Mechanism of DNA Binding by the DnaB Helicase of *Escherichia coli*: Analysis of the Roles of Domain γ in DNA Binding[†]

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ABSTRACT: We have analyzed the mechanism of single-stranded DNA (ssDNA) binding mediated by the C-terminal domain γ of the DnaB helicase of *Escherichia coli*. Sequence analysis of this domain indicated a specific basic region, "RSRARR", and a leucine zipper motif that are likely involved in ssDNA binding. We have carried out deletion as well as in vitro mutagenesis of specific amino acid residues in this region in order to determine their function(s) in DNA binding. The functions of the RSRARR domain in DNA binding were analyzed by site-directed mutagenesis. DnaBMut1, with mutations $R_{328}A$ and $R_{329}A$, had a significant decrease in the DNA dependence of ATPase activity and lost its DNA helicase activity completely, indicating the important roles of these residues in DNA binding and helicase activities. DnaBMut2, with mutations $R_{324}A$ and $R_{326}A$, had significantly attenuated DNA binding as well as DNA-dependent ATPase and DNA helicase activities, indicating that these residues also play a role in DNA binding and helicase activities. The role(s) of the leucine zipper dimerization motif was (were) determined by deletion analysis. The DnaBΔ1 mutant with a 55 amino acid C-terminal deletion, which left the leucine zipper and basic DNA binding regions intact, retained DNA binding as well as DNA helicase activities. However, the DnaBΔ2 mutant with a 113 amino acid C-terminal deletion that included the leucine zipper dimerization motif, but not the RSRARR sequence, lost DNA binding, DNA helicase activities, and hexamer formation. The major findings of this study are (i) the leucine zipper dimerization domain, $I_{361}-L_{389}$, is absolutely required for (a) dimerization and (b) ssDNA binding; (ii) the base-rich RSRARR sequence is required for DNA binding; (iii) three regions of domain γ (γI, γII, and γIII) differentially regulate the ATPase activity; (iv) there are likely three ssDNA binding sites per hexamer; and (v) a working model of DNA unwinding by the DnaB hexamer is proposed.

DNA helicases play an important role in the replication of chromosomal DNA. The DnaB helicase is the principal replicative DNA helicase of *Escherichia coli*, although other DNA helicases have been characterized in *E. coli* with diverse functions and mechanisms of action (reviewed in refs *1* and *2*). DnaB helicase participates in the initiation of DNA replication once the *ori*C region of *E. coli* is bound by the DnaA initiator protein (*1*). It continues to act as the lagging strand helicase during the priming and elongation phases of DNA replication, and it migrates on the DNA template with a strict 5′ → 3′ direction (*3*, *4*). DnaB helicase also plays an identical role in the initiation of bacteriophage λ DNA replication (*3*, *4*). DnaB helicase interacts functionally and perhaps structurally with DNA primase, DnaC, and λP proteins (*1*, *3*−*6*). In the replication fork, interaction of DnaB helicase with the τ subunit of the DNA polymerase III holoenzyme leads to highly processive migration of the replication fork (*7*).

The DnaB helicase is a hexamer of 52 kDa[1] identical monomers (*8*, *9*), and electron microscopic studies are suggestive of a doughnut-shaped rosette structure with an internal diameter of ∼40 Å (*10*). Recent studies suggest that the DnaB and T7 helicase hexamers are trimers made up of apparent dimeric units (*10*−*13*). The hexameric structure appears to be a universal feature of all primary replicative DNA helicases (*14*) including T7 gene 4 helicase (*11*, *15*), T4 gene 41 helicase (*16*), SV40 T-antigen (*17*, *18*), and the recently discovered hexameric yeast DNA helicase, HcsA (*19*, *20*). The ATP binding appears to be mediated by a "type I ATP/GTP binding motif" (*21*). Although there are six theoretical ATP/nucleotide triphosphate binding sites, our previous studies using fluorescent ATP analogue binding to DnaB helicase have demonstrated that only three nucleotide molecules appear to bind to the hexamer with high affinity (*22*). This observation was later extended by Bujalowski and

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; ATPase, adenosine 5′-triphosphatase; AMPPNP, 5′-adenylyl imidodiphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-thiogalactoside; kDa, kilodalton; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; ssDNA, single-stranded DNA.

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Table 1: Sequences of Oligonucleotides Used in This Study[a]

oligonucleotide	sequence
1	5′-TCTCATATGGCAGGAAATAAACC-3′
2	5′-CTCGGATCCAGTTCACGAAGACG-3′
3	5′-TCTTCTGGATCCTCAGATCAAGTCCGCATCCTGC-3′
4	5′-TCTTCTGGATCCTCACAGCGAGCGAGAGATTTC TG-3′
5	5′-CGTTCCCGCGCA***GC***C***GC***TATTGCCCGTGAA-3′,
6	5′-TTCACGGGCAATA***GC***G***GC***TGCGCGGGAACG-3′;
7	5′-CTGACGCCAACGGAAGT***AGC***TTC***AGCT***GCACGCCGTATTGCCCGT-3′
8	5′-ACGGGCAATACGGCGTGC***AGCT***GAA***GCT***ACTTCCGTTGGCGTCAG-3′
9	5′-AGCACTGGCGAAAGAA***GCA***AACGTGCCGGTGGTGGC-3′
10	5′-GCCACCACCGGCACGTT***TGC***TTCTTTCGCCAGTGCT-3′
11	5′-AGCACTGGCGAAAGAA***GCA***AACGTGCCGGTGGTGGCA***GCA***TCCCAGTTGAACCGTT-3′
12	5′-AACGGTTCAACTGGGA***TGC***TGCCACCACCGGCACGTT***TGC***TTCTTTCGCCAGTGCT-3′

[a] Mutated codons are shown in italics.

Klonowska (*23*) to include the possibility of three additional low-affinity binding sites per hexamer as a consequence of a negative cooperativity of ATP binding to the hexamer. Using a nitrocellulose filter binding assay, Patel and Hingorani (*24*) demonstrated that the hexameric T7 DNA helicase also binds only three NTP molecules per hexamer, and thus this "half of sites binding" may be a general feature of all hexameric helicases. It appears that there is likely only one physiologically important ATP binding site per dimeric unit in the DnaB hexamer.

We have earlier proposed that a "leucine zipper" domain at the C-terminus plays a role involved in the dimerization of DnaB protein leading to hexamer formation (*12*). Leucine zippers are most well-known in eukaryotic transcription factors such as cFOS, cJUN, GCN4, and C/EBP (*25*−*32*). Leucine zippers are encoded in a small polypeptide segment (∼50 amino acids), and they have dimerization and DNA binding regions at the C- and N-termini, respectively. Normally these two regions are fused together. However, in C/EBP there is a seven amino acid gap between these two regions. DNA binding by the leucine zipper involves, first, dimerization of two leucine zipper motifs of the two proteins bringing the two DNA binding regions in close proximity, followed by binding to DNA by a scissor-grip mechanism (*33*).

The DNA binding domain(s), mechanism of DNA binding, and subsequent unwinding by the DnaB helicase remain unknown. We have analyzed the structural and functional roles of a leucine zipper like motif in the DnaB helicase by a combination of deletion and site-directed mutagenesis studies. On the basis of the findings reported here, we have presented a model for the mechanism of DnaB helicase unwinding of duplex DNA.

MATERIALS AND METHODS

Nucleic Acids, Enzymes, and Other Reagents. The plasmid pKA1 containing the wild-type DnaB gene was a kind gift from Drs. N. Nakayama and K. Arai of Kyoto University, Kyoto, Japan (*34*). Ultrapure deoxynucleotides were obtained from Amersham Pharmacia Biotech (Piscataway, NJ) and were used without further purification. [α-^{32}P]ATP and [γ-^{32}P]ATP were obtained from DuPont NEN (Boston, MA). T4 polynucleotide kinase was obtained from New England Biolabs Inc. (Beverly, MA). Poly(ethylenimine)−cellulose TLC strips were from J. T. Baker Chemical Co. (Pittsburgh, PA). Oligonucleotides (Table 1) were synthesized by Integrated DNA Technologies (Coralville, IO) and were of high purity (≥95%), as determined by autoradiography of the phosphorylated products.

Buffers. Buffer A contained 25 mM Tris-HCl (pH 7.9), 10% sucrose, and 0.25 M NaCl. Buffer B contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 1 mM DTT, and NaCl as indicated. Buffer C was analogous to buffer B, except that it contained 25 mM Hepes (pH 7.5) instead of Tris-HCl. Buffer D contained 25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/mL BSA, and 5 mM DTT. 1× TBE buffer was 89 mM Tris−borate and 2.5 mM EDTA (pH 8.3). Low ionic strength gel loading buffer contained 25 mM Hepes, pH 7.5, 50 mM KCl, 10% glycerol, 0.1 mM EDTA, and 0.5 mM DTT.

In Vitro Site-Directed Mutagenesis of the DnaB Gene. All site-directed mutageneses were carried out using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) that employs higher fidelity *Pfu* DNA polymerase. The DnaB helicase expression vector pET29bDnaB was used as template; there were 12 cycles of PCR, and each cycle was 30 s at 95 °C, 30 s at 50 °C, and 15 min at 68 °C using complementary oligonucleotides 5 and 6 (Table 1) as mutagenic primers for DnaBMut1, oligonucleotides 7 and 8 (Table 1) as mutagenic primers for DnaBMut2, oligonucleotides 9 and 10 (Table 1) as mutagenic primers for DnaBMut3, and oligonucleotides 11 and 12 (Table 1) as mutagenic primers for DnaBMut4, respectively. The mutants were selected by sequencing the *Nco*I/*Bam*HI region. The *Nco*I/*Bam*HI fragment of the mutants was subcloned in the pET29bDnaB in the same sites to generate the final mutants for further studies. The resultant plasmids were introduced in BL21(DE3) cells for expression of DnaB helicase mutants. The expression was as described (*35*).

DNA Sequencing. Determination of the DNA sequence of the mutant DnaB genes described in this study was carried out using the Sequanase kit (U.S. Biochemicals, Cleveland, OH) which employs a thermal cycler based dideoxy sequencing method (*36*) and a modified T7 DNA polymerase.

Construction of Deletion Clones. The deletion clones for DnaBΔ1 (Scheme 2) and DnaBΔ2 (Scheme 2) were generated by PCR amplification with oligonucleotide primers. DnaBΔ1 was constructed by PCR using oligonucleotides 1 and 3 (Table 1) as left and right primers, respectively. DnaBΔ2 was constructed by PCR using oligonucleotides 1 and 4 (Table 1) as left and right primers, respectively. In either case, a B*am*HI site was incorporated after the stop

codon in the right primer and an *Nde*I site in the left primer. The PCR-amplified DNA fragments were subcloned in pET29B expression vector as described above. The plasmid pET29bDnaBΔ2 was digested with *Nde*I and *Spl*I, and a wild-type *Nde*I and *Spl*I fragment was incorporated to remove any mutation arising from PCR. The plasmid pET29bDnaBΔ2 was digested with *Nde*I and *Bcl*I, and a wild-type *Nde*I and B*cl*I fragment was incorporated to remove any mutation arising from PCR.

Purification of Wild-Type DnaB and Its Mutants. The extraction and all purification steps were carried out at 4 °C as described (*35*). Extracts were prepared by sonication in buffer A containing 0.5 M NaCl, and the lysates were then centrifuged at 40000*g* for 30 min. Protein in the supernatant (fraction I) was precipitated with 0.18 g/mL $(NH_4)_2SO_4$. After centrifugation at 40000*g*, the resultant protein pellet was resuspended in buffer B containing 0.1 M NaCl (B100) and then loaded on a high-performance Q-Sepharose column equilibrated with buffer B containing 100 mM NaCl (A100). The column was washed with buffers B100 and B300. The column was then eluted isocratically with buffers B350 and B400, and fractions were collected. The fractions were analyzed for protein and by SDS−PAGE. DnaBmut1 and wtDnaB proteins eluted in the B400 fractions, and DnaBMut2 eluted in the B350 fractions. These proteins were further purified by SE-HPLC using a Bio-Rad TSK3000SW gel filtration column in buffer C150. The peak fractions were pooled and used for further studies.

ATPase and Helicase Assays. These assays were performed as described previously (*35*).

Mobility Shift DNA Binding Assays. To assess the DNA binding of DnaB and its mutant proteins, gel retardation assays that measure the mobility shift of an enzyme-bound versus a free 5′-P^{32} labeled oligonucleotide were carried out. Binding reactions were carried out in a 25 μL final volume of buffer D containing 10 mM $MgCl_2$, 200 pg of 5′-P^{32} labeled oligo$(dT)_{50}$, 3.4 mM ATP, and protein as indicated. The binding reactions were allowed to proceed for 10 min at room temperature, after which time 2 μL of 0.1% bromophenol blue in low ionic strength loading buffer was added. A fraction (85%) of the reaction mixtures was immediately loaded onto a 4−8% polyacrylamide gel containing 1× TBE. Electrophoresis was carried out to separate enzyme-bound oligonucleotides from free oligonucleotides at 30 mA for 1 h at ambient temperature in 1× TBE buffer. After electrophoresis, the gel was dried and autoradiography was carried out using Fuji-RX film at −80 °C for 12 h. Quantitation was carried out by scintillation counting of the corresponding excised enzyme-bound and free oligonucleotide regions of the gel.

RESULTS

We have described three distinct functional domains of the DnaB helicase: α, β, and γ (*35*). These studies indicated that the DNA binding site is located in the C-terminal domain γ. In addition, a leucine zipper motif that we identified earlier in DnaB helicase is also located in domain γ. We have carried out selective deletions of this region and *in vitro* mutagenesis of specific amino acid residues of the putative DNA binding site to further elucidate the mechanism of DNA binding.

Scheme 1: Alignment of the DnaB Leucine Zipper Region with cFOS and cJUN Leucine Zipper Sequences

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..BB.B.B....B.B.BB......L......L......L......L......L. Consensus
IRRRERNKMAAAKCRNRRRELTDTLQAETDQLEDEKSALQTEIANLLKEKEKLE   cFOS
.RKRMRNRIAASKCRKRKLERIARLEEKVKTLKAQNSELASTANMLREQVAQLK   cJUN
...SSGLTPTEVRSRARR......IAEISRSLKALAKELNVPVVALSQLNRSLE   DnaB
DNA Binding Region      Zipper Region
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In Vitro Mutagenesis of the DNA Binding Domain of DnaB Helicase. Classic eukaryotic leucine zipper proteins, such as cFOS and cJUN transcription factors (*25*−*32*), have a basic DNA binding motif located at the N-terminal of the dimerization domain as shown in Scheme 1. The consensus DNA binding motif is bipartite with one-half containing BBXBXB (where B represents Arg or Lys and X, any amino acid) and the other half containing an inverted repeat (BXBXBB) with the two regions separated by a four amino acid spacer (*27*). X-ray crystallographic studies of the yeast GCN4 leucine zipper by O'Shea et al. (*31*, *32*) demonstrated that a rigid Y-shaped structure of the leucine zipper domain dimer binds the DNA double helix through its BBXBXB and BXBXBB peptide domains. In the case of the DnaB helicase, only one-half of the leucine zipper consensus DNA binding motif, the RSRARR sequence (Scheme 1), is present at the N-terminus of the leucine zipper and is separated by 31 amino acid residues. The DnaB helicase is a hexamer which appears to be composed of three dimeric units, and in each unit, the two subunits could likely be held together by leucine zipper dimerization (*10*, *12*).

We wanted to determine unequivocally the role(s) of the putative DNA binding motif, RSRARR, and the dimerization domain of the proposed leucine zipper in the structure and function of the DnaB helicase. Therefore, we have carried out oligonucleotide-based site-directed mutagenesis of key amino acid residues in the leucine zipper motif as shown in Figure 1A. The mutations were carried out using high-fidelity *Pfu* DNA polymerase to minimize the risk of unwanted mutations. In addition, a 510 bp 3′-fragment (*Nco*I/*Bam*HI) was excised from the resultant mutant and subcloned into an expression vector containing the wild-type DnaB (wtDnaB) gene. The nucleotide sequence and the corresponding gel picture of each of the mutated regions in the mutants, DnaBMut1, DnaBMut2, and DnaBMut3, are shown in Figure 1B.

We specifically mutated the arginine residues in the putative DNA binding motif (RSRARR) or the leucine residues in the dimerization motif ($IX_6LX_6LX_6LX_6L$; Scheme 1 and Figure 1A). DnaBMut1 was mutated as follows: Arg-328 and Arg-329 to Ala-328 and Ala-329. DnaBMut2 was mutated as follows: Arg-324 and Arg-326 to Ala-324 and Ala-326. DnaBMut3 was mutated at Leu-374 to Ala-374, and DnaBMut4 was mutated as follows: Leu-374 and Leu-381 to Ala-374 and Ala-381. Each of these mutants was expressed in *E. coli* and purified to homogeneity (>99%) before the assays were carried out. An SDS−PAGE analysis of the purified proteins used in this study is shown in Figure 2. DnaBMut4 protein was insoluble, and no further analysis could be carried out. The mutation of the leucine zipper as in DnaBMut3 and DnaBMut4 appeared to invariably result in lower solubility due to protein aggregation similar to that observed by San Martin et al. (*10*). Structural and functional analysis of the soluble mutants was carried out as described below with purified proteins.

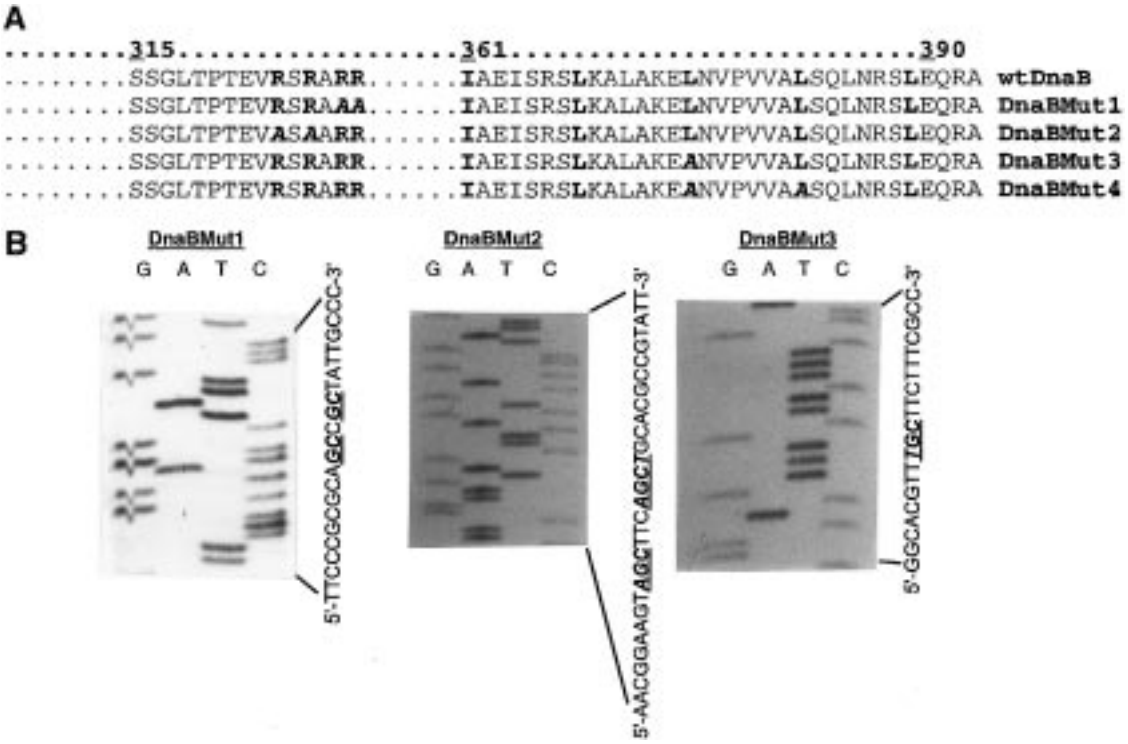


FIGURE 1: Site-directed mutagenesis of the putative leucine zipper domain of DnaB helicase. (A) The amino acid residues that are likely involved in dimerization and/or DNA binding are shown in bold. The residues that are mutated in each of the mutants are shown in italics. (B) DNA sequence analysis of the region of mutagenesis for each mutant gene is presented. The sense-strand sequence of DnaBMut1 and DnaBMut2 and the opposite-strand sequence of DnaBMut3 are shown.

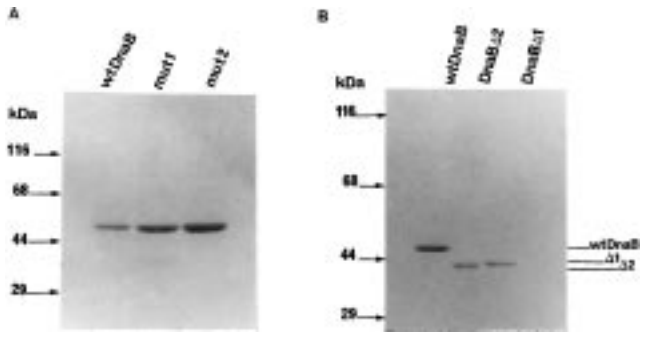


FIGURE 2: SDS−PAGE analysis of proteins. Following overexpression of the various mutants, the proteins were purified as described in Materials and Methods. Aliquots of the indicated protein (~4 μg) samples were analyzed by 10−18% SDS−PAGE, and following electrophoresis the proteins were visualized by Coomassie blue R-250 staining. The molecular weights of the protein standards are as indicated. (A) Analysis of wtDnaB, DnaBMut1, and DnaBMut 2 proteins. (B) Analysis of wtDnaB, DnaBΔ1, and DnaBΔ2 mutant proteins.

DNA-Dependent ATPase Activities of the Mutant DnaB Proteins. DnaB protein has a potent ATPase activity that is absolutely required for its helicase function. One of the most important features of its ATPase activity is its strong dependence on binding to DNA, particularly ssDNA (*34*, *37*, *38*). We have analyzed the DNA-dependent ATPase activity and determined the K_d for DNA binding.

The ATPase activity of wtDnaB helicase was stimulated approximately 20-fold by DNA (Figure 3A). This observation is consistent with results of past studies (*34*). In contrast, highly purified DnaBMut1 protein displayed a comparable or slightly higher ($V_{max} = 6.8 \times 10^5$ pmol min^{-1} mg^{-1}) level of ATPase activity in the absence of DNA (Figure 3B, Table 2), but the stimulation with ssDNA was significantly attenuated ($V_{max} = 9.4 \times 10^5$ pmol min^{-1} mg^{-1}). In the linear protein concentration range, the stimulation was insignificant (Figure 3B) when compared to that observed with wtDnaB protein. Thus, mutations in R328 and R329 essentially abolished the DNA binding and DNA-dependent ATPase activity, indicating important roles for these two amino acid residues in DNA binding. DnaBMut2 protein displayed low ATPase activity, in the absence of ssDNA, similar to that observed with wtDnaB protein (Figure 3A, C). The ATPase activity was stimulated ~2-fold by ssDNA (Figure 3C). The V_{max} were 2.4×10^5 and 5.3×10^5 pmol min^{-1} mg^{-1} in the absence and presence of ssDNA, respectively. Consequently, these mutations appeared to reduce the DNA stimulation of ATPase activity of DnaBMut2 significantly, but they did not eliminate it. The data suggest that although this mutant interacted with DNA, the interaction was strongly attenuated, pointing to a significant role of the R324 and R326 residues in the DNA−protein interaction.

The results obtained with the DnaBMut1 and DnaBMut2 mutants clearly indicated that the RSRARR motif of the DnaB helicase is essential for DNA binding.

DNA Binding by Mutant DnaB Proteins As Measured by Mobility Shift DNA Binding Assay. We have assessed the DNA binding properties of wtDnaB and mutant DnaB proteins directly by mobility shift DNA binding (gel shift) assays using 5′-^{32}P labeled oligo(dT)$_{50}$. The gel shift assay provides a direct demonstration of ssDNA binding as shown in Figure 4. DNA binding by wtDnaB and mutant DnaB proteins was measured, and a comparison of the DNA binding of these proteins is presented in Figure 4. In each case, increasing amounts of purified protein, which ranged between 50 and 500 ng, were added to the binding reaction. For example, in the case of wtDnaB, the amount of the

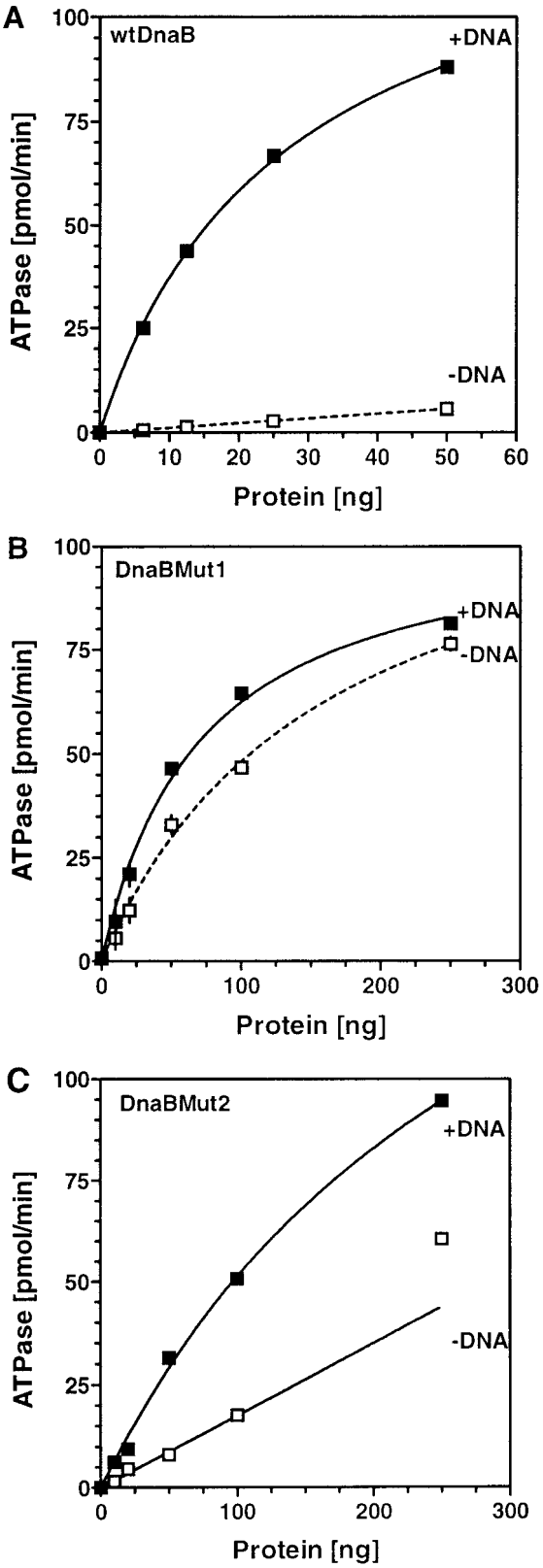


FIGURE 3: DNA-dependent ATPase activities of DnaB protein and its mutants. (A−C) Titration of purified DnaB proteins, 0−250 ng, in a standard ATPase assay in the presence (■) and absence (□) of 200 pmol of M13mp19 ssDNA: (A) wtDnaB protein; (B) DnaBMut1 protein; and (C) DnaBMut2 protein. All of the reactions were carried out at 37 °C for 15 min.

protein−DNA complex increased with increasing amounts of protein (lanes 2−5 of Figure 4) and saturated at amounts greater than 500 ng (data not shown). The DNA binding

Table 2: Comparison of ATPase Activities of DnaB Protein and Mutants in the Presence and Absence of DNA

	−DNA		+DNA		
DnaB	V_{max}, pmol min^{-1} mg^{-1}	K_m, μM	V_{max}, pmol min^{-1} mg^{-1}	K_m, μM	K_d, μM
wtDnaB	3.7×10^5	20	6.4×10^6	80	0.02
DnaBMut1	9.8×10^5	110	9.4×10^5	100	ND[a]
DnaBMut2	2.4×10^6	80	5.3×10^6	150	1.2
DnaBΔ1	1.3×10^4	29	1.1×10^6	79	0.04
DnaBΔ2	4.1×10^4	140	4.1×10^4	140	ND
DnaBαβ	3.2×10^4	75			

[a] ND: not determined.

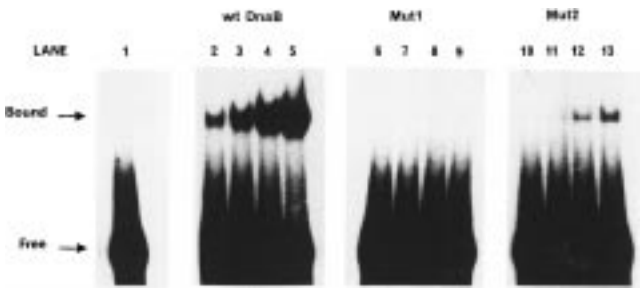


FIGURE 4: Analysis of DNA binding activities of DnaB protein and its mutants. Mobility shift DNA binding (gel shift) assays were carried out as described in Materials and Methods using purified mutant or wild-type protein as indicated and 5′-^{32}P end-labeled oligo(dT)$_{50}$. Lanes: 1, probe alone; 2−5, wtDnaB, 50, 100, 250, and 500 ng, respectively; 6−9, DnaBMut1, 50, 100, 250, and 500 ng, respectively; 10−13, DnaBMut2, 50, 100, 250, and 500 ng, respectively.

ability of DnaBMut2 protein was significantly reduced (~8-fold) when compared to wtDnaB. No significant DNA binding was observed with DnaBMut1 protein. The DNA binding results were consistent with those obtained in comparisons of DNA-dependent ATPase and DNA helicase activities. Thus, in the case of DNA-dependent ATPase activity, we found a 20-fold stimulation of the activity for wtDnaB, and a less than 2-fold stimulation was observed with DnaBMut2 protein (Figure 3). The ATPase activity of DnaBMut1 protein was virtually DNA independent and displayed a lack of significant stimulation in the presence of DNA (Figure 3), which can be attributed to a lack of detectable DNA binding as demonstrated here (Figure 4).

Analysis of the Helicase Activities of DnaB Helicase Mutants. The helicase activities of various mutants were analyzed using a standard DNA helicase assay and were compared with wtDnaB protein. In each case, the wild-type and the mutant proteins were titrated in the range of 25−400 ng per assay (Figure 5). For the DNA binding domain mutant, DnaBMut1, where Arg-328 and Arg-329 were both mutated, DNA helicase activity was not detectable (Figure 5A). Even at the highest concentration of protein used in our study, we did not observe any measurable helicase activity. However, the helicase activity of DnaBMut2, where Arg-324 and Arg-326 were mutated, was observed but was significantly reduced when compared to wtDnaB helicase (Figure 5B). A comparison of the time course of DNA helicase activities of DnaBMut2 and wtDnaB proteins is shown in Figure 5C. The extent of DNA unwinding was significantly reduced in the case of DnaBMut2 protein. Consequently, the mutations in Arg-324 and Arg-326 did not eliminate the DNA helicase activity altogether; rather,

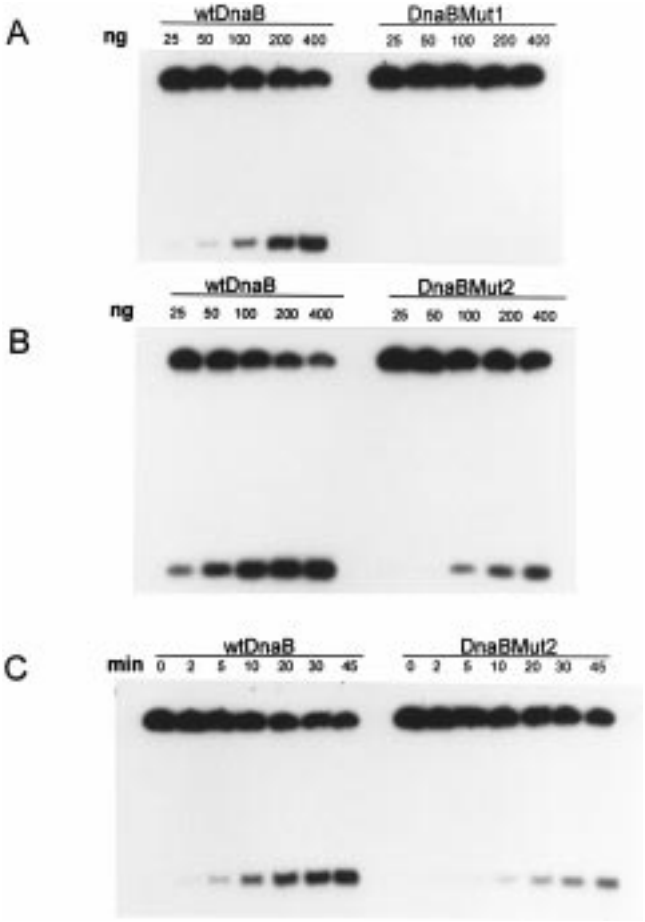


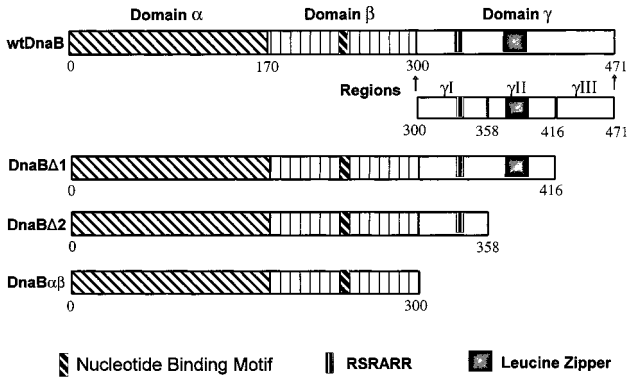
FIGURE 5: DNA helicase activities of DnaB protein and its mutants. (A, B) Titrations of purified DnaB and mutant proteins were carried in the protein concentration range of 25−400 ng in a standard DNA helicase assay (see Materials and Methods). The reactions were carried out for 30 min at 30 °C. In each section DNA helicase activities of both wild-type (left panels) and the mutant DnaB proteins (right panels) are directly compared under identical conditions: (A) DnaBMut1 protein; (B) DnaBMut2 protein. (C) Time course analyses of helicase activities of wtDnaB and DnaBMut2 proteins were carried out for the period 0−45 min using 200 ng of the purified proteins at 30 °C.

they resulted in a significant lowering of unwinding of duplex DNA.

Oligomeric Structures of Mutants of DnaB Helicase. Both DnaBMut1 and DnaBMut2 proteins retained the same hexameric structure as wtDnaB protein as determined by size-exclusion HPLC (SE-HPLC). It should be noted that the SE-HPLC analysis of DnaBMut3 protein indicated that this mutant formed aggregates (data not shown). It is perhaps possible that, in the absence of the leucine zipper domain, the protein cannot form a hexamer and as a result it is forced into nonsense aggregation which leads to insolubility. Earlier, San Martin et al. (*10*) reported that the leucine zipper mutants containing three Leu → Val mutations at residues 367, 374, and 381 also displayed limited solubility and formed dimers but not hexamers by electron microscopic analysis.

Deletion Analysis of the Dimerization Motif of the Leucine Zipper. It is clear from studies presented here and those of San Martin et al. (*10*) that site-specific mutagenesis of the leucine zipper dimerization motif itself leads to lower solubility and aggregation, making further analysis difficult. Consequently, we prepared a series of deletions in domain

Scheme 2: Structure of the Deletion Clones and Regions of Domain γ



γ that would allow unequivocal assessment of the role of the leucine zipper part of this domain in the DnaB helicase mechanism. On the basis of the amino acid sequence and structural motifs, we have divided domain γ into three regions: γI, amino acids 300−358; γII, amino acids 357−416; and γIII, amino acids 417−471. The approximate boundaries of these three regions uniquely correspond to endogenous restriction sites in the DnaB gene: γI, *Nco*I to *Spl*I; γII, *Spl*I to *Bcl*I; and γIII, *Bcl*I to *Bam*HI. It should be noted that *Bcl*I is not a unique site, as another *Bcl*I site exists at the 5′ terminus of the gene, and thus, the *Bcl*I site utilized here is the 3′-*Bcl*I site. All subcloning involving the *Bcl*I site was done by careful partial digestion.

As shown in Scheme 2, the DnaBΔ1 mutant contained a deletion of 55 C-terminal amino acid residues, yet the leucine zipper motif and basic DNA binding domain remained intact. The DnaBΔ2 mutant contained a deletion of 113 C-terminal residues and the zipper motif was removed, but the basic DNA binding motif was still present. The DnaB$\alpha\beta$ mutant contained a deletion of 171 C-terminal amino acid residues. In this case, the RSRARR and the zipper motifs were eliminated as described in the previous paper. These three deletions were designed to carry out a complete evaluation of the role(s) of RSRARR and the leucine zipper motif in DnaB helicase. Each of these deletion mutants was expressed in *E. coli* and purified to homogeneity (Figure 2B). Gel filtration (SEHPLC) studies indicated that the DnaBΔ2 mutant was a dimer similar to that observed with DnaB$\alpha\beta$ protein (*35*) whereas the DnaBΔ1 mutant protein formed a broad peak, indicating the formation and equilibrium of a number of associated states including dimer and hexamer (data not shown). Thus, the zipper motif is involved in the protein−protein interaction leading to hexamer formation. It should be mentioned that the deleted C-terminal end is likely to be required for absolute stability of the hexamer.

DNA-Dependent ATPase Activities of the Deletion Mutants. We have shown in the accompanying paper that the ATPase active site is contained within domain β, and its activity appears to be regulated by domain γ in terms of the rate of ATP hydrolysis as well as DNA stimulation. The ATPase activities and the DNA dependence of the deletion mutants, DnaBΔ1 and DnaBΔ2, were analyzed to determine the role(s) of different elements of domain γ in ATP hydrolysis. The ATPase activities of DnaBΔ1 (panels A and B) and DnaBΔ2 (panel C) are shown in Figure 6. The kinetic parameters of ATPase activities of wild-type DnaB and

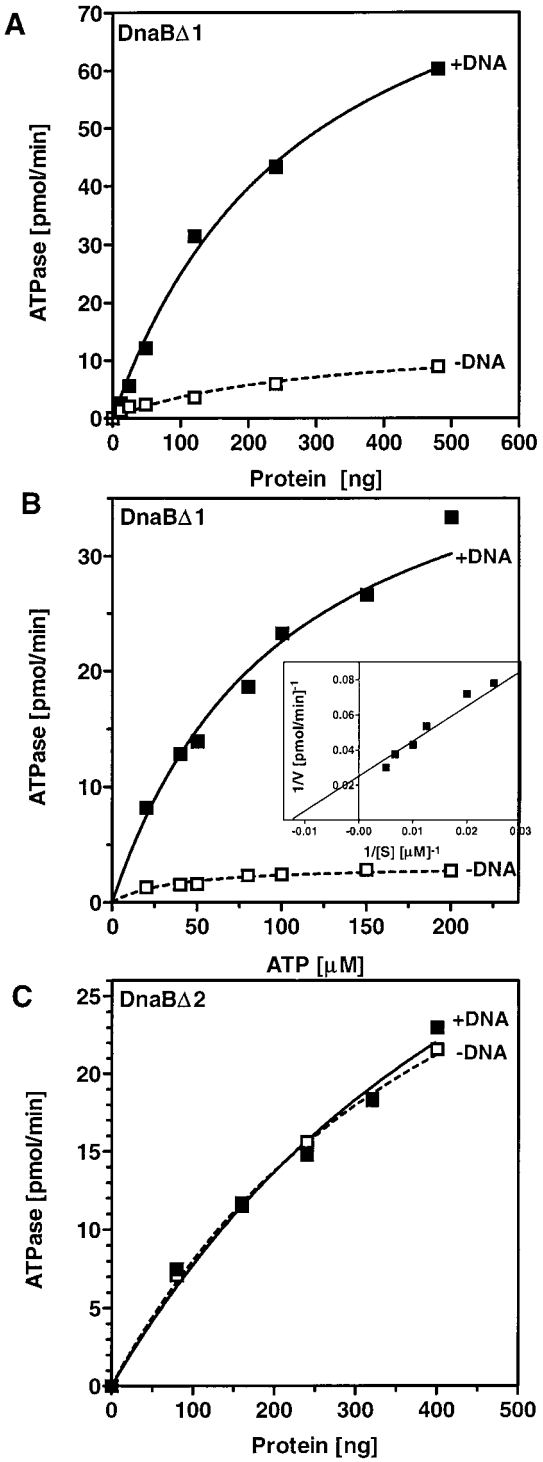


FIGURE 6: DNA-dependent ATPase activities of the DnaB deletion mutants. (A) Protein titration of DnaBΔ1 in the presence and absence of 200 pmol of M13mp19 DNA. (B) Plot of the ATPase for DnaBΔ1, *V* vs [S], demonstrating the difference in the influence of substrate concentration on the initial rate of ATP hydrolysis in the presence and absence of ssDNA. The corresponding Lineweaver−Burk plot in the presence of ssDNA is shown as an inset. (C) Protein titration of DnaBΔ2 in the presence and absence of 200 pmol of M13mp19 DNA.

deletion mutants are presented in Table 2. The ATPase activity of DnaBΔ1 was DNA dependent (Figure 6A). The K_m values for ATP hydrolysis in the presence and absence of ssDNA for DnaBΔ1 were comparable to that observed with wtDnaB protein (Figure 6B, Table 2). On the other hand, the ATPase activity of DnaBΔ2 was absolutely DNA independent (Figure 6C) and was comparable in terms of K_m and V_{max} to that observed with the DnaBαβ or the DnaBβ proteins (Table 2). Thus, the presence of the RSRARR motif and region γI in the absence of the zipper motif (region γII) did not facilitate DNA binding. In addition, this portion of domain γ did not appear to influence the ATPase activity of the domain β either. However, the differences in the ATPase activities of DnaBΔ2 ($V_{max} = 4.1 \times 10^4$ pmol min^{-1} mg^{-1}) and DnaBΔ1 ($V_{max} = 1.1 \times 10^6$ pmol min^{-1} mg^{-1}) proteins in the presence of DNA were striking. It should be noted that the structural difference between these two deletions was only 58 amino acids (region γII), comprising the zipper domain only. The ATPase activity of DnaBΔ1 was absolutely ssDNA dependent (Table 2, Figure 6A). The rate (V_{max}) of ATPase activity was comparable to that of the wild-type protein except that the rate of ATP hydrolysis was lower in both the presence and absence of DNA. This difference in the rate of ATP hydrolysis may be due to the deletion of the C-terminal 55 amino acid γIII region in DnaBΔ1. In the accompanying paper, we have demonstrated that the role of domain γ in the stimulation of ATPase activity is two-fold. The studies presented here indicated that region γIII is likely involved in the stimulation of the basal ATPase activity by domain β. Region γII in conjunction with region γI appeared to be involved in the DNA-dependent stimulation of the ATPase activity. Region γI alone did not appear to stimulate the ATPase activity. Together, these data appeared to explain well the overall regulation of ATPase activity by domain γ.

The titration of ssDNA in a standard ATPase assay was carried out to determine the DNA concentration required for attaining 50% of V_{max} or an effective K_d for ssDNA binding. The K_d values for wtDnaB, DnaBΔ1, and DnaBMut2 were as follows: 0.02, 0.04, and 1.2 μM respectively (Table 2). The K_d of DnaBMut2 appeared to be significantly different from the wtDnaB and DnaBΔ1. It should be pointed out that K_d for ssDNA binding can also be determined from Scatchard analysis of gel shift data. However, such analyses cannot be carried out in the presence of the native substrate ATP because the steady-state level of the DnaB·ssDNA complex is very low due to rapid conversion of ATP to ADP. Therefore, it must be carried out in the presence of a nonhydrolyzable analogue such as AMPPNP which leads to a dead-end complex of DnaB·ssDNA·AMPPNP. The dissociation constant of this complex could be significantly different from the true K_d. In addition, the measurements are normally carried out at lower than optimal temperature (37 °C) in order to have a more stable complex. As a result, the K_d of ssDNA binding determined from the ATPase analysis is far more realistic than various DNA binding assay techniques and Scatchard analysis. However, the DNA binding assay can be used to determine the maximum number of binding sites per hexamer for ssDNA under the experimental conditions as described below, and this parameter cannot be obtained by other means.

DNA Binding and DNA Helicase Activities of the Deletion Mutants. On the basis of the findings of ATPase activity studies as described above, we have examined the ssDNA binding by gel shift and DNA helicase assay. The results of gel shift experiments are presented in Figure 7. It is clear that the DnaBΔ2 mutant protein at various protein concentrations did not have any detectable ssDNA binding activity (lanes 7−11, Figure 7). In contrast, the DnaBΔ1 mutant

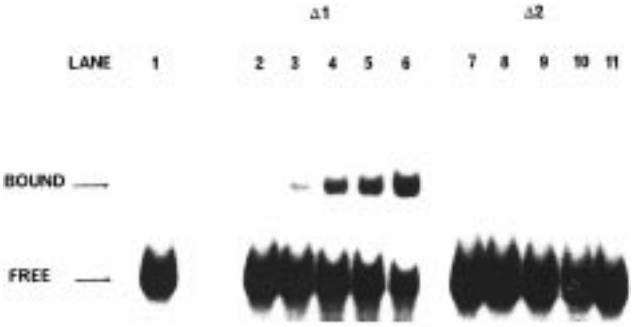


FIGURE 7: Analysis of DNA binding by the DnaB and its deletion mutants by gel shift assay. Details of the assay procedure is given in Figure 4. Protein titrations of oligo(dT)$_{50}$ ssDNA binding by DnaBΔ1 and DnaBΔ2 were carried out in a standard assay. Lanes: 1, no enzyme; 2−6, DnaBΔ1, 0.15, 0.3, 0.48, 0.64, and 0.8 pmol, respectively; 7−11, DnaBΔ2, 0.15, 0.3, 0.48, 0.64, and 0.8 pmol, respectively. Bound and free refer to the enzyme·oligo-(dT)$_{50}$ complex and free oligo(dT)$_{50}$, respectively.

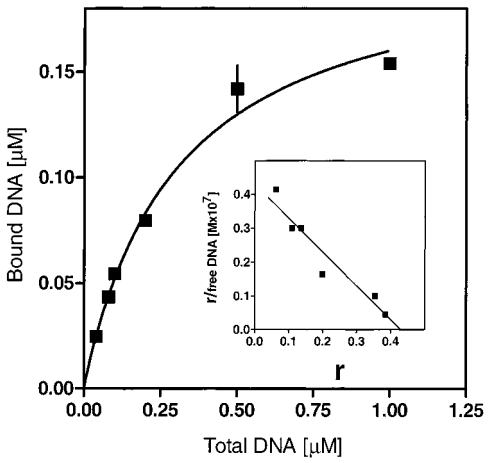


FIGURE 8: Scatchard analysis of oligo(dT)$_{50}$ binding by DnaB protein. DNA binding over a range of 0.4−10 pmol of oligo(dT)$_{50}$ was examined. The binding was quantitated by scintillation counting of excised bands corresponding to bound and free probe. A plot of bound versus free is presented. The *r* and *n* denote the average number of oligo(dT)$_{50}$ molecules bound/DnaB protomer and number of binding sites per protomer, respectively.

protein had significant ssDNA binding activity (lanes 2−6, Figure 7). Thus, the most noteworthy feature of the DNA binding assays is the presence of DNA binding in DnaBΔ1 but absolute absence of DNA binding in DnaBΔ2. This experiment provided strong support to the hypothesis that the leucine zipper region is essential for ssDNA binding. A Scatchard analysis (Figure 8) of ssDNA titration in a gel shift assay indicated that the number of binding sites per protomer is 0.45 which supports the notion that there are three ssDNA binding sites per DnaB hexamer. The structural and functional features of these proteins have been compared and summarized in Table 3.

In the previous paper, we have demonstrated that the DnaBαβ with a 169-residue C-terminal deletion did not have any helicase activity. Deletion mutant DnaBΔ2 was completely devoid of the helicase activity (Figure 9), which is similar to that observed with DnaBαβ and correlated well with the findings of the DNA binding studies (Figure 7). Deletion mutant DnaBΔ1, on the other hand, was capable of DNA unwinding (Figure 9). The helicase activity of DnaBΔ1 was ∼15% of that observed with wtDnaB. However, our studies, thus far, indicated that DNA helicase

Table 3: Summary of Structural and Functional Characteristics of Wild-Type and Mutant DnaB Proteins

DnaB mutants	hexameric	ATPase	DNA dependent	DNA helicase
wtDnaB	+	+	+	+
DnaBMut1	+	+	−	−
DnaBMut2	+	+	±	±
DnaBΔ1	+	+	+	+
DnaBΔ2	−	+	−	−
DnaBαβ	−	+	−	−

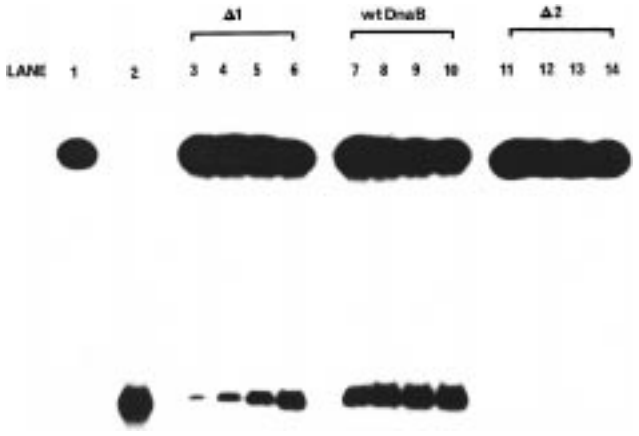


FIGURE 9: DNA helicase activity of DnaB protein and its deletion mutants. Protein titration in a standard helicase assay (Materials and Methods). In each case 1, 2, 4, and 8 pmol of enzyme was added to the assay. Lanes: 3−6, DnaBΔ1; 7−10, wtDnaB; 11−14, DnaBΔ2.

activity has stringent structural requirements, and all of the other deletions that we have studied thus far did not have any helicase activity. Therefore, even though the helicase activity of DnaBΔ1 is reduced, this activity is quite significant because it demonstrated the absolute requirement of the leucine zipper moiety in the DNA unwinding as we have shown earlier with DNA binding (Figure 7). In addition, it should be noted that the ssDNA binding activity of DnaBΔ1 was comparable to that observed with the wtDnaB. The difference in these two activities may be due to weakening of the hexameric structure due to deletion of region γIII that also resulted in a decrease in the ssDNA-dependent ATPase activity (Table 2). The decrease in the ATPase and DNA helicase activities appeared to be comparable, pointing to a link of these two activities in the energy transduction mechanism of DnaB helicase.

DISCUSSION

DnaB helicase of *E. coli* is a hexameric DNA helicase. Our previous studies (*22*) first demonstrated that there are three nucleotide binding sites in the hexamer. This has been shown subsequently in other DNA helicases such as T4 and T7 DNA helicases (*11*, *16*, *24*). Consequently, this phenomenon may have a special significance in the mechanism of action of hexameric DNA helicases. However, the nature of the DNA binding site in the hexameric DNA helicases remains unknown. Our recent studies (*12*, *35*) indicated that a leucine zipper in the C-terminus of DnaB protein is perhaps involved in the dimerization of DnaB protomers. This dimerization domain of DnaB helicase is very similar to that observed with various eukaryotic leucine zipper containing transcription factors (Scheme 1). Electron microscopic studies

of San Martin et al. (*10*) have further suggested the role of this domain in the oligomerization of DnaB helicase.

Roles of the Leucine Zipper Motif in the DNA Binding, Helicase, and DNA-Dependent ATPase Activities. Sequence comparison of the putative leucine zipper region of DnaB helicase to other leucine zipper proteins indicated that the RSRARR sequence, 31 amino acids upstream from the leucine zipper dimerization motif, has certain similarities to the DNA binding domain of other leucine zippers (Scheme 1). Our studies involving *in vitro* mutagenesis indicated that the arginine residues in the RSRARR sequence are required for DNA binding (Figures 4 and 7) and DNA helicase (Figures 5 and 9) activities. In addition to the RSRARR sequence, the role(s) of the putative leucine zipper dimerization motif in the DNA binding must be evaluated as point mutations in this domain resulted invariably in aggregated protein. As a result, selective deletions at the C-terminal end of DnaB helicase were carried out. Deletion of 55 C-terminal amino acid residues (DnaBΔ1, Scheme 2) in which the leucine zipper motif remained intact resulted in the formation of a weak hexamer but retained both DNA binding and DNA helicase activities. However, further deletion of 58 residues in DnaBΔ2 (Scheme 2) that removed only the zipper motif but not the RSRARR sequence resulted in a complete loss of DNA binding and DNA helicase activities as well as hexamer formation. These results indicated that the zipper dimerization motif is required for hexamer formation and DNA helicase activities. This motif is also required in addition to the upstream RSRARR sequence for ssDNA binding. Mutagenesis of the RSRARR sequence attenuated or eliminated the DNA binding as described earlier. The ssDNA binding is, thus, likely mediated through the formation of a dimeric leucine zipper motif in a manner similar to that observed for the eukaryotic GCN4 and FOS/JUN leucine zipper motifs.

In the accompanying paper, we have demonstrated that the ATPase active site resides completely within domain β, and domain γ plays an important role in stimulating the ATPase activity and it appeared to be 2-fold. We have divided domain γ in three regions: γI, γII, and γIII. Analysis of the DNA-dependent ATPase activities of various deletion and point mutants of DnaB helicase allowed delineation, in part, of the roles of various regions of domain γ in the regulation of the ATPase activity (Figures 3 and 6, Table 2). Our deletion studies described here essentially divided domain γ in these three regions. Region γIII, the C-terminal end containing 55 residues (deleted in DnaBΔ1), appeared to be involved in stimulating the basal or overall ATPase activity in the presence or absence of DNA (Table 2). Region γII, 58 residues preceding this region (deleted in DnaBΔ2), that contained the zipper motif (Schemes 1 and 2) was not involved in the stimulation of the basal ATPase activity but was clearly involved in the ssDNA stimulation of the ATPase activity (Figures 6 and 7). Region γI, 58 residues preceding the zipper that contained the RSRARR sequence, appeared to mediate in part the ssDNA stimulation of ATPase, presumably through the RSRARR sequence, because mutations in this sequence as in DnaBMut1 and DnaBMut2 either eliminated or attenuated the DNA-dependent ATPase activity as well as the DNA binding and DNA helicase activities. Thus, region γI and the RSRARR sequence are likely the DNA binding motif and contributed to DNA binding. Region γII, containing the leucine zipper motif, is also essential for DNA binding, which is likely due to the dimerization of the zipper. Dimerization of the zipper is known to be required for formation of the DNA binding site in classical leucine zippers. Finally, region γIII is likely required in addition to the leucine zipper motif for a stable dimer formation and for stimulation of the ATPase activity. A summary of the structural and functional features of the wtDnaB and mutants described here is presented in Table 3.

Transcription factors that contain leucine zippers are normally required to bind a specific duplex DNA sequence in a static manner with strong affinity in order to activate the gene. However, DNA helicases must bind ssDNA according to a different set of criteria. For example, (i) the binding should be sequence independent, (ii) the binding should not be of too high affinity, and (iii) it should allow mobility of the helicase bound to the DNA. The attenuated or half-site RSRARR sequence (Figure 1) may provide the DnaB helicase precisely with these required characteristics. Unlike transcription factors cJUN and cFOS, the DNA binding and dimerization motifs of DnaB helicase are separated by a 31 amino acid spacer region (or hinge region). This spacer region could allow for a very flexible movement of the DNA binding domain which is not needed in transcription factors. Furthermore, this may allow the helicase to twist the DNA for unwinding purposes as well as to allow rapid binding and release of DNA. The downstream leucine zipper dimerization motif ($I{\cdot}X_6{\cdot}L{\cdot}X_6{\cdot}L{\cdot}X_6{\cdot}L{\cdot}X_6{\cdot}L$) allows the two DNA binding sequences of the two subunits to come together for efficient ssDNA binding similar to that observed in classical leucine zippers such as GCN4 (*30*−*32*). Therefore, each of the three dimeric units of the hexameric helicase would form a single ssDNA binding site. Thus, the hexamer may have three ssDNA binding sites, which is in agreement with the number of ssDNA binding sites per promoter of 0.45 determined from Scatchard analysis (Figure 8).

The Leucine Zipper Domain Is Required for a Flexible DNA Binding Domain That Facilitates DNA Unwinding. The results presented here along with our previous studies (*12*) demonstrated that this domain contained a dimerization motif and an attenuated DNA binding motif that closely resembles the classical eukaryotic leucine zippers such as the GCN4 protein (*25*−*32*). Site-specific mutations in the DNA binding or deletion of the dimerization site of this domain resulted in a loss or severe attenuation of the ssDNA binding and DNA helicase activities (Table 1, Figure 2B). As anticipated, the loss or attenuation of DNA binding as determined by gel shift assay and stimulation of ATPase activity was always parallel to that observed with the DNA helicase activity of each mutant. This indicated that domain γ is required for both hexamer formation and interaction with ssDNA and, thereby, plays an important role in the mechanism of DNA helicase.

Together, these results strongly suggest that the RSRARR domain is the DNA binding domain of the DnaB protein which functions in both its DNA-dependent ATPase and helicase activities. The mediation of DNA binding through the leucine zipper like domain is also supported by these data. Mutation of this domain, in a protein whose RSRARR domain remains wild type, eliminated DNA binding, DNA dependence of the ATPase, and helicase activity. Although it is probably likely that other neighboring residues in the

native protein influence DNA binding and associated activities, results obtained in this study clearly indicated two domains pivotal to DNA binding and define the roles of these individual domains in the DNA binding event.

Topology and Orientation of Nucleotide and DNA Binding Sites in the DnaB Hexamer. Earlier studies have established that three of the six nucleotide binding sites participate actively in DnaB function (*22*). It is possible that negative allosteric regulation reduces the "nucleotide affinity" of the two subunits adjacent to a nucleotide-bound subunit in the hexamer. Thus, in the hexameric doughnut, if we assign numbers to all six subunits from 1 to 6, then the occupied subunits would be either 1−3 and 5 or 2−4 and 6 (Figure 10). Three dimeric units, formed by the attachment of the two monomers by the leucine zipper as shown here, should be 1−2 (A), 3−4 (B), and 5−6 (C), and each of these dimeric units contains one ssDNA binding site and presumably one active NTP hydrolysis site. If we consider binding of ssDNA to site A between subunits 1 and 2, then the NTP hydrolysis in response to DNA binding would take place and the ssDNA would move to the next binding site.

Proposed Model of DNA Unwinding by the DnaB Hexamer. Electron microscopic studies of the DnaB hexamer by San Martin et al. (*10*) clearly demonstrated that the basic structure of the DnaB hexamer is a doughnut with an ~4 nm (~40 Å) diameter hole in the center. Similar doughnut structures have been observed with the T7 DNA helicase (*15*). The results of this study extended our earlier proposal of the leucine zipper domain as one of the two dimerization sites in the DnaB hexamer (*12*). It appears from the electron microscopic studies that three leucine zippers are positioned at a 120° angle with respect to each other and that the N-terminal RSRARR sequences would be located in the internal part of the doughnut hole in relatively close proximity. Yu et al. (*13*) have recently shown that in T7 DNA helicase one ssDNA molecule is bound to the dimer in the internal part of the doughnut hole.

Previous studies (*4*, *10*, *12*, *22*, *35*) and those presented here have delineated the following unique features of the DnaB helicase: (i) the hexamer is composed of three apparently dimeric units; (ii) out of six possible NTP binding/hydrolysis sites, only three sites are involved in nucleotide binding and hydrolysis; (iii) as demonstrated here, there are only three ssDNA binding sites; and (iv) the polarity of movement is fixed and it is 5′ → 3′. Taken together, a tentative working model of DNA unwinding by the DnaB helicase has been developed as shown in Figure 10.

The important features of this model (Figure 10) are as follows: (i) the three leucine zippers in the DnaB hexamer create three independent DNA binding sites which are oriented at 120° angles to each other; (ii) due to a 120° angle between the neighboring DNA binding sites, movement of ssDNA from one to the other binding site must involve exactly a 120° rotation of the plane of the ssDNA molecule; (iii) hydrolysis of one molecule of ATP will result in the rotation of the plane of ssDNA by 120°; (iv) the DnaB hexamer in each complete cycle should hydrolyze three ATP molecules and should rotate the plane of the ssDNA by 3 × 120° or 360°; and (v) in order to unwind DNA, the DnaB hexamer should move the ssDNA in a clockwise manner. It should be noted that the DnaB hexamer would be prohibited from moving DNA in an anticlockwise manner, due to the fact that it will lead to further twisting of DNA, and the resultant strain would hinder further movement in that direction. Therefore, the preferred direction of movement of ssDNA by the DnaB hexamer would be in a clockwise direction.

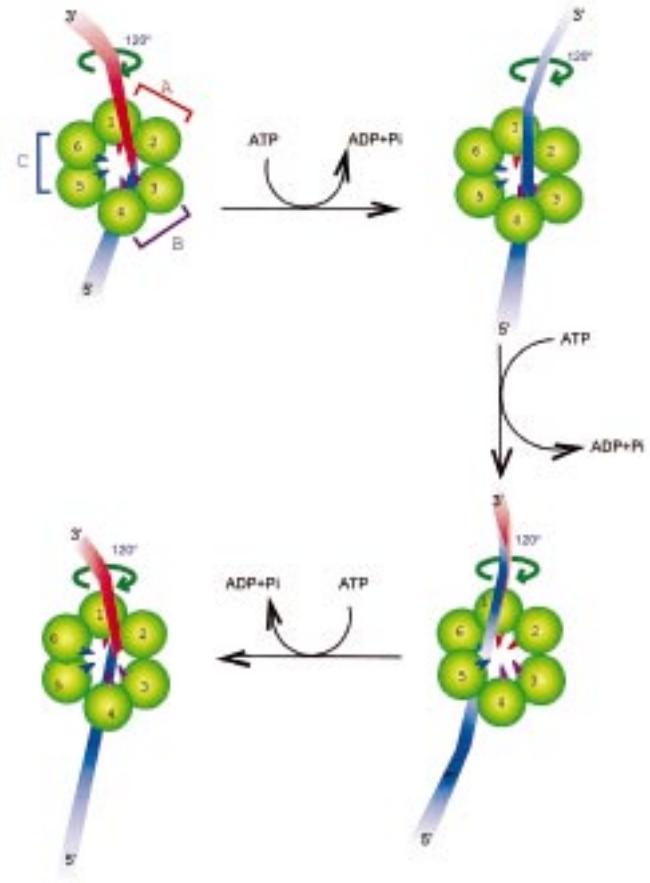


FIGURE 10: Conceptual model of DNA unwinding by DnaB helicase in the lagging strand of the replication fork. The proposed model is based on our finding that there are three ssDNA binding sites and three nucleotide binding sites per DnaB hexamer and suggests the possible roles of the three binding sites in DNA unwinding. The view of the hexamer−DNA complex is from the top or 3′ → 5′ direction. The DNA strand shown here is the lagging strand of the replication fork and is presented as a ribbon and colored red on one side and blue on the other. The monomers are labeled consecutively in a clockwise manner from 1 to 6, and this order is maintained throughout this diagram. A, B, and C refers to putative dimeric units of monomers 1 + 2, 3 + 4, and 5 + 6, respectively, each containing a leucine zipper dimerization and DNA binding motif. Due to a 120° angle between the neighboring DNA binding sites, movement of ssDNA from one binding site to the other must involve an exact 120° rotation of the plane of the ssDNA molecule; hydrolysis of ATP molecule(s) provides the energy for the rotation of ssDNA by 120°; the DnaB hexamer in each complete cycle should hydrolyze at least three ATP molecules and should rotate the plane of the ssDNA by 3 × 120° or 360°. In order for unwinding to continue, the direction of rotation of ssDNA must be in a clockwise manner.

DnaB helicase has been shown to have a strict 5′ → 3′ polarity of movement. The lagging strand is oriented in a 5′ → 3′ polarity in the replication fork; thus, DnaB helicase would bind to the lagging strand only. The mechanism of unwinding proposed here requires binding of DnaB helicase only to the lagging strand of the fork. DnaB helicase interacts with the DNA primase and the DNA polymerase III

holoenzyme and functions as the lagging strand DNA helicase. Previous studies by Arai and Kornberg (*39*) and recently by Marians and co-workers (*6*, *7*) have established functional and perhaps structural interactions of the DnaB helicase with DNA primase and the pol III holoenzyme τ subunit.

In the chromosomal replication fork with a closed circular DNA, uninterrupted helicase action would require the function of a DNA topoisomerase upstream of the helicase. The DnaB helicase is a potent ssDNA-dependent ATPase, and consequently, it is capable of attaining a very rapid rate of DNA unwinding. The rate of DNA unwinding will be dependent on other replication proteins such as DNA polymerase III holoenzyme, topoisomerase, and ssDNA binding proteins. The model presented here can be applied equally well to other hexameric DNA helicases. Recent data from various laboratories suggest that the T7 gene 4 DNA helicase and T4 gene 41 helicase act in a manner identical to that of DnaB helicase. Indeed, T7 DNA helicase has a very similar hexameric structure, nucleotide (dTTP) binding properties, and perhaps DNA binding properties as judged by electron microscopic studies (*13*, *24*). Notarnicola et al. (*11*) have recently demonstrated that mutants of T7 DNA helicase defective in hexamer formation are also defective in nucleotide hydrolysis, ssDNA binding, and DNA unwinding, which is in excellent agreement with results presented here. These data perhaps indicate that the ssDNA binding also requires two adjacent subunits, suggesting a possible three ssDNA binding sites per hexamer. We proposed earlier that the DnaB hexamer is a trimer of dimeric units (*12*), and in the preceding paper (*35*) we have reported putative sites for protein−protein interactions in the DnaB hexamer. In this regard, Notarnicola et al. (*11*) have also shown that the T7 helicase can also form dimers and proposed two separate and distinct protein−protein interfaces. Our studies presented here suggest a similar feature with the DnaB hexamer possessing two interfaces: one in the N-terminal and the other in the C-terminal leucine zipper. The leucine zipper motif may be unique to DnaB helicase. In other hexameric helicases this region could possibly be replaced by other DNA binding and dimerization motifs.

The proposed working model presented here is consistent with and brings together our current knowledge regarding the ATP hydrolysis, DNA binding, and oligomeric structure of DnaB helicase as they relate to DNA unwinding in the double helix. Further studies will be needed to obtain a detailed understanding of the coupling of energy transduction to DNA unwinding.

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