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The Carboxyl Terminal Sequence of Nucleolar Protein B23.1 Is Important in Its DNA Polymerase α-Stimulatory Activity

Hayato Umekawa,*,[1] Keiji Sato,* Masaharu Takemura,† Yoshinori Watanabe,* Satomi Usui,* Takao Takahashi,* Shonen Yoshida,† Mark O.J. Olson,‡ and Yukio Furuichi*

*Department of Sustainable Resource Science, Faculty of Bioresources, Mie University, Tsu, Mie 514-8507, †Research Institute for Disease Mechanism and Control, Nagoya University School of Medicine, Nagoya, Aichi 466-8550, and ‡Department of Biochemistry, University of Mississippi Medical Center, Jackson, Mississippi 39216-4505, USA

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The protein B23 is a major nucleolar phosphoprotein comprising two isoforms, B23.1 and B23.2, which differ only in their carboxyl-terminal short sequences, the N-terminal 255 residues being identical in both forms. Both B23.1 and B23.2 stimulated immunoaffinity-purified calf thymus DNA polymerase α in a dose-dependent manner. The stimulatory effect of protein B23.1, the longer isoform, was found to be 2-fold greater than that of B23.2. Purified DNA polymerase α bound tightly to a protein B23.1-immobilized column, while it bound weakly to a protein B23.2-immobilized column. Surface plasmon resonance studies by BIAcore further showed that protein B23.1 bound to the DNA polymerase α-(dA)·(dT) complex more tightly than did protein B23.2. The protein B23 isoforms appear to interact directly with the DNA polymerase α protein and not through the bound nucleic acid. These observations indicated that protein B23 physically bound to the DNA polymerase α and stimulated the enzyme activity. Product analyses showed that protein B23 greatly enhanced the reaction both in amount and length of product DNA, whereas it did not significantly alter the processivity of polymerization. In contrast, protein B23 effectively protected DNA polymerase α from heat inactivation. These results suggest that protein B23 stabilizes DNA polymerase α that is detached from product DNA, allowing the enzyme to be recruited for further elongation. Moreover, experiments using various C-terminal deletion mutants of protein B23 indicated that 12 amino acids at the C-terminal end of B23.1, which are absent in B23.2, may be essential for the full stimulation of the DNA polymerase α.

Key words: DNA polymerase α, protein B23, protein isoforms.

Protein B23 is a conserved phosphoprotein (pI = 5.1, molecular mass = 38 kDa) that is localized to the granular and fibrillar regions of the nucleolus, where rRNA synthesis and ribosome assembly take place (*1*, *2*), and also to the nuclear matrix, where DNA synthesis might be performed (*3*). It is 20 times more abundant in tumor cells than in normal cells (*4*) and is found to be elevated in mitogen-stimulated normal lymphocytes (*5*, *6*). Protein B23 is phosphorylated by CDK2/CyclinE to initiate centrosome duplication (*7*) and is dephosphorylated by the δ isoform of protein phosphatase type I (*8*). Protein B23 also has the ability to shuttle between the nucleolus and the cytoplasm (*9*). The nuclear import function of protein B23 is supported by its ability to bind proteins containing nuclear localization signals (*10*, *11*), including the HIV-1 Rev protein (*11*, *12*) and the HTLV-1 Rex protein (*13*). Rat protein B23 is expressed in two isoforms, designated as B23.1 and B23.2, which are polypeptides of 292 and 257 amino acids, respectively (*14*). The untranslated 5′ regions of the two mRNAs and the N-terminal 255 residues are identical in the two isoforms. However, the 3′ untranslated regions and sequences coding for the C-terminal ends of the two proteins are completely different. Protein B23.1 is found almost exclusively in the nucleolus, and B23.2 appears to be present in the cytoplasm and/or nucleoplasm (*15*). Under native conditions, B23 has an apparent molecular mass of 230–350 kDa, suggesting that the protein forms oligomers (*12*, *16–18*). Recently, we have reported that protein B23.1 stimulated the activity of DNA polymerase α, but it showed little effect on the activities of DNA polymerase β, γ, and primase (*19*). This finding suggested that protein B23.1 directly affects the activity of DNA replication enzyme. In the present study, we examined the interaction of protein B23 with DNA polymerase α and analyzed the DNA products. We also generated C-terminal deletion mutants of protein B23 to determine the regions required for the stimulation of DNA polymerase α. It was found that protein B23 physically associated with DNA polymerase α, and that the C-terminal 12 amino acids sequence that is specific to protein B23.1 was required for maximum stimulation of DNA polymerase α.

MATERIALS AND METHODS

Recombinant Proteins—Rat recombinant proteins B23.1 and B23.2 used in immobilized affinity columns were expressed in the transformed *Escherichia coli* using pKK223-

[1]To whom correspondence should be addressed. Phone: +81-59-231-9608, Fax. +81-59-231-9684, E-mail. umekawa@bio.mie-u.ac.jp

3 vector and purified essentially as previously described (*12*). The construction and expression of protein B23 and its deletion mutants were performed as follows. The PCR primers for protein B23.1 and its deletion mutants were. BN (5′-GCCTCGGATCCATGGAAGACTCGATG-3′) and B1C (5′-AACCGGATCCTTAAAGAGACTTCCTCC-3′) for full-length B23.1; BN and B2C (5′-TCAAGCTTTTACTC-CTGGTCAGTCATCCGGA-3′) for ΔC12; BN and B3C (5′-AAAAGCTTTTACACATAATTAATGAACTTGGC-3′) for ΔC22; BN and B4C (5′-TAAAGCTTTTACTTGGCTTCCAC-TTTGGGAAG-3′) for ΔC27; BN and B5C (5′-GCAAGCTTT-TAGGGAAGAGAACCACCTTTTTC-3′) for ΔC32; BN and B6C (5′-AGAAGCTTTTATTTTTCTATACTTGCTTGCA-3′) for ΔC37. ΔC indicates C-terminal mutants and the number specifies the number of amino acids deleted from the C-terminal end. PCR amplification was performed using rat B23.1 cDNA as a template. Protein B23.2 was prepared by using rat B23.2 cDNA as a template, and BN and B7C (5′-AAGCTTTCAATGCGCTTTTTCTATACTTGCTT-3′) as primers. PCR products were excised from the gel and ligated into pGEM-T EASY vector (Promega). All constructs were sequenced and subsequently subcloned into pQE-30 vector (Qiagen), with the N-terminal His tag. The fragments were digested with *Bam*HI and *Hin*dIII and ligated into pQE-30 vector. The recombinant plasmids were transformed into *E coli* M15, and the recombinant proteins were purified by a nickel-chelating affinity column chromatography. The histidine-tagged fusion protein B23 and its deletion mutants were used in DNA polymerase α assays, surface plasmon resonance experiments, product analysis, and activity protection assay.

Purification of DNA Polymerase α–Primase Complex—DNA polymerase α–primase complex was purified from calf thymus extract by immunoaffinity column chromatography, as described previously (*20*). The phosphocellulose fraction was passed through a column conjugated with bovine serum albumin, then applied to an immuno-affinity Sepharose 4B column conjugated with monoclonal antibody (MT17) directed against calf thymus DNA polymerase α, and successively eluted with 3 M NaCl and 3.2 M $MgCl_2$ in the buffer containing 50 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol, and 10% glycerol. Activity was recovered in $MgCl_2$ fractions.

DNA Polymerase Assay—DNA polymerase α activity was determined by the incorporation of radioactive dNTPs into the acid-insoluble materials with activated calf thymus DNA. The standard reaction mixture for DNA polymerase α (25 μl) contained 80 mM Tris-HCl, pH 7.5, 8 mM 2-mercaptoethanol, 80 μM each of dATP, dGTP, and dCTP, 40 μM [^{3}H]dTTP (18.5 kBq), 8 mM $MgCl_2$, 5 μg of bovine serum albumin (BSA), and 200 μg/ml of activated calf thymus DNA. Incubations were carried out at 37°C for 30 min, then acid-insoluble radioactivity was measured as described (*21*).

B23 Affinity Column Chromatography—B23.1 (1 mg) or B23.2 (1 mg) was conjugated with 3 ml of CNBr-activated Sepharose (Pharmacia) according to the manufacturer's manual. Most of the applied B23 isoforms was immobilized in the gel. An aliquot of the gel was packed into a column, then equilibrated with 50 mM Tris-HCl (pH 7.5), 2 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, and 1 mg/ml BSA (buffer A). For the control column, 1 mg of BSA was conjugated to the gel. Immunoaffinity-purified calf thymus DNA polymerase α (2.4 μg) was applied to these columns, which were then incubated at 4°C without flow for 10 min to allow it to be absorbed. The columns were then washed successively with six volumes of buffer A and three volumes each of buffer A containing 0.15 M KCl, 0.3 M KCl, 0.6 M KCl, and 1.0 M KCl.

Surface Plasmon Resonance Experiments—Surface plasmon resonance measurements were done by using a BIAcore™ 2000 apparatus (Pharmacia Biosensor). The 5′-biotinylated (dA)100 (Sawady Technology) (10 μg/ml) in 120 μl was injected across a streptavidine-pretreated dextran sensor *in situ* in the BIAcore apparatus at 5 μl/min in 10 mM sodium phosphate (pH 7 5) and 0.5 M NaCl at 25°C. Hybridization of oligo $(dT)_{12\text{-}18}$ (10 μg/ml) was carried out in the same buffer at 25°C by flowing 120 μl at 5 μl/min across the immobilized oligo $(dA)_{100}$. After washing the sensor chip with 80 mM Tris-HCl (pH 7.5) containing 8 mM $MgCl_2$, binding of DNA polymerase α (0.1 unit) to the (dA)·(dT) hybrid surface was done in the same buffer at flow rate of 10 μl/min at 25°C. The sensor chip was then washed with the same buffer, and no decline of the signal was confirmed. Finally, protein B23 was applied at various concentrations to the immobilized surface in 80 mM Tris-HCl (pH 7.5) containing 8 mM $MgCl_2$ at a flow rate of 10 μl/min at 25°C. The kinetic constants (k_{on} and k_{off}) and association constant (K_A) were calculated by use of the evaluation program BIAevaluation 3.0.

Product Analysis—The reaction was performed as described previously (*21*, *22*) with some modifications. DNA polymerase α (2 units) and B23.1 (6.25 μg/ml) were preincubated together for 20 min at 4°C, then with 1.24 μg of M13 singly-primed DNA (M13mp2/15-mer universal primer, labeled with ^{32}P at 5′-end) in 40 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 0.1 mg/ml of BSA for 20 min at room temperature. Reactions were initiated by adding all four dNTPs (80 μM each) and 4 mM $MgCl_2$ with or without excess amount of sonicated calf thymus DNA (1 mg/ml) as a trap. Incubation was carried out at 37°C, and 5-μl aliquots of reaction mixtures were withdrawn at 10, 20, and 40 min and mixed with 1 μl of loading buffer containing 98% deionized formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol FF, and 0.025% bromophenol blue. The samples were then heated at 100°C for 10 min and cooled immediately in ice-water. DNA products were electrophoresed on a sequencing gel of 8% polyacrylamide in 8 M urea at 2,000 V for 1.5 h. The gel was exposed to X-ray film overnight at −80°C.

Activity Protection Assay—DNA polymerase α was mixed with 20 μg/ml of protein B23 1 or BSA and the mixture (5 μl) was preincubated at 37°C for 5 min, then transferred to 43°C. After incubation at 43°C for the time indicated, the DNA polymerase α was chilled and assayed as described above.

Others—SDS–polyacrylamide gel electrophoresis was done in a 12.5% gel according to the method of Laemmli (*23*). Protein concentration was measured by the method of Bradford (*24*).

RESULTS

Dose-Dependent Stimulation of DNA Polymerase α by Protein B23 Isoforms—We have previously demonstrated

that the rat recombinant protein B23.1 as well as natural protein B23 (predominantly B23.1) stimulated the immunoaffinity-purified calf thymus DNA polymerase α activity about 3-fold. The stimulatory effect of protein B23.1 was specific to DNA polymerase α, since B23.1 showed no effect on the activity of either DNA polymerase β or DNA polymerase γ (*19*). On the other hand, protein B23.2, which is

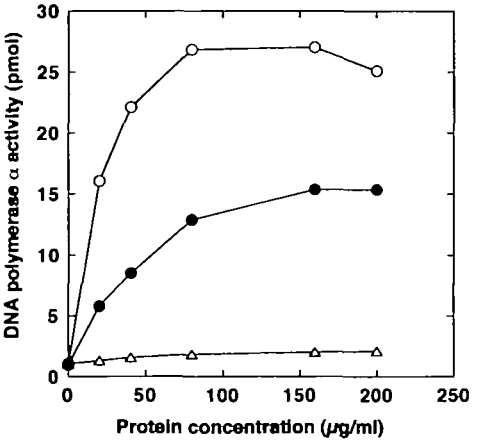

Fig 1 **Dose-dependent stimulation of DNA polymerase α by protein B23.** Activity of immunoaffinity-purified DNA polymerase α (0 48 unit) was measured as described under "MATERIALS AND METHODS" with various amounts of protein B23 (○, B23 1, ●, B23 2) or BSA(△), as indicated

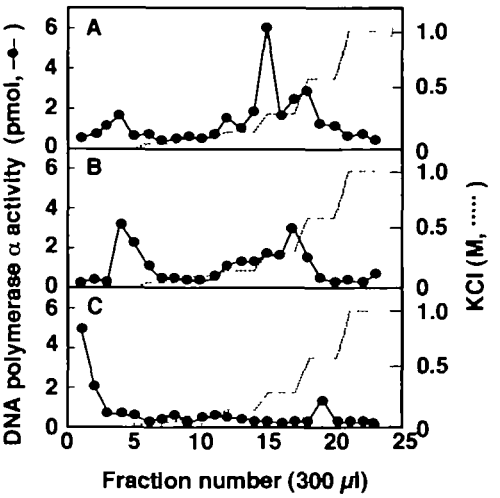

Fig 2 **Binding of immunoaffinity-purified calf thymus DNA polymerase α to protein B23-immobilized affinity columns.** Protein B23 1–immobilized (A), protein B23 2–immobilized (B), and BSA-immobilized (C) columns (volume 300 μl) were prepared and equilibrated as described under "MATERIALS AND METHODS." Immunoaffinity-purified calf thymus DNA polymerase α (2 4 μg) was applied to each affinity column After incubation of the column for 10 min at 4°C, unadsorbed enzyme was recovered by washing the column with 1 ml of buffer A, followed by stepwise elution of adsorbed enzyme with buffer A containing the indicated concentrations of KCl Fractions (300 μl) were collected and assayed for DNA polymerase α activity

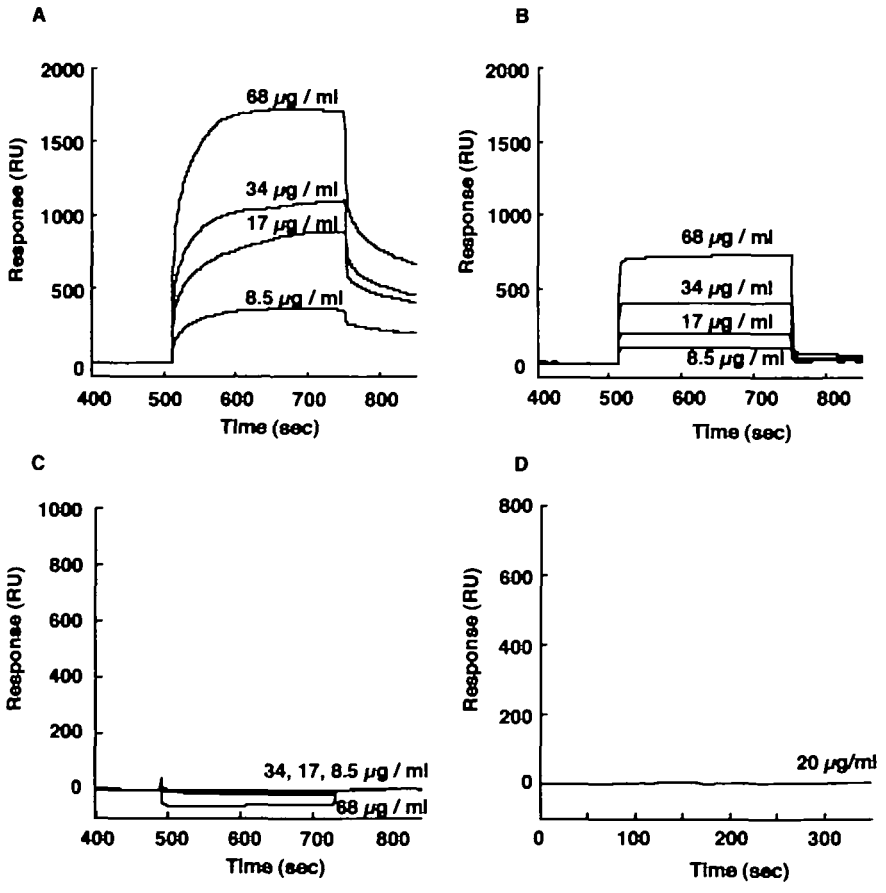

Fig. 3. **Surface plasmon resonance analysis of the interaction of protein B23 with DNA polymerase α–poly(dA)-oligo(dT) complex.** Hybridization of oligo $(dT)_{12\text{-}18}$ to the immobilized $(dA)_{100}$ on the sensor chip and binding of DNA polymerase α to the (dA)-oligo(dT) were carried out as described under "MATERIALS AND METHODS." Different concentrations (8 5, 17, 34, and 68 μg/ml) of B23 1 (A), B23 2 (B), and BSA (C) were injected over the complex of DNA polymerase α–(dA)-oligo-(dT) The background resulting from injection of running buffer alone was subtracted from the data before plotting. D, 20 μg/ml protein B23.1 was injected over (dA)-oligo-(dT) alone and other experimental conditions were same as in A.

35 amino acid residues shorter than protein B23.1 in its C-terminal end, showed little effect on DNA polymerase α activity at concentrations up to 5.5 μg/ml (*19*, *25*). Figure 1 shows a marked stimulation of DNA polymerase α by the addition of protein B23.1. The enzyme activity increased with increasing concentration of protein B23.1 and reached a plateau level of stimulation (about 25-fold). A histidine-tagged fusion protein of B23.1 showed a similar stimulatory effect to natural protein B23 or recombinant protein B23.1 produced by pKK233-2 protein expression vector (*19*). Under the conditions used, about 2 μg of protein B23.1 was required to fully stimulate 38 ng of DNA polymerase α. The molar ratio of DNA polymerase α/protein B23.1 was 1:28, based on the assumpation that the molecular masses of DNA polymerase α and protein B23.1 are 240 and 350 kDa, respectively. A higher concentration of the histidine-tagged protein B23.2 also stimulated DNA polymerase α activity, and the rate of stimulation by protein B23.2 was almost half of that by protein B23.1 Protein B23.2 also forms an oligomer, and its molecular mass is calculated to be 350 kDa (*18*). The minimum amount of protein B23.2 required to reach the plateau level of stimulation was about 3.8 μg. The molar ratio of protein B23.2 required to attain full stimulation was about 52, which was almost two times higher than that of protein B23.1. The stimulation of DNA polymerase α by protein B23 was also observed with the synthetic DNA template-primers, such as poly(dA)-oligo$(dT)_{12\text{-}18}$ and poly[d(A-T)], suggesting that the stimulation was not specific for a given class of DNA template-primer (data not shown).

Physical Binding between DNA Polymerase α and Protein B23—To elucidate the physical interaction between DNA polymerase α and protein B23, we employed B23-immobilized affinity column chromatography. Immunoaffinity-purified calf thymus DNA polymerase α (2.4 μg) was dialyzed against buffer A and loaded onto B23.1-, B23.2-, and BSA-immobilized columns that had been pre-equilibrated with the same buffer. As shown in Fig. 2, the DNA polymerase α activity was adsorbed on the B23.1-immobilized column and eluted with 0.3 to 0.6 M KCl (Fig. 2A). About 84% of the applied DNA polymerase α was recovered in the adsorbed fraction, and the remainder was found in flow-through fraction The overall recovery of the activity was almost 100%. In contrast, a weak interaction was observed with DNA polymerase α and B23 2-immobilized column, about 41% of the applied enzyme was recovered in the adsorbed fraction (Fig. 2B). Since these two isoforms differ only in 37 amino acid residues at C-terminus, this region seems to be important for the strong interaction with DNA polymerase α. Almost all the DNA polymerase α activity was recovered in the unadsorbed fraction when BSA-immobilized column was used (Fig. 2C).

Binding of protein B23 1 to DNA polymerase α was also demonstrated using the BIAcore system The conditions employed for BIAcore experiment were similar to those of the assay system for the DNA polymerase α. The 5′-biotinylated $(dA)_{100}$ was covalently immobilized *via* a biotin group at its 5′ end to the streptavidine-dextran surface. The amount of 5′-biotinylated $(dA)_{100}$ corresponded to 2000 resonance unit (RU). Oligo $(dT)_{12\text{-}18}$ was applied to the immobi-

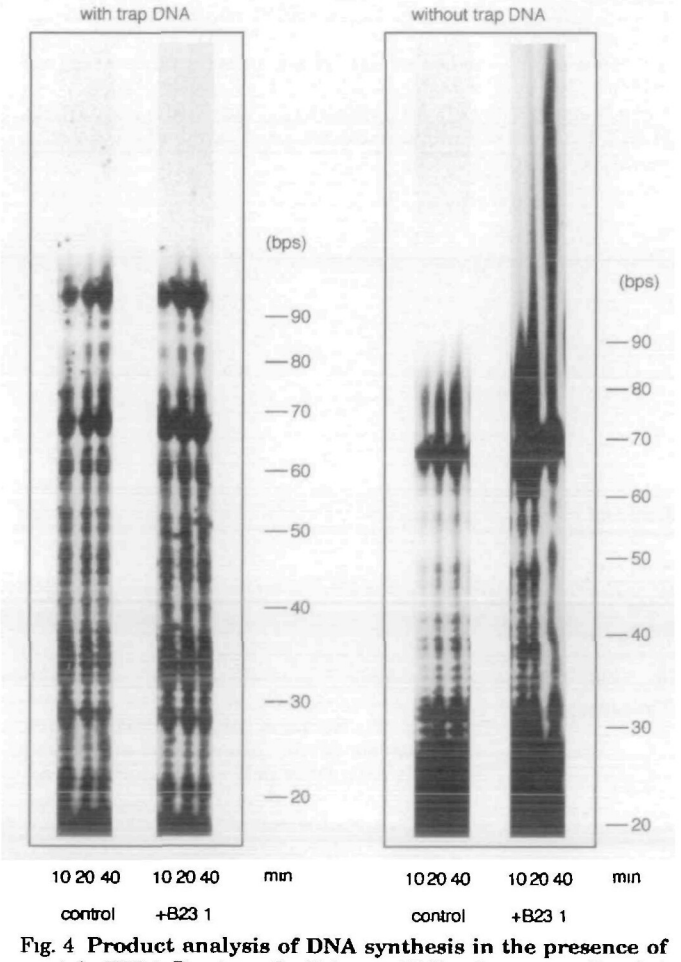

Fig. 4 **Product analysis of DNA synthesis in the presence of protein B23.1.** Reaction of calf thymus DNA polymerase α (2 units) on the singly primed M13 DNA was carried out as described in "MATERIALS AND METHODS" section, in the presence or absence of protein B23.1 (6.25 μg/ml), with (left panel) or without (right panel) excess amount of sonicated calf thymus DNA (1 mg/ml) as a trap. Aliquots of reaction mixture (5 μl) were withdrawn at 10, 20, and 40 min DNA products were then electrophoresed on a sequencing gel followed by autoradiography.

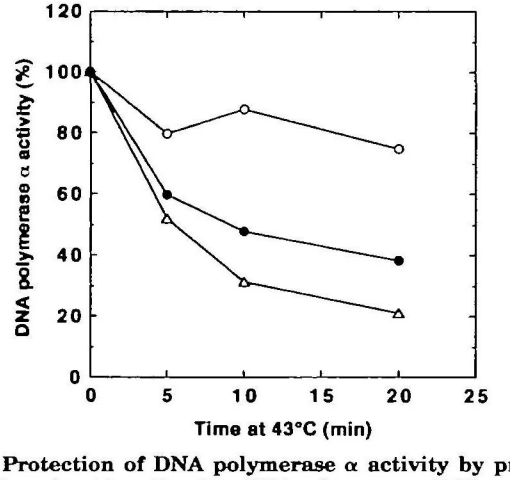

Fig. 5 **Protection of DNA polymerase α activity by protein B23.1 from heat-inactivation.** DNA polymerase α (0 28 unit) was incubated for the indicated times at 43°C with 20 μg/ml of protein B23.1 (○), with 20 μg/ml BSA (●) or without proteins (△) then the remaining activity was assayed as described in "MATERIALS AND METHODS"

lized $(dA)_{100}$. Optimum hybridization was achieved after 17 min by using 10 μg/ml of $oligo(dT)_{12-18}$ at a flow rate of 5 μl/min and the change of the RU was 1367 RU. The hybrid of (dA)·(dT) corresponded to a substrate for DNA polymerase α. Then, DNA polymerase α (0.1 unit) was injected at flow rate of 10 μl/min and was accumulated on the hybrid. The bulk effect and background binding were subtracted from the data. An increase of 180 RU was obtained by the injection of DNA polymerase α The signal caused by DNA polymerase α binding did not decline following flow of running buffer for 30 min, indicating stable association (data not shown). The protein B23 isoform was passed over the DNA polymerase α–(dA)·(dT) complex. The analyte buffer containing no protein B23 was simultaneously injected into a separate flow-cell, and the resulting sensograms were subtracted from the data as background. When the protein B23.1 was injected into the flow cell, an increase in surface plasmon resonance (SPR) signal was observed. After end of injection, a significant amount of protein B23.1 remained bound to the surface, as evidenced by the difference between the sensograms and the base line (Fig. 3A). The SPR signal due to binding of protein B23.1 increased in a dose-dependent manner. The k_{on} and k_{off} rates for protein B23.1 were 9.42×10^4 M^{-1} s^{-1} and 2.30×10^{-3} s^{-1}, respectively. The association constant (K_A) as determined by the kinetic analysis (on and off rates) for B23.1 was 4.10×10^7 M^{-1}. Protein B23.1 did not significantly interact with the hybrid ligand in the absence of DNA polymerase α (Fig. 3D). Thus, protein B23.1 appears to interact directly with the DNA polymerase α protein and not through the bound nucleic acid. On the other hand, the binding of the protein B23 2 to the DNA polymerase α–(dA)·(dT) complex was also observed, but the on and the off rates for the interaction were too fast to be amenable to the kinetic analysis (Fig. 3B) Therefore, the K_A of protein B23.2 (1.10×10^6 M^{-1}) was calculated from measured steady-state binding levels of the analyte. BSA did not interact with the DNA polymerase α–(dA)·(dT) complex (Fig. 3C) We also coupled DNA polymerase α covalently to a CM5 sensor chip. However, we failed to demonstrate the interaction between DNA polymerase α and protein B23 with this system, for reasons unknown.

Stabilization of DNA Polymerase α Activity by Protein B23—The DNA product of the reaction with M13 phage DNA hybridized with synthetic primer was analyzed on a sequencing gel. To confirm that we were observing dNTP incorporation resulting from a single DNA binding of enzyme, we performed DNA synthesis in the presence of an excess amount of nonradiolabeled, sonicated calf thymus DNA as a trap, as described previously (*22*). In the left panel of Fig 4, the reactions were performed by first incubation of DNA polymerase α with M13 singly primed substrate, and then excess amount of sonicated DNA, $MgCl_2$, and four dNTPs were added to initiate the reaction. As a result, protein B23.1 did not increase the size of DNA elongation products by DNA polymerase α in the presence of trap DNA. Protein B23.1 did not directly bind to the trap DNA (activated calf thymus DNA), which was revealed by BIAcore experiment (data not shown) On the other hand, as shown in the right panel of Fig. 4, protein B23.1 stimulated the synthesis of long DNA chains by DNA polymerase α in the absence of trap DNA. A similar result was obtained with protein B23.2, but the degree of elongation was lower (data not shown). These results suggest that the

TABLE I **C-terminal amino acid sequences of residues 256–292 of protein B23 deletion mutants.**

Protein	Amino acid sequence
B23 1	G G S L P K V E A K F I N Y V K N C F R M T D Q E A I Q D L W Q W R K S L
ΔC12	G G S L P K V E A K F I N Y V K N C F R M T D Q E - - - - - - - - - - - -
ΔC22	G G S L P K V E A K F I N Y V -
ΔC27	G G S L P K V E A K -
ΔC32	G G S L P -
ΔC37	- -
B23 2	A H -

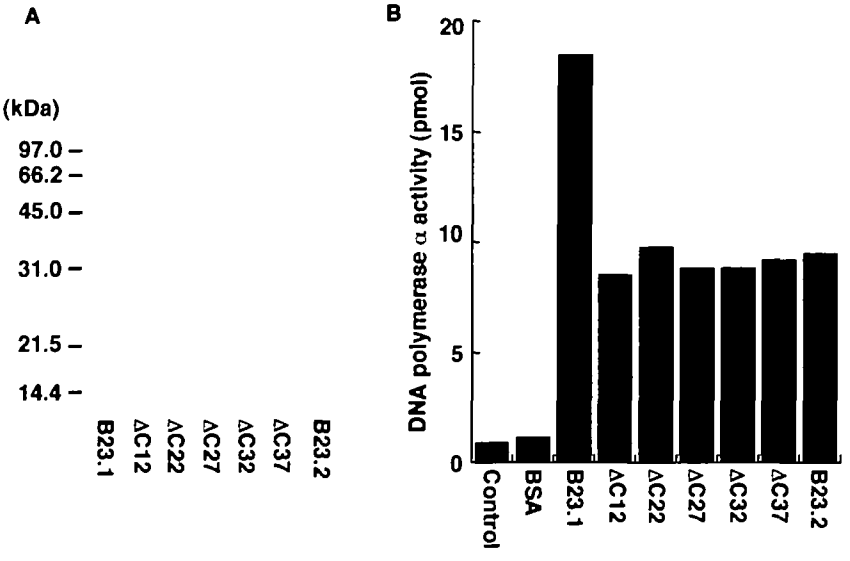

Fig 6 **Effect of B23 deletion mutant proteins on the activity of DNA polymerase α.** A: Electrophoretic analyses of protein B23 deletion mutants (structures, see Table I) Purified His-tagged proteins were run on the 12.5% SDS-PAGE Molecular markers and the different mutant forms of protein B23 were loaded on the lanes indicated B Effect of mutant proteins on the activity of DNA polymerase α Activity of DNA polymerase α (0 48 unit) was measured as described under "MATERIALS AND METHODS" with various mutant proteins (20 μg/ml) as indicated.

DNA polymerase α-stimulatory activity of B23.1 is not due to the raising of its processivity.

Then we measured the stabilizing effect of protein B23 on DNA polymerase α activity. In the buffer alone, DNA polymerase α was rapidly inactivated by heat treatment at 43°C (Fig. 5). Addition of protein B23 greatly protected the enzyme activity from heat inactivation, while the same amount of BSA did not stabilize the activity (Fig. 5). Therefore, the protective effect may be due to specific interaction of protein B23 with DNA polymerase α, and not to a non-specific effect of the elevated protein concentration. These results suggest that the enhanced activity (Fig. 4) is due to stabilization of DNA polymerase α. In the presence of protein B23, the enzyme would be stable even after its dissociation from product DNA and be recruited again on template DNA for further elongation of the product.

C-Terminal Region of Protein B23.1 That Stimulates DNA Polymerase α—To determine the domain involved in the stimulation of DNA polymerase α, various C-terminal deletion mutants of protein B23 1 were examined with respect to the stimulatory effect (Table I and Fig 6). Deletion of the C-terminal 12 amino acids (ΔC12) decreased the stimulating activity of the protein by 2-fold. The mutant proteins lacking longer regions from the C-terminal, ΔC22, ΔC27, and ΔC32 showed almost the same stimulation as ΔC12 (Fig. 6B). The mutant ΔC37, which is the common region of protein B23.1 and B23.2, also showed same stimulation as ΔC12. These observations indicate that the common region of the two isoforms and residues 280–292 are both essential for maximum stimulation of DNA polymerase α by protein B23.1.

DISCUSSION

The study reported here indicated that protein B23 stimulated immunoaffinity-purified calf thymus DNA polymerase α activity through a direct protein–protein interaction We have previously reported that protein B23.1 stimulated DNA polymerase α activity, but protein B23.2, the shorter of the two isoforms, was not capable of accelerating of DNA polymerase α activity (*19*, *25*). However, the previous study indicated that the activity of DNA polymerase α was slightly stimulated by the addition of 10 μg/ml of protein B23.2 (*19*). The effect of the histidine-tagged fusion protein B23.2 on the DNA polymerase α activity was similar to that of the recombinant protein B23.2 without the histidine tag that was produced by pKK223-3 vector at the concentration for 10 μg/ml (data not shown). These observations indicated that the DNA polymerase α activity was not affected by the histidine tag itself. The effects of higher concentration of the two isoforms on the enzyme activity were not examined, because it was difficult to obtain them in large amount by using pKK233-2 protein expression vector. Employing pQE-30, with N-terminal His tag, gave the recombinant protein in high yield. The present study indicated that addition of high concentration of protein B23.2 also enhanced the activity of DNA polymerase α. It is interesting that the maximum stimulation by protein B23.2 was 2-fold lower than that by protein B23.1. The minimal molar ratios of protein B23.1 and B23.2 to the DNA polymerase α required to fully stimulate the activity were 28:1 and 52:1, respectively. It has been reported that protein B23 is predominantly a monomer with small amounts of oligomer in solutions of low ionic strength (*26*) such as the assay conditions for DNA polymerase α. It may be considered that the oligomeric form was active and the monomeric form was inactive or less active in the stimulation of DNA polymerase α activity. On the other hand, protein B23 has been reported to be phosphorylated *in vivo* by CDK2/cyclin E (*7*). Since the bacterially produced protein B23 is unphosphorylated, it is likely that stimulation of the DNA polymerase α is affected by the phosphorylation of protein B23. This possibility is now being pursued in our laboratory.

An earlier study showed that factor T from calf thymus stimulated the activity of immunoaffinity-purified DNA polymerase α and might be lost during the immunoaffinity chromatography (*21*). Protein B23 was also found in partially purified DNA polymerase α fraction by a biochemical method (*27*), and it might be separated from the enzyme by immunoaffinity column chromatography. Thus, protein B23 resembles factor T in its interaction and the stimulation. However, factor T increased the capacity of DNA polymerase α to read through the pausing structure of the template, while protein B23 did not affect the processivity of the enzyme (Fig. 4) and protected the enzyme from heat-inactivation (Fig. 5). Therefore, protein B23 may not be identical to factor T.

BIAcore experiments indicated the dose-dependent association of protein B23.1 with DNA polymerase α (Fig. 3A). Protein B23.1 bound significantly to the DNA polymerase α–(dA)·(dT) complex, but not to (dA)·(dT) alone (Fig 3, A and D). These observations suggest that protein B23.1 acts directly through DNA polymerase α to enhance the DNA synthesis B23 2-immobilized column chromatography (Fig 2B) and BIAcore studies (Fig. 3B) indicated that protein B23.2 has a weak affinity for DNA polymerase α. These observations confirm the stimulatory effect of protein B23.2 on the activity of DNA polymerase α.

It is known that some nucleolar and viral proteins associate with protein B23. The nucleolar proteins C23 (nucleolin) and p120 interacted with protein B23 and the amino acids 194–239 and 186–239 of protein B23 were necessary for binding to C23 and to p120, respectively (*28*). Two highly acidic regions of protein B23 (amino acids 120–132 and 161–188) were acceptor regions of the human T-cell leukemia virus Rex protein (*13*). Moreover, both B23 isoforms interacted with the human immunodeficiency virus Rev protein (*12*). These observations imply that the common region of the isoforms might be involved in the association with these nucleolar and viral proteins. Szebeni and Olson (*29*) suggest that protein B23 has a molecular chaperone function. In this case, both proteins B23.1 and B23.2 inhibited temperature-dependent aggregation of HIV-1 Rev protein, suggesting that the C-terminal region of protein B23.1 did not play a role in this activity (*18*). In contrast, protein B23.1 was shown to have a greater affinity for DNA polymerase α (Figs. 2 and 3). The C-terminal end of protein B23.1 might be important for stronger interaction. ΔC12, the C-terminal deletion mutant of protein B23.1, shows a significant decrease in DNA polymerase α–stimulatory activity (Fig. 6B), suggesting that the C-terminal 12 amino acids are important for the stimulatory activity. However, the involvement of the common region cannot be ruled out because the common region of the two isoforms still possesses 50% of the stimulatory activity of protein B23.1. Thus, protein B23 may exhibit its multiple functions by

interacting with other proteins and enzymes via various domains on the protein molecule.

Borggrefe *et al.* indicated that protein B23 formed a multiprotein complex with nucleolin, poly(ADP-ribose) polymerase, and SWAP-70 which preferentially recombined sequences derived from immunoglobulin heavy chain switch regions (*30*) This observation suggests a possible involvement of protein B23 not only in DNA replication but also in DNA recombination. The biological implication of the polymerase stimulation by protein B23, especially in the nucleolus, must await further study.

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