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Characterization and Application to Hot Start PCR of Neutralizing Monoclonal Antibodies against KOD DNA Polymerase[1]

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DNA polymerase from *Pyrococcus kodakaraensis* KOD1 (KOD DNA polymerase) is one of the most efficient thermostable PCR enzymes exhibiting higher accuracy and elongation velocity than any other commercially available DNA polymerase [M. Takagi *et al.* (1997) *Appl. Environ. Microbiol.* 63, 4504-4510]. However, even when KOD DNA polymerase was used for PCR, troubles with nonspecific DNA amplification and primer dimer formation still remain because of undesirable DNA polymerase activity during the first denaturing step of PCR. In order to inhibit this undesirable DNA polymerase activity (hot start PCR), two neutralizing monoclonal antibodies (mAbs), 3G8 and βG1, to KOD DNA polymerase were obtained. Both of these antibodies belong to subclass IgG_1, κ. K_d values were 7.3×10^{-8} for 3G8 and 1.1×10^{-6} for βG1. Nucleotide sequencing of cDNAs of these monoclonal antibodies revealed their sequences to differ in their CDRs (complementarity determining region). Exonuclease activity measurement and epitope mapping revealed that the epitope for 3G8 is located in conserved regions among α-like (family B) DNA polymerases (Region II), and the epitope for βG1 is located in the 3′-5′ exonuclease domain. When hot start PCR with each of these mAbs was performed, the specificity of target gene amplification became much higher than in reactions without monoclonal antibody. Furthermore, this method can easily be applied to long distance PCR (>17.5 kbp).

Key words: DNA polymerase, epitope, 3′-5′ exonuclease, hot start PCR, monoclonal antibody.

The melding of a technique for repeated rounds of DNA synthesis with the discovery of a thermostable DNA polymerase has given scientists the very powerful technique known as PCR (*1*). PCR is based on three simple steps required for any DNA synthesis reaction; (i) denaturation of the template into single strands; (ii) annealing of primers to each original strand for new strand synthesis; (iii) extension of the new DNA strands from the primers (*2*). To perform more than one round of synthesis, the templates must again be denatured at high temperature. *Taq* polymerase from *Thermus aquaticus* (*3*) and *Tth* polymerase from *Thermus thermophilus* (*4*) are thermostable DNA polymerases conventionally used in PCR that are classified in the pol I-like bacterial DNA polymerase family (family A) (*5*). However, recently, archaeal DNA polymerases classified in the α-like DNA polymerase family (family B DNA polymerases) from *Pyrococcus furiosus, Pyrococcus* GB-D, and *Thermococcus litoralis* are often used in PCR because of their high fidelity of DNA synthesis based on their 3′-5′ exonuclease activity for proofreading misincorporated nucleotides (*6-8*). Indeed, high fidelity is ideal for PCR, but these family B polymerases often require longer reaction times (at least two minutes) because of their slow elongation speed. We have recently reported a new thermostable family B polymerase, KOD DNA polymerase from hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 (*9*), that is an efficient PCR enzyme with high fidelity and fast extension rate (*10*). Although the KOD DNA polymerase is an accurate and time-saving PCR enzyme, non-specific amplification and primer dimer formation during PCR are still the most serious problems encountered in the amplification of a target DNA, especially when only a limited amount of template DNA is available. The most likely reason for the low specificity of PCR is undesirable DNA polymerase activity during the first cycle of temperature elevation.

In order to inhibit this undesirable DNA polymerase activity at low temperature, several methods of "hot start PCR" were developed (*11*). There are three major ways to perform hot start PCR. The simplest method is called "manual hot start"; an easy method to apply a preheated DNA template and DNA polymerase into a preheated reaction mixture. The second method use solid oil and is

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called the "wax method" (*12*). This method separates the PCR mixture into two fractions, the DNA template and DNA polymerase, using solid oil. The disadvantages of these methods are the high risk of contamination and complicated manipulation steps.

The third method, hot start PCR using a neutralizing monoclonal antibody, was developed for *Taq* DNA polymerase (*13*). This method is based on the principle that the *Taq* DNA polymerase does not have activity until the bound monoclonal antibody is heat-denatured. Indeed this method of hot start PCR using a monoclonal antibody is much easier than other two methods described above. However, *Taq* DNA polymerase exhibits a lower fidelity compared to archaeal family B polymerases. Therefore, the hot start method using a neutralizing monoclonal antibody should be applied for the most efficient PCR enzymes such as KOD DNA polymerase.

In this paper, we describe the application of two neutralizing mAbs to hot start PCR, and discuss the biochemical and physicochemical properties of these mAbs.

MATERIALS AND METHODS

Establishment of Hybridomas Producing mAb Specific to KOD DNA Polymerase—BALB/c mice were immunized biweekly with 50 μg of purified KOD DNA polymerase in complete Freund's adjuvant (Difco, Detroit, Michigan, USA) until anti-KOD DNA polymerase antibodies could be detected in the serum. The final immunization was performed with incomplete Freund's adjuvant (Difco). Spleen cells were fused with mouse myeloma SP2/0 cells utilizing polyethylene glycol 1500 (Boehringer Mannheim, GmbH, Germany), and hybridomas were established by HAT selection methods as reported elsewhere. Hybridomas producing anti-KOD polymerase antibodies were selected by ELISA.

Assay for DNA Polymerase Activity—DNA polymerase activity was determined by a TCA precipitation assay (*7*). The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 μg/ml BSA, 0.15 mM of each dNTP, [8-^{3}H(N)]-dATP (0.13 μCi/nmol final concentration), 150 μg/ml activated calf thymus DNA, and 7.5 mM dithiothreitol. One unit of enzyme activity is defined as the amount of enzyme required to incorporate 10 nmol of dNTP into an acid-insoluble form at 75°C in 30 min.

Inhibition of DNA Polymerase Activity by anti-KOD DNA Polymerase Monoclonal Antibodies—KOD DNA polymerase was preincubated (25 U/ml, 60.5 nM) with each monoclonal antibody (mAb) (50-500 μg/ml, 13.3-133 μM) in buffer containing 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$ 0.1% Triton-X100, and 10 μg/ml BSA (equal to PCR buffer) for 10 min. Then 20 μl of the mixture was mixed with 80 μl of 120 mM Tris-HCl (pH 8.0), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1 mM $MgCl_2$, 0.1% Triton-X100, and 10 μg/ml BSA, 150 μg/ml activated calf thymus DNA, and 0.2 mM each dCTP, dGTP, and dTTP, 0.1 mM [8-^{3}H(N)]dATP (0.13 Ci/nmol), and incubated at 42°C for 150 min. The reaction was terminated by the addition of 30% trichloroacetic acid, cooled on ice 30 min, and aspirated to a prewet 25 mm GFC filter disk (Whatman International, Maidstone, England). The precipitated reaction products were washed with 10% trichloroacetic acid. The filter was dried and the amount of incorporated ^{3}H was counted with a liquid-scintillation counter (Beckman Instruments, Furellton, California, USA).

Inhibition of 3′-5′ Exonuclease Activity by Anti-KOD DNA Polymerase Monoclonal Antibodies—The 3′-5′ exonuclease assay of DNA polymerase quantified the release of [8-^{3}H(N)]dTTP from the 3′ end of labeled λ-DNA/*Hin*dIII fragments as detected by a liquid scintillation counter (*10*).

The inhibition of the 3′-5′ exonuclease activity by mAbs was examined as follows. KOD DNA polymerase (5 units) and antibodies (0-11 μg) were mixed in reaction buffer [0.2 mg of λ-DNA/*Hin*dIII fragments, 20 mM Tris-HCl (pH 8.5), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 0.1% Triton-X100, 10 μg/ml BSA] and the reaction mixture was incubated at 45°C for 1 h. The enzyme reaction was terminated by the addition of 50 μl of 1% BSA and 100 μl of 10% TCA. The mixture was kept on ice for 10 min and then centrifuged for 10 min. The supernatant was obtained and radioactivity was measured by a liquid scintillation counter.

Strains, Plasmids, and Media—*Escherichia coli* JM109 was used for plasmid propagation and nucleotide sequencing. *E. coli* BL21(DE3) was used for the overexpression of the cloned genes. pUC18 was used as a vector for cloning cDNA for the antibody Fab regions. pET-32a (*14*) was used for the expression of recombinant proteins. *E. coli* JM109 was grown in L-broth and *E. coli* BL21(DE3) was grown in NZCYM medium at 37°C (*10*). Competent *E. coli* JM109 and BL21(DE3) cells were prepared as described before (*10*). Ampicillin (final concentration 50 μg/ml) was added to the plasmid-harboring *E. coli* cells.

mRNA Extraction and cDNA Synthesis—Total mRNA was extracted from 1×10^7 hybridoma cells with a Quick-Prep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden) by the method described previously (*10*). Reverse transcriptase RAV-2 (Takara Shuzo, Kyoto) was used to synthesize a cDNA library from the total mRNA. The library was used as templates for PCR of cDNA encoding the monoclonal antibody Fab region.

Nucleotide Sequence Accession Numbers—The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank nucleotide sequence databanks with accession numbers AB017091(3G8 L chain)-AB-17092(3G8 H chain), AB017093(βG1 L chain), and AB-17094(βG1 H chain).

Determination of Dissociation Constants Using Surface Plasmon Resonance (SPR)—The specificity of the mAbs was characterized by SPR (BIAcore X, Biacore, Uppsala, Sweden). A solution of 10 μg/ml of KOD DNA polymerase was used to immobilize the enzyme onto a CM5 sensor chip by the standard amine coupling method as recommended by the manufacturer. For binding analysis, mAbs at concentrations ranging from 40 nM to 5 μM were allowed to interact with KOD DNA polymerase at a flow rate of 10 μl/min. The running buffer was 10 mM HEPES (pH 7.4), 3.4 mM EDTA, 150 mM NaCl, and 0.0005% v/v surfactant P20 (Biacore). The rate binding constants were determined using BIAevaluation version 3.0 software (Biacore).

Proteolytic Digestion of KOD DNA Polymerase—For partial proteolytic fragmentation, KOD DNA polymerase (100 μl of a 1 mg/ml sample) was incubated with trypsin (2 μg) at 37°C for 180 min, or with *S. aureus* V8 protease (3 μg) at 37°C for 30 min. Buffers used were 50 mM Tris-HCl

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(pH 8.0), 10 mM $CaCl_2$ (trypsin), and 50 mM sodium phosphate (pH 7.8), 0.1% SDS (V8 protease). Peptide fragments obtained by proteolytic digestion of KOD DNA polymerase were applied to SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and isolated by an electroelution method. The fragments were then transferred onto a PVDF membrane and subjected to N-terminal amino acid sequence determination (Model 476A , Applied Biosystems, Foster CA, USA).

Polymerase Chain Reactions—KOD DNA polymerase, *Pfu* DNA polymerase, and *Taq* DNA polymerase were purchased from Toyobo (Osaka). Deep Vent DNA polymerase and Vent DNA polymerase were obtained from New England Biolabs (Beverly, MA, USA). KOD Dash (Toyobo) was used for long distance PCR (>5 kbp).

Hot start PCR by KOD DNA polymerase was performed in 100 μl reaction mixtures containing 2 μg of each mAb, 2.5 units of KOD DNA polymerase, 1.4 ng of IL-6 cDNA (555 bp), 50 pmol of each primer for IL-6 cDNA [Forward (5′-ATGAACTCCTTCTCCACAAGCGC-3′) and reverse (5′-GAAGAGCCCTCAGGCTGGACTG-3′)], and 3.8 μg *Pyrococcus kodakaraensis* KOD1 (*9*) chromosomal DNA as a background DNA to increase the possibility of non-specific DNA amplification. PCR was performed by 1 cycle of 95°C for 5 min, 55°C for 30 s, 74°C for 30 s, followed by 35 cycles of 95°C for 15 s, 55°C for 30 s, 74°C for 30 s by a Thermal cycler PJ2000 (Perkin Elmer, Norwalk, CT, USA).

Hot start PCR by KOD Dash was performed in 100 μl reaction mixtures containing 2 μg of each mAb, 2.5 units of KOD Dash, and 1 ng of human placental cDNA pool with the addition of 2 μg of genomic DNA mixture (Clontech, Palo Alto, CA, USA), 20 pmol of each primer for the human transferrin receptor gene (1.3 kbp) [forward (5′-CCACCATCTCGGTCATCAGGATTGCCT-3′) and reverse (5′-TTCTCATGGAAGCTATGGGTATCACAT-3′)]. PCR was achieved by 35 cycles of 95°C for 15 s , 61°C for 30 s, 74°C for 30 s.

For long distance PCR, different primers [forward (5′-TGCACCTGCTCTGTGATTATGACTATCCCACAGTC-3′) and reverse (5′-ACATGATTAGCAAAAGGGCCTAGCTTGGACTCAGA-3′)] were used. The PCR conditions were as follows: 200 ng of human genomic DNA, 0.1-2.5 μg mAb, 2.5 U KOD Dash, and primers for the human β-globin gene fragment (17.5 kbp) in PCR buffer. Step Down PCR (*15*) was performed to achieve more efficient amplification of the specific DNA fragment. The reaction conditions were 94°C for 1 min, 5 cycles of 94°C for 30 s, 74°C for 10 min, 5 cycles of 94°C for 30 s, 72°C for 10 min, 5 cycles of 94°C for 30 s, 70°C for 10 min, and 15 cycles of 94°C for 30 s, 69°C for 10 min.

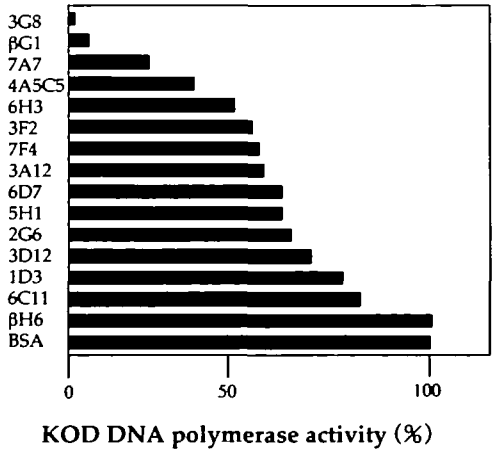

Fig. 1. **Inhibition of KOD DNA polymerase activity by mAbs.** Neutralizing mAbs against KOD DNA polymerase were screened by measuring DNA polymerase activity. The incorporation of [^{3}H]dATP into newly synthesized DNA was quantified with a liquid scintillation counter. DNA polymerase activity with the addition of BSA (50 μg/ml) was defined as 100%.

RESULTS AND DISCUSSION

Anti-KOD DNA Polymerase Monoclonal Antibodies—Two mice were immunized with purified KOD DNA polymerase (*16*) and the production of antibody against the DNA polymerase was examined by ELISA. Many positive cells were obtained and 40 clones were established by limiting dilution. Among 40 positive clones, fifteen clones were selected and their culture supernatants containing monoclonal antibodies (mAbs) were subjected to a KOD DNA polymerase inhibition test. Two monoclonal antibodies that exhibited strong inhibition of the DNA polymerase activity were selected (3G8 and βG1, Fig. 1). The isotypes of these two mAbs were determined and both 3G8 and βG1 belong to IgG_1, k. The mAbs were purified from the supernatant of their hybridoma cell cultures on a proteinA column, and purity was examined by SDS-PAGE. Inhibition of the DNA polymerase activity was confirmed using the purified monoclonal antibodies.

Nucleotide Sequence Analysis of cDNA for Monoclonal Antibodies—cDNAs for these mAbs were amplified by PCR with primers based on the conserved N- and C-terminal regions of antibody Fab fragments (IgG_1, κ). Using the cDNA pool obtained by the above method, the PCR products of 650 bp DNA fragments corresponding to the H and L chains were purified and cloned into plasmid pUC18. Nucleotide sequences of the 663 bp (221 amino acids) H chain Fab region and 654 bp (218 amino acids) L chain of 3G8, and the 648 bp (216 amino acids) H chain and 654 bp (218 amino acids) L chain of βG1 were determined (AB017091-AB017094). The deduced amino acid sequences of 3G8 and βG1 in their CDRs were compared (Fig. 2). Except for the L chain CDR2, no similarity in the CDRs was found, indicating that these two antibodies are not identical and might recognize different epitope regions on the KOD DNA polymerase.

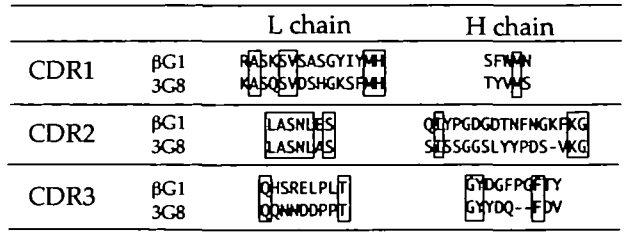

Fig. 2. **Amino acid sequences of CDRs for 3G8 and βG1.** The amino acid sequences deduced from nucleotide sequence analysis were compared at the CDRs (complementarity determining regions) of the H and L chains. Conserved amino acid residues are boxed.

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Determination of Dissociation Constants—Dissociation constants (K_d) of the two mAbs were determined using surface plasmon resonance (SPR) as described above. The mAb 3G8 at concentrations ranging from 40 nM to 330 nM and mAb βG1 at concentration from 500 nM to 5 μM were used for interaction with KOD DNA polymerase. Dissociation constants were calculated to be 7.3×10^{-8} for 3G8 and 1.1×10^{-6} for βG1. These antibodies exhibited different physical properties in their K_d against KOD DNA polymerase although they showed similar inhibitory effects on polymerase activity.

The binding properties of the two mAbs were studied further as follows. The binding sites for one of the mAbs (βG1) on KOD DNA polymerase were saturated by several washes with the mAb. Then the other mAb (3G8) was introduced and the resonance unit profiles were compared with those without prior saturation of one set of binding sites. If the epitopes of the two mAbs are the same or very close on the KOD DNA polymerase structure, then the binding of 3G8 should be hindered by the prebound βG1. The results shown in Fig. 3 clearly show that binding by 3G8 is not affected by prebound βG1, indicating that the two mAbs recognize different epitope regions.

Epitope Mapping of mAbs—In order to predict the epitope region of each mAb, the inhibition of the 3′-5′ exonuclease activity of KOD DNA polymerase by the mAbs

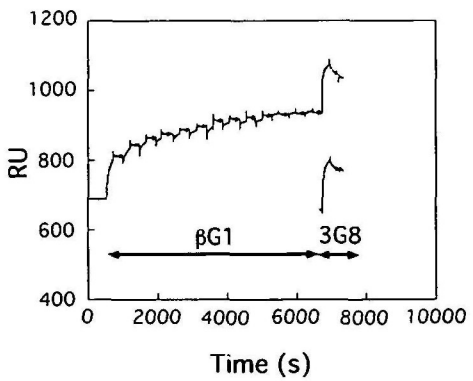

Fig. 3. **Characterization of two antibodies using surface plasmon resonance (SPR).** Binding profiles of two mAbs were analyzed using SPR. A monoclonal antibody (βG1) was flowed through thirteen times until the binding position of the KOD DNA polymerase was saturated. The saturation process was monitored by the increase in resonance units (RU). Then 3G8 was flowed and the increase in RU was examined (upper sensorgram). The lower sensorgram is for the control flow of 3G8 onto KOD DNA polymerase without prebound mAb. The names of the monoclonal antibodies used in each flow are indicated below the RU profiles.

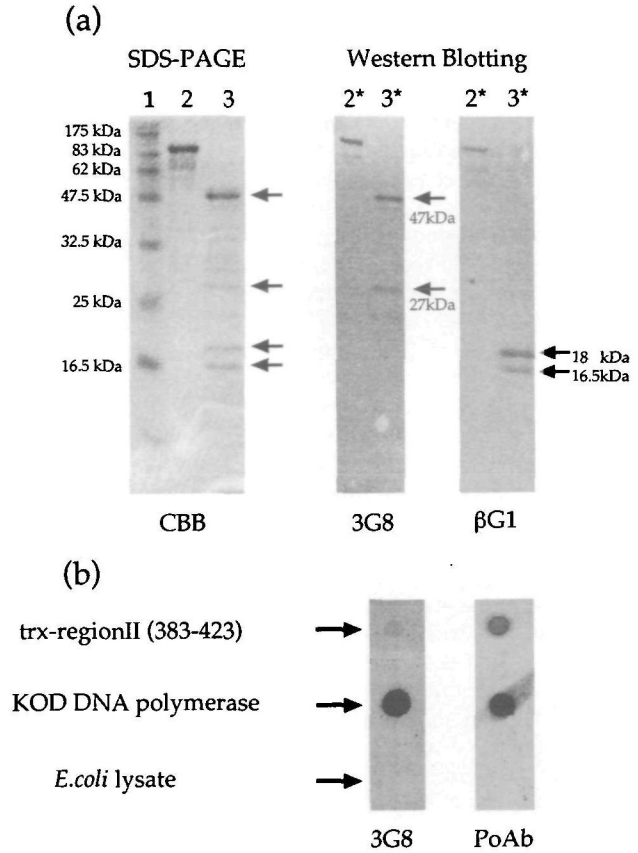

Fig. 4. **Immunoblot analysis of fragmented KOD DNA polymerase.** (a) KOD DNA polymerase was digested with V8 protease as described in "MATERIALS AND METHODS." KOD DNA polymerase (lane 2) and digested fragments of KOD DNA polymerase (lane 3) were separated by SDS-PAGE and the protein bands were detected by Coomassie Brilliant Blue staining. Western analysis using 3G8 and βG1 was also performed after blotting the protein bands (lanes 2 and 3 with asterisks). Highlighted bands and their sizes are indicated by arrows. (b) TrxA-Region II fusion protein, KOD DNA polymerase, and *E. coli* lysate were applied to dot blot analysis. Positive signals were detected from KOD DNA polymerase and the trx-Region II fusion protein but not from *E. coli* lysate.

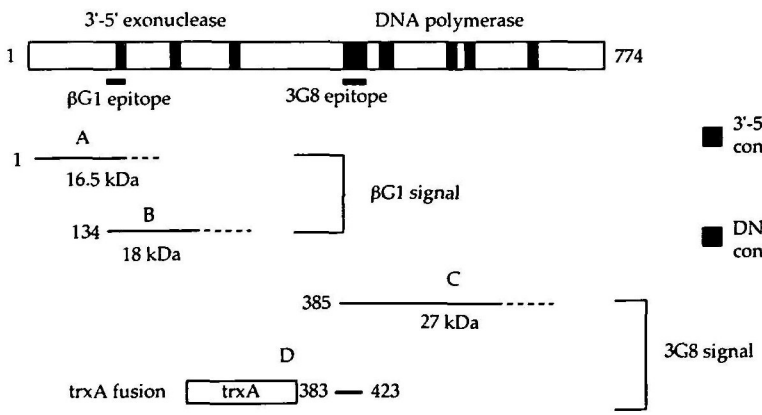

Fig. 5. **Epitope regions for 3G8 and βG1.** Areas of the PVDF membrane containing peptide fragments highlighted by βG1 (16.5 and 18 kDa) and 3G8 (27 kDa) were subjected to N-terminal amino acid sequence determination in order to specify possible epitope regions. A possible epitope region of βG1 was located between residues 134 and 150 based on two positive signals (fragments A and B) and N terminal amino acid sequence determination. The epitope for 3G8 was possibly located between residue 385 and 423 based on two positive signals from fragments C and fragment D (383-423) fused to trxA.

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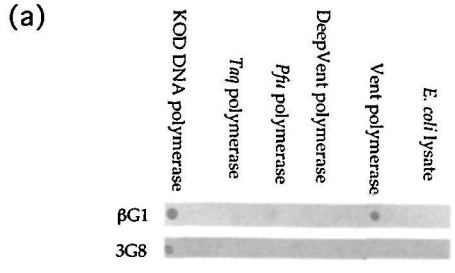
was examined. When 3G8 was added, the remaining exonuclease activity was 18%, while the addition of βG1 resulted in a remaining activity of only 4.6%. βG1 inhibited the exonuclease activity more completely than 3G8, indicating that the epitope of βG1 might be located in the exonuclease domain of KOD DNA polymerase and the epitope of 3G8 might lie in another region of DNA polymerase.

A precise examination of the epitope regions of these two mAbs was made by a combination of partial proteolytic digestion and Western blot analyses followed by N-terminal amino acid sequence determination of the generated peptide fragments. KOD DNA polymerase was digested with V8 protease and trypsin as described in "MATERIALS AND METHODS." From the results of Western blotting, protein fragments with molecular masses of 16.5 and 18 kDa were reacted with βG1 and a fragment with a molecular mass of 27 kDa reacted with 3G8 (Fig. 4a). Areas of the PVDF membrane containing these highlighted peptide fragments were used for N-terminal amino acid sequence determination. The most likely epitope regions were specified by the results of N-terminal sequencing (fragments A to C, Fig. 5) and molecular size estimation by SDS-PAGE. Fragment A (16.5 kDa), starting from amino acid number 1, and fragment B (18 kDa), starting from amino acid number 134 from the N-terminal of KOD DNA polymerase, were generated by V8 protease digestion, and both fragments were recognized by βG1. Therefore, the data suggest that the epitope of βG1 is located downstream of amino acid number 134 to around 170 (Fig. 5). This region is located in the conserved region of 3′-5′ exonuclease activity. Indeed, βG1 inhibits the exonuclease activity more than 3G8. The amino acid sequences of DNA polymerases from *Pyrococcus furiosus* (Pfu polymerase), *Thermococcus litoralis* (Vent polymerase), *Pyrococcus* GB-D (Deep Vent polymerase), and *Thermus aquaticus* (*Taq* polymerase) were compared and dot blot analysis was performed (Fig. 6a). Interestingly, βG1 interacted with both KOD DNA polymerase and Vent polymerase but not with the other DNA polymerases, indicating that βG1 recognizes identical amino acid residues on KOD DNA polymerase and Vent polymerase. The amino acid sequence alignment in Fig. 6b suggests that arginine 169 (R169) of KOD DNA polymerase is conserved in the amino acid sequence of Vent polymerase but not in the other DNA polymerases. Therefore, the epitope for βG1 is located in a region that includes R169.

A protein fragment with a molecular mass of 27 kDa (fragment C) generated by V8 protease was recognized by 3G8 (Fig. 5). The N-terminal amino acid sequence of fragment C starts from residue 385 (Fig. 5). In order to specify the epitope region for 3G8, a partial peptide region of KOD DNA polymerase (383-423, fragment D) was expressed as a trxA fusion protein using the pET-32a expression system (*14*), and recognition of the fragment by 3G8 was examined. A weak positive signal that was significantly stronger than in the case of *E. coli* lysate was detected for the trx-fusion protein by dot blot hybridization (Fig. 4b). When a control experiment using a polyclonal antibody (PoAb) was performed, a similar weak signal could be observed for the trx-Region II fusion protein (Fig. 4b). Therefore, we consider that the weak signals are attributable to structural factors involved in the recognition of the trx-fused protein by antibodies. A possible epitope region for 3G8 (383-423) is located in Region II, a sequence that is well conserved among family B DNA polymerases and considered to be important for the catalytic activity of DNA elongation (*18-20*). Therefore, the data suggest that the epitope for mAb 3G8 is located in the catalytic center of the DNA polymerase (Region II) and that the mAb inhibits the DNA polymerase activity directly.

As supported by the comparison of the deduced amino acid sequences of CDRs and the inhibition test of exonuclease activity by mAbs, the epitopes for βG1, and 3G8 are located at different positions. Although the epitope for βG1 was shown to be located in the exonuclease region, the mAb inhibited DNA polymerase activity. Steric hindrance between DNA and the DNA polymerase around the exonuclease region was brought about by the binding of βG1, and eventually the DNA polymerase activity as well as the exonuclease activity was inhibited.

Hot Start PCR with Neutralizing Monoclonal Antibodies to KOD DNA Polymerase—Hot start PCR was attempted with the addition of the monoclonal antibodies 3G8 and βG1. The result of agarose gel electrophoresis clearly show



(b)

KOD DNA polymerase	134-ELKMLAFDIETLYHEGEEFAEGPILMISYADEEGARVITWKNV-176
Pfu polymerase	134-ELKILAFDIETLYHEGEEFGKGPIIMISYADENEAKVITWKNI-176
Vent polymerase	134-ELKLLAFDIETFYHEGIEFGKGEIIMISYADEEEAKVITWKNI-176
Deep Vent polymerase	134-ELKLLAFDIETLYHEGEEFAKGPIIMISYADEEEAKVITWKKI-176
KOD DNA polymerase	383-SYEGGYVKEPERGLWENIVYLDFRSLYPSIIITHNVSPDTL-426
Pfu polymerase	384-SYIGGFVKEPEKGLWENIVYLDFRALYPSIIITHNVSPDTL-427
Vent polymerase	386-IYLGGYVKEPEKGLWENIIYLDFRSLYPSIIVTHNVSPDTL-429
Deep Vent polymerase	384-SYKGGYVKEPEKGLWEGLVSLDFRSLYPSIIITHNVSPDTL-427

Fig. 6. **Dot blot analysis and amino acid sequence comparison.** (a) Dot blot analysis of two mAbs (βG1 and 3G8) was performed using four DNA polymerases (*Pyrococcus furiosus*; Pfu polymerase, *Thermococcus litoralis*; Vent polymerase, *Pyrococcus* GB-D; Deep Vent polymerase and *Thermus aquaticus*; Taq polymerase) and a lysate of *E. coli* cells. (b) Amino acid sequence comparison of conserved regions among archaeal family B polymerases. Amino acid sequences of the KOD DNA polymerase, including possible epitope regions for βG1 (134-176) and 3G8 (383-426), are aligned with the corresponding amino acid sequence regions of commercially available family B DNA polymerases (Pfu polymerase, Vent polymerase, Deep Vent polymerase). Amino acid residues not conserved in KOD DNA polymerase are indicated on a gray background (■) and amino acid residues conserved only in KOD DNA polymerase and Vent polymerase are shown on a black background (■).

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that the specific amplification of the target IL-6 gene was enhanced by the presence of either the 3G8 or βG1 monoclonal antibody, possibly because these mAbs inhibited the DNA polymerase activity during the time that the KOD polymerase was added to the tube and through the first heat denaturation step (Fig. 7).

Hot Start PCR by KOD Dash—KOD Dash is a mixture of intact KOD DNA polymerase and its mutant that lacks the 3′-5′ exonuclease activity because of an amino acid substitution in the exonuclease region (asparagine 210 to asparaginate). This enzyme mixture was designed for PCR of longer fragments and a similar technique has been used previously by mixing family B DNA polymerase (*Pfu*), with a 3′-5′ exonuclease activity, and *Taq* DNA polymerase without a 3′-5′ exonuclease activity (*17*). However, KOD Dash shows a higher accuracy of PCR while maintaining a comparable elongation speed to that of KOD DNA polymerase than the mixture of *Pfu* and *Taq* DNA polymerases. Furthermore, when hot start PCR using a monoclonal antibody is attempted, it will be much easier with KOD Dash because the system contains basically a single type of DNA polymerase. Therefore, the two monoclonal antibodies can easily be applied to hot start PCR using KOD Dash. As a control experiment, *Taq* DNA polymerase and a monoclonal antibody against *Taq* DNA polymerase were used under the same conditions except for the reaction temperature (72°C). After PCR, the reaction samples were subjected to 1.5% agarose gel electrophoresis (Fig. 8). When *Taq* polymerase (without mAb) and *Taq* polymerase with anti-*Taq* mAb (Clontech) were used, no clear DNA band near the 1.3 kbp size that corresponds to the amplified DNA products could be detected. When KOD Dash (without mAb) was used, only a small amount of DNA was amplified. However, when KOD Dash with either 3G8 or βG1 was used, significant amounts of an amplified 1.3 kbp DNA fragment could be detected. Therefore, we conclude that hot start PCR of KOD Dash using these two neutralizing mAbs is effective.

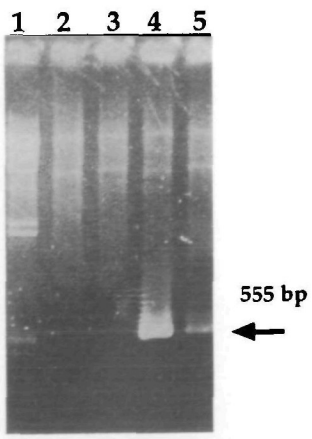

Fig. 7. **Hot start PCR by KOD DNA polymerase and mAb.** Hot start PCR using KOD DNA polymerase (2.5 U) and mAbs (2 μg) was attempted using IL-6 cDNA (555 bp) mixed with an excess amount of genomic DNA from *Pyrococcus kodakaraensis* KOD1 as the template: lane 1, λ-*Hin*dIII marker; lane 2, KOD DNA polymerase; lane 3, KOD DNA polymerase and BSA (50 μg/ml); lane 4, KOD DNA polymerase with mAb 3G8; lane 5, KOD DNA polymerase with mAb βG1.

Long Distance PCR by Hot Start PCR—If amplification of a longer DNA fragment is possible, PCR would be a more useful technique. We attempted to amplify a longer DNA fragment (human β-globin gene, 17.5 kbp) using KOD Dash and neutralizing mAbs (3G8 and βG1). Amplification of the target fragment was detected, especially when both mAbs were used for hot start PCR. The total amount of mAbs required for efficient PCR was less (0.1 μg) than in the case where an individual monoclonal antibody was used (Fig. 9).

This experimental result is consistent with the results of SPR and epitope mapping mentioned above. These two monoclonal antibodies can cooperatively inhibit DNA

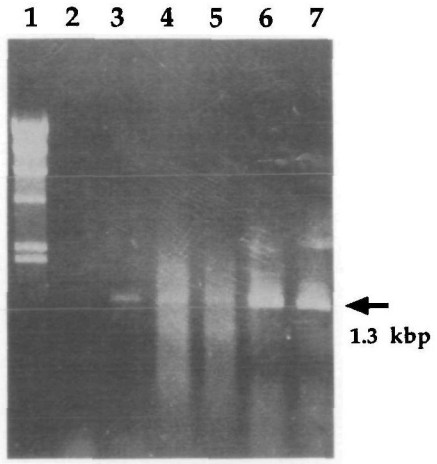

Fig. 8. **Comparison of hot start PCR of KOD Dash with that of *Taq* polymerase.** Hot start PCR by KOD Dash (2.5 U) with each mAb (2 μg) was tested. Human transferrin receptor gene (1.3 kbp) mixed with genomic DNA from human placenta used as a template. Lane 1, λ-*Hin*dIII marker; lane 2, *Taq* polymerase; lane 3, *Taq* polymerase with anti-*Taq* polymerase mAb (Clontech); lane 4, KOD Dash; lane 5, KOD Dash with BSA; lane 6, KOD Dash with 3G8; lane 7, KOD Dash with βG1.

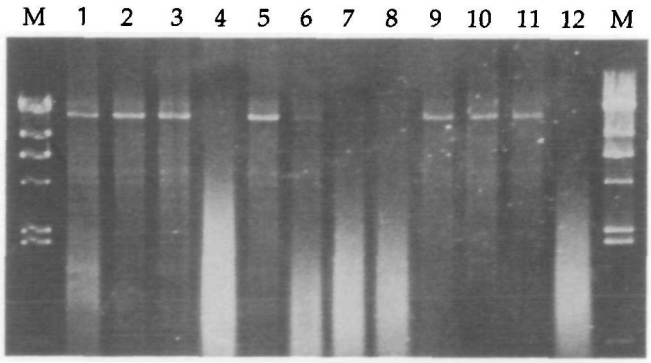

Fig. 9. **Hot start PCR for long distance PCR with KOD Dash.** Amplification of a part of the β-globin gene (17.5 kbp) was attempted to test long distance PCR by hot start PCR with the mAb and KOD Dash (2.5 U) in a reaction volume of 100 μl. The reactions were performed with the addition of both 3G8 and βG1 [0.1, 0.25, 0.5, and 1.2 μg (lane 1, 2, 3, and 4, respectively)] and 3G8 [0.25, 0.5, and 1.0 μg (lane 5, 6, and 7, respectively)] and βG1 [0.25, 0.5, 1.0, and 2.5 μg (lane 8, 9, 10, and 11, respectively)]. Lane 12 corresponds to KOD Dash without mAb and lane M to the λ-*Hin*dIII molecular weight marker.

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polymerase by binding at two different epitopes. Accordingly, more efficient hot start PCR could be achieved by the addition of two different mAbs.

Recently, we crystallized the KOD DNA polymerase (*21*) and the analysis of the tertiary structure is now in progress. The structural information will provide information about the high velocity and high fidelity of KOD DNA polymerase in DNA strand elongation and the precise mode by which the polymerase is inhibited by these mAbs.

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