–335[c]	yes
12 (trypsin)	149–252 or 234–335[d]	yes
8 (trypsin)	3–75[c]	no
16 (CNBr)	18–154[c]	no
18 (NTCB)	178–335[d]	yes
11 (endoproteinase Lys-C)	235–335[e]	yes

[a] Determined by SDS–PAGE analysis. [b] Determined by Western blot analysis. [c] Determined by Kumar et al. (1990a). [d] Determined by computer cleavage analysis of amino acid sequence. [e] Determined by direct amino acid sequence analysis (see Table IV).

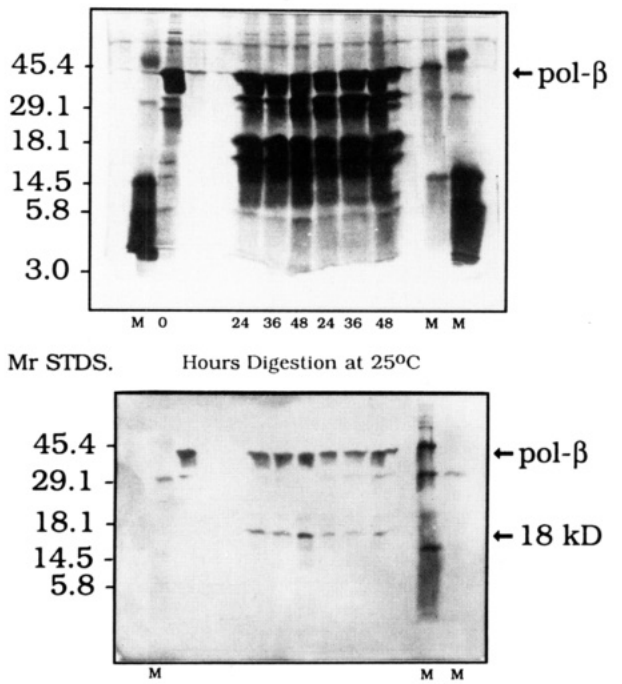

FIGURE 4: Digestion of polymerase-β with 2-nitro-5-thiocyanobenzoic acid (NTCB). (Upper panel) Recombinant polymerase-β was resuspended in 0.2 M Tris-HCl, pH 9.0, 8 M urea and 5 mM DTT and incubated at 37 °C for 1 h at time 0. NTCB was then added to final concentrations of 1.6 mM (lanes 3–5) and 16 mM (lanes 6–8) and at the times indicated, 4.25 μg of pol-β was removed and run on a 15% SDS–polyacrylamide gel. Digestion products were visualized by silver staining. Lanes labeled "M" are either prestained or biotinylated M_r standards denoted in kDa to the left of the gel. (Lower panel) Western blot analysis immunostained with mAb AJR5D10-β-6. Arrows mark the position of intact polymerase-β and of an immunoreactive 18-kDa digestion fragment.

responded either to amino acids 149–252 or to amino acids 234–335 (Table III).

(*B*) *Cyanogen Bromide (CNBr) Cleavage at Methionines.* Cleavage by CNBr yields a major degradation product of 16 kDa which does not react with pol-β mAb (data not shown). Low M_r fragments generated by CNBr cleavage were difficult to resolve and, therefore, may not have been present in high enough concentrations to be detected by Western blot analysis. On the other hand, the epitope may have been destroyed by CNBr cleavage. Thus, the only conclusion that can be drawn is that the mAb epitope does not reside within the 16-kDa fragment.

(*C*) *Cleavage at Cysteines with 2-Nitro-5-thiocyanobenzoic Acid (NTCB).* The concentrations of NTCB and DTT in the digestion reaction were extensively tested, and those presented in Figure 4 were found to be optimal. Polymerase-β degradation products present at time 0 are due to the freeze–thaw

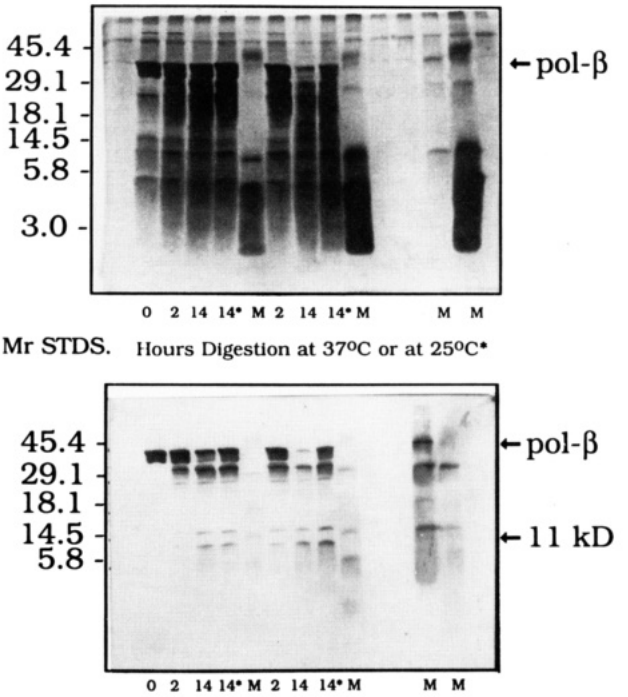

FIGURE 5: Endoproteinase Lys-C digestion of polymerase-β. (Upper panel) Recombinant polymerase-β was digested with endoproteinase Lys-C at substrate:enzyme ratios (w/w) of 100:1 (lanes 2–4) and 50:1 (lanes 6–8) for 2 and 14 h at 37 or 25 °C, respectively, as indicated. At each time point, 5 μg was removed and subsequently run on a 15% SDS–polyacrylamide gel. Lanes labeled "M" are either prestained or biotinylated M_r standards denoted in kDa to the left of the gel. Lanes 10 and 11 (unlabeled) are mock reactions containing endoproteinase Lys-C at the concentrations used in the 100:1 and 50:1 digestion reactions without the addition of polymerase-β. (Lower panel) Western blot analysis immunostained with mAb AJR5D10-β-6. Arrows denote the position of intact polymerase-β and of an immunoreactive ~11-kDa digestion product.

lability of pol-β in 0.15 M NaCl and due to the presence of 8 M urea. As can be seen in the Western blot, digestion in 1.6 mM NTCB for 48 h yielded optimal results. By analysis of NTCB cleavage sites in the pol-β primary sequence, it was determined that the immunoreactive fragment of approximately 18 kDa corresponds to amino acid residues 178–335 (Table III). The 29-kDa immunoreactive protein seen in the first and last M_r standard lanes is carbonic anhydrase.

(*D*) *Digestion at Lysines with Endoproteinase Lys-C.* During digestion, a pol-β to endoproteinase Lys-C ratio of 50:1 at either 37 or 25 °C digested for 14 h was found to be optimal in the generation of the smallest possible immunoreactive fragments (Figure 5). Low M_r bands present in the time 0 sample are the result of breakdown due to the freeze–thaw lability of pol-β. The major immunoreactive digestion products are polypeptides of 31 (as seen in trypsin digestion), ~15, and ~11 kDa. The Western blot analysis shown in Figure 5 was performed with mAb AJR5D10-β-6; however, the other five mAb gave essentially the same pattern of immunoreactive bands in experiments run under the same conditions.

(*E*) *Amino Acid Composition and N-Terminal Sequencing of an Immunoreactive Peptide.* The first four N-terminal amino acids of the immunoreactive 11-kDa endoproteinase Lys-C fragment were determined to be Phe-Met-Gly-Val (amino acids 235–238), and the C terminus was determined to be Glu-335 according to the amino acid composition analysis (Table IV). The theoretical M_r of this peptide is 11.9 kDa; however, the apparent M_r from SDS–PAGE analysis was ~11 kDa. Therefore, the amino acid composition comparison was made with the other two possible fragments (Phe-235 to

Table IV: Amino Acid Composition Analysis of the 11-kDa Endoproteinase Lys-C Fragment

	observed[a]		calculated[b]	calculated[c]
amino acid	pmol	no. of residues[d]	no. of residues	no. of residues
His (H)	59	2.0	2	2
Met (M)	43	1.5	2	2
Asp, Asn (D,N)	237	8.1	13	8
Glu, Gln (E,Q)	326	11.1	13	6
Ser (S)	133	4.5	4	2
Gly (G)	210	7.1	7	3
Arg (R)	235	8.0	8	4
Thr (T)	125	4.3	4	1
Ala (A)	114	3.9	3	2
Pro (P)	150	5.1	7	3
Tyr (Y)	110	3.7	7	4
Val (V)	146	5.0	5	2
Ile (I)	174	5.9	8	4
Leu (L)	208	7.1	6	4
Phe (F)	100	3.4	5	3
Lys (K)	79	2.7	4	3

[a] Data obtained from PTH-amino acid anlaysis of PVDF-bound sample. [b] Assuming the C terminus is Glu-335. [c] Assuming the C terminus is Lys-289. [d] The number of residues/molecule was calculated assuming that the number of His was 2.

Lys-289 and Phe-235 to Lys-331) to confirm that Glu-335 was at the C terminus. The results clearly rule out Lys-289 (Table IV) and appear to rule out Lys-331, since this would result in one less arginine and one less serine residue. A summary of the polymerase-β digestion analyses for immunoreactive polypeptides is presented in Table III. All experimental results are consistent with the determination that the mAb epitope is within amino acid 235–335 of pol-β.

Sequences within the 11-kDa Fragment of Pol-β Have Sequence Homology to Other Proteins. The complete amino acid sequences (if known) for each protein to which pol-β mAb showed cross-reactivity were obtained from the SWISS-PROT sequence library. The complete sequence of rat polymerase-β was compared to each of the proteins listed in Table V by the method of Lipman (Lipman & Pearson, 1985; Wilbur & Lipman, 1983) and by the method of Myers and Miller (1988). Homology comparisons were done on areas of at least 5–7 amino acids in length (the theoretical minimal size of an epitope). Each protein examined had at least one area sharing significant sequence homology with an area within amino acids 235–335 of pol-β. The majority of the homology seems to correspond to the region of pol-β consisting of amino acids 283–320, suggesting that at least a significant portion of the mAb epitope is within this region. In contrast, some proteins tested which did not interact with pol-β mAb also share some sequence homology with pol-β. However, this homology is outside the region of the putative dNTP site of DNA pol-β (data not shown).

DISCUSSION

Production of Monoclonal Antibodies to Polymerase-β. It is not surprising that all of the monoclonal antibodies recovered were of the IgM class. This is basically a limitation of the in vitro system related to the amount of time that the spleen cell culture is immunized with the antigen. Under the conditions of the system used, a longer immunization period would significantly reduce the number of hybridoma colonies recovered, since the viability of the spleen cell culture drops drastically after day 5. Attempts to modify the system to yield IgG antibodies were unsuccessful. In addition, our attempts at in vivo immunization in mice was unsuccessful, presumably due to the high degree of conservation of pol-β amino acid residues in mammals (Chang et al., 1982a). Rabbit polyclonal antiserum prepared against calf thymus pol-β was of very low titer when tested for its ability to inhibit enzyme activity of pol-β preparations various species (Chang & Bollum, 1981). Other investigators were able to generate a more useful polyclonal antibody by immunizing rabbits with chicken pol-β (Yamaguchi et al., 1982).

All six mAb appear to recognize the same epitope on pol-β even though the hybridoma cell lines were derived from two separate immunization/fusion events, and all cell lines were derived from individual single colonies. It would be expected that each cell line would produce an antibody that recognizes a separate and distinct epitope. However, the fact that pol-β is so weakly immunogenic presents the possibility that perhaps only one region of the protein can elicit a detectable immune response when using ELISA and immunoblot analysis to measure for the presence of antibody.

Neutralization of Polymerase-β Activity and Immunoprecipitation. Each of the six mAb to pol-β was capable of neutralizing enzyme activity 40–50% when assayed on activated DNA with a [dNTP] of 3.75 μM in the reaction mixture. As the results in Table I indicate, the detection of mAb neutralizing activity is strictly dependent on the dNTP concentration in the pol-β reaction mixture. Preincubation of pol-β with dNTPs blocks the ability of the mAb to neutralize pol-β enzymatic activity (Table II). Together, these data suggest that the mAb recognizes an epitope at or near the dNTP binding site; however, the affinity of the mAb for this site appears to be rather weak, since dNTP can apparently titrate off the mAb. By comparing the ratio of the K_m (7.5 μM) of pol-β for dNTP in our activated DNA assay system (Stalker et al., 1976) and the K_d (1.8×10^{-8} M, Figure 3) of the mAb to pol-β, and by assuming that approximately a 100-fold excess of dNTP would be needed to completely abolish mAb activity, we can predict that dNTP would need to be present in a 4.2×10^4-fold greater concentration than IgM in our neutralization assays. The data in Table I for assays on the activated DNA substrate indicate that pol-β activity can be restored in the presence of 15 μM dNTP in the enzyme assays. Comparing this concentration to the concentration of IgM mAb (3.8×10^{-10} M) present in the assay corresponds to a 3.9×10^4-fold greater amount of dNTP in the assay. This agrees well with the above calculation and, thus, strongly supports the specificity of the mAb interaction with the dNTP site of pol-β.

The low affinity of the mAb was also apparent in the immunoprecipitation experiments. No greater than 70% of the polymerase-β, as judged by the amount of enzyme activity remaining in the supernatant, could be precipitated regardless of the amount of mAb used. A Scatchard plot of the data (Figure 3) predicts a dissociation constant (K_d) which indicates a weak binding affinity. For immunoprecipitations in particular, a mAb with a K_d of 10^{-7} M gives a weak signal, while a mAb with a K_d of 10^{-9} M produces a strong signal (Harlow & Lane, 1988).

Mapping of the mAb Epitope on Polymerase-β. Examination of the ATP binding site of adenylate kinase supports the idea that the mAb epitope is at or near the dNTP binding site of pol-β. This is of particular interest since there do not appear to be any reports in the literature demonstrating the production of mAb to a nucleotide binding site for any enzyme. Exhaustive studies using mAb to probe structure–function relationships of F_1-ATPases (Aggeler et al., 1990) as well as studies on mAb raised to calf thymus DNA-dependent ATPase A (Mesner et al., 1991) have yet to produce a mAb which recognizes a nucleotide binding domain. Ten additional

Table V: Proteins That Interact with Monoclonal Antibodies to Polymerase-β Share Amino Acid Sequence Homology with Rat DNA Polymerase-β[a]

protein	amino acid residues	amino acid residues in rat pol-β	no. of residues identical	identical and similar residues[b]
DNA polymerase I (*E. coli*)	107–112	301–306	4/6	5/6
DNA polymerase III (*E. coli*)				
α-subunit (130 kDa)	1046–1052	299–305	4/7	5/7
	1153–1159	244–250	3/7	5/7
β-subunit (41 kDa)	7–11	283–287	3/5	3/5
τ-, γ-subunits (71, 52 kDa)	65–69	288–292	3/5	3/5
	465–469	286–290	4/5	4/5
	637–643	295–301	4/7	6/7
ϵ-subunit (28 kDa)	64–73	302–311	6/10	7/10
terminal transferase (calf)	173–177	248–252	3/5	3/5
	499–504	315–320	4/6	5/6
	511–517	327–333	4/7	5/7
SSB (*E. coli*)	48–54	328–335	3/7	5/7
	67–72	306–311	4/6	4/6
	72–76	287–291	3/5	3/5
carbonic anhydrase (bovine)	139–144	301–306	4/6	4/6

[a] Amino acid sequences were obtained from the Swiss-Prot data bank and analyzed by the method by Lipman, Pearson, and Wilbur (Lipman & Pearson, 1985; Wilbur & Lipman, 1983) and the method of Myers and Miller (1988). [b] Similar residues are those which when interchanged rarely modify the biological activity of a protein. Similar residues are A, S, T; D, E; N, Q; R, K; I, L, M, V; F, Y, W.

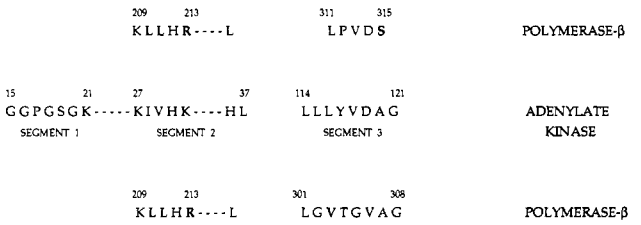

FIGURE 6: Comparison of amino acid sequences of a putative nucleotide binding domain of DNA polymerase-β and three segments of the nucleotide binding domain of adenylate kinase. The three peptides in the nucleotide binding domain of adenylate kinase (Fry et al., 1986) are labeled as segments 1–3. Residues which are similar to those of adenylate kinase are typed in boldface. Similar residues are those which when interchanged rarely modify the biological activity of a protein. Similar residues are A, S, T; D, E; N, Q; R, K.; I, L, M, V; F, Y, W.

mAb raised to DNA-dependent ATPase A also fail to react with a nucleotide binding site (J. Hockensmith, personal communication). To our knowledge, none of the mAb raised to DNA pol-α (Tanaka et al., 1982) recognize a nucleotide binding domain. The MgATP binding site of adenylate kinase was located by a combination of NMR and X-ray diffraction studies on porcine and rabbit muscle enzyme (Fry et al., 1986). The binding site was found to be near three protein segments that have high sequence homology to segments found in other nucleotide-binding phosphotransferases such as myosin and F_1-ATPase, *ras* p21 and transducin GTPases, and cAMP-dependent and src protein kinases (Fry et al., 1986; also see Figure 6). The conservation of these residues suggests equivalent mechanistic roles in the above proteins.

Segments within rat polymerase-β have significant homology with segments 2 and 3 of adenylate kinase (Figure 6). A region consisting of residues 209–213 in pol-β has two positions of identity and conservative substitutions at the other three positions when compared with segment 2 (residues 27–31) of adenylate kinase. Amino acids 311–315 of pol-β share three identical residues, including the highly conserved aspartate, and one similar residue with a portion of segment 3 (amino acids 116–120) in adenylate kinase. In addition, amino acids 301–308 of pol-β share three identical residues and one similar residue with segment 3 (amino acids 114–121). Although pol-β does not have any segments with a high degree of homology to adenylate kinase segment 1, pol-β residues 301–308 comprise the most glycine-rich octapeptide within the protein. Perhaps residues 301–308 and 311–315 in pol-β can, together, provide similar function(s) as do segments 1 and 3 in adenylate kinase. It is interesting to note that the segment 2 and segment 3 "homologues" of pol-β are approximately the same number of residues apart as are segment 2 and segment 3 of adenylate kinase. Secondary structure analysis of pol-β by the method of Garnier et al. (1978) predicts that the region of residues 209–213 is mainly α-helix, and residues 301–308 are predicted to be in an extended or β-sheet conformation. This is analogous to what has been determined for segments 2 and 3 of adenylate kinase.

It should also be noted that the area of pol-β (amino acids 68–72), identified as part of the dNTP binding pocket in pyridoxal 5′-phosphate (PLP) affinity labeling studies (Basu et al., 1989), shows some sequence homology (three of five residues identical) with segment 2 of adenylate kinase. This suggests that this segment may also be part of a nucleotide binding domain. However, this region has been shown to be a template binding domain (Kumar et al., 1990a). A subsequent report (Kumar et al., 1990b) shows that the catalytic activity of the enzyme is within the C-terminal (residues 85–335) 31-kDa fragment. The authors then propose a speculative model of pol-β, predicting that the dNTP binding site is within the 31-kDa domain near the carboxy terminus of the protein with the amino-terminal 8-kDa domain being involved in template binding. These results seem to rule out the possibility that the 8-kDa domain is involved in dNTP binding; however, this cannot be conclusively determined until the three-dimensional structure of the protein is elucidated.

Sequence Homology between Polymerase-β and Proteins Which Interact with Monoclonal Antibodies Raised against Polymerase-β. Cross-reactivity to DNA polymerase-α, DNA polymerases I and III, and terminal transferase was not surprising since all of these enzymes share the capability of incorporating nucleotides into a DNA substrate and, therefore, may all share some evolutionarily conserved functional domain(s). The cross-reactions with carbonic anhydrase and SSB were not anticipated and do not appear to relate to any functional similarities. This led to the search for possible "epitope sequence homology" to explain these results (Table V).

Cross-reactivity with terminal transferase was not surprising due to the homology shared by the two enzymes (Matsukage et al., 1987). Photoaffinity labeling studies have mapped two peptides involved in the nucleotide binding domain of calf terminal transferase (Evans et al., 1989). The authors point out that an area of the major photolabeled peptide has some

the polymerase consensus sequence, a region of eight highly conserved residues in vaccinia virus, adenovirus 2, and Epstein–Barr virus DNA polymerases which was suggested to relate to a conserved function, such as nucleotide binding (Earl et al., 1986). Flanking regions of terminal transferase show sequence homology with adenylate kinase, thus supporting the photoaffinity labeling experiments (Evans et al., 1989). The authors also proposed that residues 188–195 of polymerase-β may have a role in nucleotide binding, since this region shows homology (four of eight residues identical) with the polymerase consensus sequence. However, this region has no significant homology with the adenylate kinase ATP binding site and is not within the epitope recognized by the mAb raised to pol-β. Moreover, the studies of Date et al. (1990), using site-directed mutagenesis of the recombinant enzyme, show that Arg-183 is involved in primer recognition. The deoxyribonucleoside triphosphate binding domain appears to be outside this region, since the K_m's for dNTP of the mutant pol-β molecules analyzed were not significantly different from the K_m for the wild-type enzyme.

In summary, the studies herein are the first to report the development and characterization of monoclonal antibodies raised to DNA polymerase-β. These antibodies surprisingly showed cross-reactivity to a number of proteins. Nearly all of these polypeptides possess nucleotide binding sites, which suggested the potential value of these antibodies in elucidating structure–function relationships within polymerase-β and other nucleotide-binding proteins. Most notably, these antibodies were able to partially inhibit pol-β enzymatic activity in vitro, which led to the identification of a putative dNTP binding site in the C-terminal region of pol-β. These studies will be integral in leading investigators to further clarify the exact location of the dNTP site through crystallographic and site-directed mutagenesis studies.

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