DNA Binding Properties of Human pol γB*

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We have recently reported the crystal structure of the accessory subunit of mitochondrial DNA polymerase, pol γB, and identified a region of the protein involved in DNA binding. The DNA employed in previous studies was presumed to be single-stranded, because it was generated by single-sided PCR. Further characterization of this DNA indicated that, due to a strand transfer event during synthesis by single-sided PCR, the DNA adopts a double-stranded hairpin conformation under native conditions. We used a series of double- and single-stranded oligonucleotides of different lengths to confirm that human pol γB prefers to bind double-stranded DNA longer than 40 bp with little apparent sequence specificity. Site-specific deletion mutagenesis identified clusters of basic residues in two surface loops required for DNA binding located on opposite sides of the symmetrical pol γB dimer. A heterodimer of pol γB that contains one mutant and one wild-type DNA binding region was shown to be unable to bind double-stranded DNA, suggesting that a single DNA molecule must contact both DNA binding sites in the pol γB dimer. The ability to bind double-stranded DNA is not essential for pol γB stimulation of pol γA activity in vitro, but may play a role in DNA replication or repair.

Mitochondrial DNA is replicated by DNA polymerase γ, an enzyme with a catalytic subunit, polγA, containing both 5′ → 3′ polymerase and 3′ → 5′ exonuclease activities, and an accessory subunit, polγB, that affects a number of key properties of the catalytic subunit (1, 2). polγB is related both in primary sequence and structure to class IIa prokaryotic aminoacyl-tRNA synthetases (aaRSs) (1, 3). Apart from its role in the stimulation of pol γ, pol γB has DNA binding activity that may reflect properties of aaRSs. All aaRSs bind specific RNAs, although one, phenylalanyl-tRNA synthetase, has been shown to bind specifically to double-stranded DNA using an atypical helix-turn-helix domain (4). The so-called b5 domain that mediates this DNA binding is not found in most aaRSs. In preliminary experiments, we found that pol γB was able to bind to a DNA substrate generated by single-sided PCR that was presumed to have a mostly single-stranded conformation (3) similar to the H-strand region that serves as origin for lagging strand mtDNA replication (O1) (5). These observations provided support for models suggesting that the DNA binding ability of pol γB might play a role in initiation of mtDNA replication (6). This sort of model has been suggested for Drosophila pol γ as well (7), although this enzyme appears to have a simple heterodimer structure with extensive contacts between the A and B subunits (8).

In this report we present the results of further experiments to characterize the nucleic acid binding properties of mammalian pol γB, by studying binding to a variety of single-stranded and double-stranded DNAs and by exploring the effects of amino acid changes on nucleic acid binding. The results show that wild-type pol γB binds only to one of two major DNA species generated by single-sided PCR extending through O1, pol γB prefers to bind to an aberrant PCR product that is substantially double-stranded due to a strand transfer event at the hairpin structure at O1. Binding titrations with a variety of single-stranded and double-stranded DNAs of different lengths confirmed that pol γB prefers to bind double-stranded DNA. We further show that clustered point mutations that convert basic residues to alanine residues in two nucleic acid binding loops alter the DNA binding properties of the protein. The pol γB dimer contains two DNA binding sites on opposite sides of the protein. We constructed a pol γB heterodimer containing one mutant and one wild-type DNA binding site and found that this heterodimer was unable to bind double-stranded DNA. Thus, we conclude that both sites are required for high affinity DNA binding, suggesting that an individual DNA molecule must wrap around pol γB to interact simultaneously with both sites.

EXPERIMENTAL PROCEDURES

DNA Clones and Oligonucleotides—The DNA template used for the synthesis of 129-mer and other single-stranded DNAs was first cloned by PCR from HeLa mitochondrial DNA using primers containing XbaI (HOL1) and XhoI (HOL2) restriction sites. This clone, containing human mitochondrial DNA sequences from position 5495 to 5920, spanning O1, was named pJAC64. Sequencing revealed a point mutation, G to A, at position 5773.

Oligonucleotides were obtained from Operon. The sequences of oligonucleotides used to generate fragments of mtDNA for binding assays are as follows: HOL1, 5′-TCTAGATATACATAAATCTTAT-3′ (used with HOL2 to make clone pJAC64); HOL2, 5′-TCTGAGCAACCGTGGC GGAACAT-3′; HOL3, 5′-C CGGCAGTTTTATCAGGATG-3′ (used to make 98-mer and 83-mer); HOL6, 5′-CCCTAGATATACATAAATCTTAT-3′ (used with HOL2 to make a double-stranded 221-bp DNA by PCR that was used as template for synthesis of the 98-mer (the 5′-end of HOL6 matches the 3′-end of 98-mer)); and HOL8, 5′-TCTGAGCAACCGTGGC GGAACAT-3′ (used with HOL2 to make a double-stranded 206-bp DNA by PCR that was used as template to prepare the 98-mer (the 5′-end of HOL8 matches the 3′-end of 98-mer)).

The following forward oligonucleotides (F) were used as single-stranded DNA in EMSA. Each forward oligonucleotide was annealed

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‘ The abbreviations used are: pol, polymerase; ds47, double-stranded 47-mer; aaRS, aminoacyl-tRNA synthetase; mtDNA, mitochondrial DNA; EMSA, electrophoretic mobility shift assay; Ni-NTA, nickel-nitrotoltriacetic acid; CBP, calmodulin binding protein; nt, nucleotide(s); O1, origin for lagging strand mtDNA replication.

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with its corresponding complement (R) to prepare double-stranded oligonucleotides: 32F, 5’-GGCGAGGAGGAGACAAATGTCTGCTTCT-GT-3’; 32R, 5’-ACAAGGAACACCATTTGCTGCATCTGCGC-3’; 40F, 5’-AGAGAATTTTCCCTAAAGAGCAGGACATCTTAACTGCA-3’; 40R, 5’-CTTCTTATGAGATTGCTCTGCTCTGAAGGGATGCTTCT-3’; 47F, 5’-TAACTTAAATGAACATTTTTTGATTCATATAT-ATCATAAGCTA-3’; and 47R, 5’-TAGCTATGATATATATATGCAAAGAGCCGTAATACTATGAT-3’. The 32F and 32R oligonucleotides were also used for site-specific mutagenesis to create mutant P1; the 40F and 40R oligonucleotides were used to create mutant P2. Oligonucleotides used to produce the NotI/Xhol cassette encoding the calmodulin binding protein tag were: CBPF, 5’-ATAAGAATGCGGCCGCAAAGCGACGATGGAAAAAG and CBPR, 5’-ATTCGAGTCTGCTCTGCTCTGAAGGGATGCTTCTC. Labeling, Purification, and Annealing of Oligonucleotides—Oligonucleotides used for PCR and EMSA were gel-purified before labeling and again after labeling as described above. Concentrations of unlabeled oligonucleotides were calculated based on UV absorption. Labeling was carried out with polynucleotide kinase (New England BioLabs) and [γ-32P]ATP, under standard conditions. Fractions of known amounts of labeled oligonucleotides were spotted onto DE-81 paper (Whatman), washed with 250 mM potassium phosphate, and counted in a scintillation counter to determine specific activities. To generate double-stranded oligonucleotides, equal amounts of complementary single-stranded oligonucleotides were mixed in a buffer containing 100 mM NaCl, 10 mM Tris, pH 8.0, 1 mM EDTA, heated at 90 °C, and then cooled slowly to room temperature.

Synthesis of Single-stranded DNA—The general approach used for synthesis of single-stranded DNA by PCR has been described previously (3). To generate the 129-mer, the 228-bp double-stranded DNA used as a template was excised by restriction digestion with enzymes XhoI and Hinfl from clone pJAC64. Primer HOL3 was used in a standard PCR reaction using either Tth DNA polymerase (Fisher) or Pfu Turbo DNA polymerase (Stratagene) with buffers supplied by the manufacturer. To prepare other single-stranded DNA species, the template was obtained by PCR using pJAC64 DNA as template and the primers described above. 25 or 30 cycles were carried out at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 2 min. The first 10–15 cycles were carried out using 100 ng of template DNA, 20 pmol of unlabeled primer, and 5 μCi of [α-32P]dATP. For 5’-end-labeled products, 5 pmol of kinase-modified 32L3 were used in a PCR reaction under the same conditions as above. The PCR products were precipitated with ethanol and collected by centrifugation, and the pellet was resuspended in formamide loading buffer, boiled, and run as 10–12% polyacrylamide–8 M urea sequencing gels. Bands were identified by autoradiography, excised from the gel, and chloro-ethyted by rotating end over end overnight in a buffer containing 0.3 M sodium acetate, 10 mM Tris, pH 8.0, 1 mM EDTA. After brief centrifugation in a microcentrifuge, the supernatant was filtered through a 0.22-μm filter, and the eluate was resuspended in formamide loading buffer, boiled, and run in 8% polyacrylamide, 0.1% bis-acrylamide, 20 mM HEPES, pH 8.0, 0.1 mM EDTA, 0.1% glycerol, 1 mM dithiobismuth, 1 mM EDTA, and 4 mM CaCl2. The homogenate was centrifuged, and the supernatant was incubated with calmodulin affinity resin on a rotator for 2 h at 4 °C. The beads were washed extensively with lysis buffer, and bound protein was step-eluted with the same buffer lacking CaCl2 and MgCl2 but containing 2 mM EGTA. The eluate was concentrated by ultrafiltration using a Centricon 30 and adjusted to 25 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20% glycerol, 2 mM β-mercaptoethanol, and 0.2 mM phenylmethylsulfonyl fluoride. Additional purification by Ni-NTA affinity chromatography was performed as described (6). Quantitation of recombinant proteins was carried out by UV absorbance or by densitometry of Coomassie Blue-stained SDS-PAGE gels using commercial glutamate dehydrogenase as a standard.

Electrophoretic Mobility Shift Assays—Reactions were carried out in 100-μl volumes containing 10 μM Tris, pH 8.5, 2.5 mM dithiothreitol, 1 mM MgCl2, 1 mM EDTA, 150 μg/ml bovine serum albumin, 10% glycerol, and 70 mM NaCl. Each reaction contained a total of 2 μl of either protein or dialysis buffer (6), which supplied the glycerol and 60 mM salt to the reaction. 1 μl of DNA was used, containing 100 μM NaCl, which was responsible for 10 mM salt in the reaction. Reactions were incubated at 30 °C for 10 min and run in native polyacrylamide gels. Gels contained 6% acrylamide, 0.1% bis-acrylaminde, 20 mM HEPES, pH 8.0, 0.1 mM EDTA. Running buffer was 20 mM HEPES, pH 8.0, 0.1 mM EDTA. Gels were pre-run at room temperature for 1 h at 80 V, using the miniprotein II system (Bio-Rad), and run under the same conditions. Gels were dried on DEAE paper (Whatman) and imaged using either Kodak XAR5 film or a phosphorimaging screen (Amersham Biosciences).

Purvis Ladders—30 flod (10,000 cpm) of end-labeled DNA was mixed with 10 μg of tRNA carrier in a total volume of 20 μl. 1 μl of 4% pyridium formate, pH 2, was added, and the mix was incubated at 48 °C for 15 min. Following ethanol precipitation, the DNA was resuspended in 20 μl of water, 80 μl of 10% piperidine was added, and the mixture was incubated at 90 °C for 10 min. After ethanol precipitation, the DNA was resuspended in formamide loading buffer, boiled, and run in 8% polyacrylamide–8 M urea sequencing gels. Gels were fixed in a solution containing 10% acetic acid, 12% methanol and dried on Whatman 3MM paper, and an autoradiogram was obtained.

RESULTS

Single-sided PCR through O1 Produces Two Major DNA Species, Only One of Which Binds Pol γB—We have previously suggested that the relationship of pol γB with some aminoacyl-tRNA synthetases could indicate a role in binding tRNA-like structures present at the mitochondrial origins of replication. To test this model we attempted to synthesize a single-stranded DNA spanning the light strand origin of replication (O1). This heavy-strand DNA fragment was expected to contain the stem-loop structure known to be required for the initiation of light strand synthesis. We chose single-sided PCR as a quick method to generate single-stranded DNA. This method involves the use of PCR to extend an oligonucleotide primer using double-stranded DNA as template, generating a run-off product. The product, labeled by incorporation of radioactive dAMP, KISSSG. Following cleavage of the PCR product with NcoI and XhoI, the resulting fragment was cloned into pET22b (+) vector cut with the same restriction enzymes. The resulting ampicillin-resistant vector was named pET22b (+)/CBP. This vector was digested with Ndel and NolI to permit it to accept Ndel/NolI DNA fragments encoding wild-type human pol γB or the P2 mutant. To permit selection for heterodimeric pol γB, Ndel/NolI cassettes encoding wild-type and P1 mutant human pol γB were inserted into the kanamycin-resistant vector pET22b (+), which supports synthesis of his-tagged proteins. Co-transfection of Escherichia coli BL21(DE3) with HGB P1 in pET29at (+) and HGB P2 in pET22b (+)/CBP and selection for both ampicillin and kanamycin resistance generated a strain capable of expressing both proteins. As a control, the two wild-type HGB clones in both pET22b (+)/CBP and pET29at (+) were also co-expressed. In each case, co-expression was expected to produce three forms of dimeric protein, the His-capped and CBP-tagged homodimers and the heterodimer bearing both His and CBP tags.

Purification of the His-CBP-tagged heterodimers was carried out first on a calmodulin column (Stratagene) and then on Ni-NTA (Qiagen). Bacterial cells were sonicated in lysis buffer containing 50 mM Tris, pH 7.4, 1 mM dithiothreitol, 150 mM NaCl, and 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μM pepstatin, 1 mM imidazole, 1 mM MgCl2, and 4 mM CaCl2. Purification of the His-capped heterodimers was carried out by UV absorbance or by densitometry of Coomassie Blue-stained SDS-PAGE gels using commercial glutamate dehydrogenase as a standard.

DNA Binding by DNA pol γB
was then separated from the template DNA using denaturing electrophoresis in polyacrylamide-urea gels. Two closely migrating species were observed near the position expected for the single-stranded DNA (Fig. 1) in addition to other shorter extension products that appeared to terminate at secondary structures (Fig. 2). Products obtained by single-sided PCR through the OL region are indicated with their one-letter code (H, C, N). O129, H129, H117, and H98 coincide with the minus species mentioned in the text (129(−), 117(−), and 98(−)). The ladder obtained with the 129(−) species interchanged their mobilities with respect to the 129(+) species at the point on, the sequence was divergent (Fig. 3). Equivalent results were obtained with end-labeled 98(+) and 98(−) PCR products, with the 98(+) ladder matching the expected sequence for the 98-mer and the 98(−) one diverging at the same point seen with 129(+) (data not shown).

As shown in Fig. 1, when the 129-mer was synthesized, a significant fraction of PCR products appeared to be aborted at a size around 89 nucleotides (upper asterisks). This is the point at which 129(+) and 129(−) sequences diverge, coinciding with the descending half of the stem at OL in the direction of synthesis. We reasoned that the 3′-region of those aborted molecules should be able to fold in a stem-loop structure, resembling the OL. The 3′-end of this stem could then prime synthesis by the polymerase, extending the size of the double-stranded stem back to the 5′-end of the primer (Fig. 4B). This would generate a large stem structure with a loop corresponding to the OL loop, i.e., a 70-bp stem with a 12-nucleotide loop. In denaturing gels, this DNA species would behave as a single-stranded DNA of 152 nt, which agrees well with the mobility of the 129(+) species seen in Fig. 3. The relative mobility of the hairpin PCR products varied somewhat with the gel temperature and the quantity loaded on gels containing 8 M urea. It is well known that urea is a rather weak denaturing agent that is not able to completely disrupt secondary structures (10). Analysis of the purine ladder generated from the 129(+) species beyond the point of sequence divergence in Fig. 3 showed that it matched the expected sequences for the 152-mer DNA predicted by the mechanism shown in Fig. 4. The 129(+) and 98(+) DNAs would be expected to behave as double-stranded DNAs of approximately half the size under native conditions. This agrees with the faster mobility of the (+) species in the native gels used for EMSA (see Fig. 2B).

We also probed the structure of these PCR products by di-
gestion with the restriction enzyme AluI. This enzyme should produce a labeled 42-nucleotide fragment from 5′-end-labeled 129(+), when analyzed in denaturing gels (Fig. 4B). AluI should not cut the single-stranded 129(−) species. This is exactly what we observed (data not shown). We also confirmed that when internally labeled 129(−) is annealed to a complementary single strand, it can be cut by AluI, generating the expected products shown in Fig. 4A (data not shown).

The reason why the polymerase stalls in the region corresponding to the descending half of the O1 stem loop is not obvious. The polymerase might be expected to stall preferentially when entering the stem structure (assuming such a structure exists during synthesis), but the products seen during synthesis replication to stalling at this site are only minor species. Instead, the polymerase appears to stall and to engage in hairpin replication at the base of the O1 loop. Interestingly, the hairpin product was only observed when the light strand was the template, not when the polymerase was moving in the opposite direction using the heavy strand as template. There may be some unusual structure resulting from the G-rich tract at the base of the O1 loop that facilitates hairpin replication by the polymerase. We have obtained different ratios of 129(+) and 129(−) species using different polymerases (Taq, Pfu) and different concentrations of nucleotides, suggesting that different conditions could produce different amounts of the hairpin species. We tested whether pol γ is similarly prone to hairpin formation at this site but did not find evidence of hairpin products.2 Collectively, these results demonstrated that the reported binding by pol γB to a single-stranded 129-mer (3) represented binding to a mostly double-stranded DNA of 70 bp, with a 12-nt loop at one end.

Pol γB Binding to Synthetic Double-stranded DNA Oligonucleotides—The data presented in Fig. 2B indicate that pol γB binds more tightly to double-stranded (H117(+), H98(+)) than to single-stranded DNA (H117(−), H98(−), L117). To confirm the double-strand DNA binding preference of pol γB, we used a series of complementary oligonucleotides of different lengths. Oligonucleotides of 32, 40, 47, and 65 nt of unrelated sequences were annealed to their complementary oligonucleotides to generate double-stranded DNA or were used alone as single strands. Binding assays proved that pol γB prefers to bind double-stranded instead of single-stranded DNA (Fig. 5). More avid binding is observed with DNAs of 47 bp or larger, which indicates an approximate minimum DNA size requirement for pol γB binding. Also, the fact that pol γB was able to bind a variety of DNA sequences indicates that there is little or no sequence specificity for this reaction, although this aspect has not been studied in detail. To calculate an approximate Kf for double-stranded DNA binding by pol γB, we measured the disappearance of free DNA as the protein concentration was increased.

2 K. G. Pinz, unpublished observation.
creased, because more than one complex can be seen. A binding titration with 1 nM ds47 DNA as shown in Fig. 6 was analyzed as a simple binding 1:1 interaction between the pol H9253B dimer and DNA, provided an apparent Kd of 8.6 ± 1.5 nM.

Identification of Residues in Pol γB Necessary for DNA Binding—We previously used deletion mutagenesis to identify two protein loops in pol γB required for DNA binding (3). We refer to these surface loops as loop I6, between strands 10 and 11, and I7, between strands 13 and 14. These two loops are closely apposed in the dimeric protein structure (3) and contain clusters of basic residues. The corresponding regions in threonyl-tRNA synthetase contribute to the RNA binding site for anticodon recognition (11). To identify residues in these loops necessary for DNA binding by pol γB, we generated alanine replacement mutants. In mutant P1, two basic residues in the I6 loop, R302K303, were replaced with alanines; in mutant P2, three residues in the I7 loop, R337K338K339, were replaced with alanines (see Fig. 7A).

We studied the abilities of the new mutants, HGB P1 and P2, as well as other mutants of pol γB, to bind the double-stranded 47-mer, ds47. Fig. 7B shows the EMSA results obtained with wild-type pol γB, deletion mutants I4, I5, I6, and I7 (described previously (3)) and point mutants, P1 and P2. These results indicate that the basic residues in loops I6 and I7 are required for pol γB binding to double-stranded DNA. The P1 and P2 mutants are able to stimulate pol γA activity in vitro (not shown), as has been shown for the I6 and I7 deletions (3), indicating that pol γB binding to double-stranded DNA is not necessary for stimulation of pol γA activity.

Double-stranded DNA Interacts with pol γB on Two Opposite Sides of the Protein—The results presented above suggest a working model to describe the DNA binding of pol γB whereby an I6 loop from one monomer and an I7 loop from the other
create a binding site for double-stranded DNA. Because pol γB is a dimer, the complex would be expected to contain two potential I6/I7 binding sites on either side of the protein. When either the I6 or I7 loop is mutated, binding sites on both sides in the pol γB dimer are affected. Thus, the foregoing results do not permit us to determine whether one I6/I7 binding site is sufficient for binding to double-stranded DNA.

To test this model directly, we generated a pol γB heterodimer containing a mutated I6 loop (P1) in one monomer and a mutated I7 loop (P2) in the other as described under “Experimental Procedures.” The strategy to accomplish this was to co-express two forms of pol γB with different C-terminal affinity tags in the same E. coli cells, as shown in Fig. 8A. We reasoned that successive chromatography on two different affinity matrices would permit purification of heterodimers containing one monomer with each type of affinity tag. We employed a C-terminal calmodulin binding protein (CBP) tag for this experiment. This is a convenient affinity tag, because the protein can be adsorbed to a calmodulin affinity column in the presence of calcium and desorbed by the replacement of calcium with the chelator EGTA (9). To provide a positive control, wild-type pol γB was cloned in the same two vectors and expressed in bacteria, and the P1/P2 heterodimer was purified by chromatography on two affinity columns as described in the text. A heterodimer containing wild-type pol γB with both tags was prepared as a positive control. B. The ability of pol γB variants to bind 47-mer dsDNA was tested by EMSA. Binding reactions contained 1 nM DNA alone (lane 1) or with 10 nM of His-tagged wild-type pol γB (lane 2) or dual-tagged heterodimer constructs of wild-type pol γB (lane 3) or the P1P2 mutant (lane 4).

**Fig. 8.** Double-stranded DNA binding by pol γB requires two sites on opposite sides of the protein. A, pol γB constructs were prepared containing point mutations P1 (His-tagged (H)) or P2 (CBP-tagged (C)) in loops I6 and I7 required for binding DNA. The proteins were co-expressed in bacteria, and the P1P2 heterodimer was purified by chromatography on two affinity columns as described in the text. A heterodimer containing wild-type pol γB with both tags was prepared as a positive control. B, the ability of pol γB variants to bind 47-mer dsDNA was tested by EMSA. Binding reactions contained 1 nM DNA alone (lane 1) or with 10 nM of His-tagged wild-type pol γB (lane 2) or dual-tagged heterodimer constructs of wild-type pol γB (lane 3) or the P1P2 mutant (lane 4).

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The accessory subunit of DNA pol γ, pol γB, stimulates the activity of the catalytic subunit under physiological buffer conditions (1). The finding that vertebrate pol γB is related to prokaryotic aminoacyl-tRNA synthetases (aaRSs) suggested that the nucleic acid binding properties of a tRNA synthetase might contribute to the function of the accessory factor. Because the sequences surrounding both origins of mtDNA replication have a high potential for forming complex secondary structures, we speculated that pol γB could be involved in the recognition of such structures, directing the polymerase to the origins of replication (6). As a first approach to test this hypothesis we studied pol γB binding to a DNA fragment derived from a region of the mitochondrial genome that contains the light strand origin of replication (O1). We initially documented binding of pol γB to a DNA substrate synthesized by single-sided PCR (3). In this report we show that the PCR product that bound tightly to pol γB in these experiments was, in fact, a largely duplex hairpin generated by fold-back priming during PCR. To confirm the double-stranded DNA binding preference of pol γB, we used a series of oligonucleotides, either in single-stranded or double-stranded form. The data in Figs. 5 and 6 show that pol γB has a poor ability to bind short duplex oligonucleotides but is able to bind with high affinity (Kd of ~8.6 nM) to a 47-mer oligonucleotide. Our measurement of the absolute affinity of this interaction is subject to technical limitations of the EMSA assay, and we are working to develop independent measurements of this affinity using other methods. Lim et al. (12) have previously observed binding of pol γB to a 34:38-mer primer-template, but the lowest protein concentration used in their experiments, 2 pmol in a 20-μl binding reaction, did not permit determination of the Kd for this interaction. Our results suggest that the presence of a 5' overhang in the primer-template used by Lim et al. (1999) probably did not influence the binding, pol γB has shown a similar ability to bind other duplex fragments longer than ds47 (data not shown), suggesting that DNAs must exceed a minimal size between 38 and 47 bp to bind. Pol γB binds very poorly to single-stranded DNA, such that only a few percent of input DNA is bound by 150 nM protein (Fig. 5). To date, we have identified no specific sequences that preferentially bind to pol γB. However, the finding that a single DNA molecule appears to interact with binding sites on both sides of the pol γB dimer suggests that DNA sequences with an intrinsic bend may be bound more avidly.

The binding of pol γB to double-stranded DNA provides a contrast to the binding of folded single-stranded RNA by tRNA synthetases. The affinity of pol γB for double-stranded DNA is much higher than that previously observed for the phenylalanyl-tRNA synthetase from Thermus thermophilus, which has been estimated to have a binding constant of 400 nM. This interaction also requires a longer minimal DNA size of ~80 bp and does not employ the same regions of the protein required for tRNA binding (4, 13). Thus, it appears that there are significant differences between the DNA binding reported for phenylalanyl-tRNA synthetase and that reported here for pol γB.

DNA binding by mammalian pol γB depends on the dimeric structure of the protein and on two superficial loops initially identified by deletion analysis, I6 and I7 (3). The corresponding
regions of threonyl-tRNA synthetase are involved in binding to the anticodon of tRNA, as depicted in Fig. 7. Both loops in pol γB contain basic lysine and arginine residues that appeared to be good candidates to play a role in DNA binding. Site-directed mutagenesis to convert these residues to alanines confirmed this model (Fig. 7). We conclude that the basic residues in the I6/I7 region are essential for the double-stranded DNA binding activity of pol γB. Because we observed that DNA binding requires a rather long segment of DNA, –38–47 bp (Fig. 5), we sought to test the model that a single DNA duplex must interact simultaneously with the I6/I7 loops on both faces of the pol γB dimer protein. We produced a heterodimer containing one pol γB polypeptide with point mutations in I6 and a second with point mutations in I7. The results shown in Fig. 8 revealed that this heterodimer was not able to bind DNA. The use of a dual-tagged control wild-type protein ruled out the trivial possibility that this deficiency was due to the nature of the tags employed in purification. Thus, we conclude that a single DNA molecule must contact basic residues on both sides of pol γB for stable binding.

The structural basis for the action of pol γB on the catalytic subunit is poorly understood. This reflects the fact that the structure of the catalytic subunit has not been determined, and the interactions between the large and small subunits have not been defined precisely. Both the mammalian pol γB, which has a dimeric structure, and its Drosophila homolog, which binds as a monomer to its cognate pol γA, resemble tRNA synthetases. Recently, Drosophila pol γB has been shown to make extensive contacts with the catalytic subunit (8). These extensive contacts may be critically important for the activity of the small subunit as a processivity factor (14). Among processivity factors, the ability of pol γB to bind duplex DNA is unusual, but not unprecedented. The toroidal “sliding clamp” processivity factors like proliferating cell nuclear antigen (PCNA) and E. coli DNA pol III β subunit do not possess intrinsic DNA binding activity and must be loaded onto DNA by additional factors. However, the herpes virus UL42 protein does bind DNA non-specifically with high affinity (15). In this case, the non-specific DNA binding activity of UL42 appears to contribute to the ability of the herpes virus DNA polymerase holoenzyme to conduct a one-dimensional scan along DNA to identify primer-template binding sites (16). Indeed, mutations in UL42 that abrogate non-specific DNA binding also impair the ability of the protein to function as a processivity factor. This provides an interesting contrast to pol γB, where mutants deficient in DNA binding, point mutants P1 and P2 and the related deletion mutants I6 and I7, are not impaired in their ability to stimulate in vitro DNA synthesis by the catalytic subunit on a poly(dA):oligo(dT) template:primer (13) and data not shown). Thus, the role, if any, that is played in mtDNA maintenance by this double-stranded DNA binding of pol γB remains to be established.

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