Purification and Characterization of a Mutant DnaB Protein Specifically Defective in ATP Hydrolysis

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The *dnaB* gene of *Escherichia coli* encodes an essential DNA replication enzyme. Fueled by the energy derived from the hydrolysis of ATP to ADP + P_i, this enzyme unwinds double-stranded DNA in advance of the DNA polymerase. While doing so, it intermittently stimulates primase to synthesize an RNA primer for an Okazaki fragment. To better understand the structural basis of these and other aspects of DnaB function, we have initiated a study of mutant DnaB proteins. Here, we report the purification and characterization of a mutant DnaB protein (RC231) containing cysteine in place of arginine at residue 231. The mutant protein attains a stable, properly folded structure that allows association of six promoters to form a hexamer, as is also true for wild-type DnaB. Further, the mutant protein interacts with ATP, the nonhydrolyzable ATP analog adenosine-5'-O-(3-thiotriphosphate) (ATP γ S), ADP, and poly(dT), and it stimulates primase action. It is, however, profoundly deficient in ATP hydrolysis, helicase activity, and replication activity at the chromosomal origin of replication. In addition, while general priming reactions with wild-type DnaB and ATP elicited the synthesis of short primers, reactions with DnaB and ATP γ S or with RC231 and either ATP or ATP γ S stimulated the synthesis of significantly longer primers. On the basis of these observations, we suggest that primase interacts directly with DnaB throughout primer synthesis during general priming, until dissociation of DnaB from DNA or ATP hydrolysis by DnaB disrupts the interaction and leads to primer termination.

The DnaB protein of *Escherichia coli* is required for replication of the bacterial chromosome and many plasmids and bacteriophage DNAs (14). Native DnaB protein is a hexamer of identical subunits encoded by the *dnaB* gene (6, 25). Its participation in DNA replication begins when a DnaB hexamer associates with up to six molecules of a specific transferase, the DnaC protein (13). This complex is competent to transfer DnaB to a region of single-stranded DNA (ssDNA) at the chromosomal origin of replication. When bound to ssDNA, DnaB exhibits a potent ATPase activity (yielding ADP + P_i) (2, 26) and is thought to migrate in a 5'-to-3' direction along the single strand to which it is bound, thereby entering the replication fork (7, 17). As this action continues, the double-stranded DNA at the fork becomes unwound, thus moving the fork. DnaB is the only helicase required to reconstitute DNA replication in vitro dependent on the chromosomal origin of replication (*oriC*), but it is possible that other helicases (Rep, UvrD, PriA) play supporting or backup roles in vivo (19).

In addition to its role as a helicase, DnaB critically influences the action of another essential replication enzyme, the DnaG primase. Primase synthesizes short RNAs that are extended by DNA polymerase (which cannot start chains de novo) to produce classical Okazaki fragments, at least on the lagging strand (4). Primase does not associate spontaneously with either single-stranded or double-stranded naked DNA; rather, other proteins, including DnaB (1, 22), must first be present on the DNA. It is thought that the association of primase with the replication fork is dynamic, occurring while DnaB continues to unwind double-

stranded DNA (31). However, there is no clear evidence that primase interacts with DnaB directly.

Although these functions of DnaB have been investigated in detail, little is known about the structural determinants of DnaB function. Partial trypsin digestion of DnaB showed that it could be digested into two relatively stable domains of 12,000 Da (N terminal) and 33,000 Da (C terminal), with loss of a 45-amino-acid linker region (23). The amino-terminal domain was missing 14 amino acids from its N-terminal end. The carboxyl-terminal domain was shown to retain nucleotide binding, ssDNA-dependent ATPase activity and the ability to form hexamers. None of the other activities of native DnaB was found in either of the two domains. The helicase activity of DnaB was unknown at the time.

With the goal of better understanding the structural basis for DnaB function, our laboratory has isolated dominant-lethal mutations in the *dnaB* gene (21). The dominant-lethal phenotype predicts that the encoded proteins, although not fully functional, are structurally intact since they interfere with host DNA replication. Such proteins are excellent candidates for analysis in vitro because they are likely to exhibit a specific alteration of function. Here, we report the overexpression, purification, and characterization of one such mutant DnaB protein, which contains cysteine in place of arginine at position 231. This protein was found to have a primary defect in ATP hydrolysis.

MATERIALS AND METHODS

Materials. Except where indicated otherwise, chemicals and enzymes were obtained from commercial suppliers and were reagent grade or equivalent. Polyclonal antibody against DnaB and a sample of purified DnaB were generous gifts from Linda Reha-Krantz, and primase was generously provided by Charles McHenry. Poly(dT) used in these experiments was characterized as having an average chain

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length of 167 on the basis of velocity sedimentation, according to the manufacturer (Pharmacia-LKB). However, this poly(dT) migrated more slowly than a 693-base polymerase chain reaction product during electrophoresis over a sequencing gel. In fact, its migration was indistinguishable from that of a separate lot of poly(dT) characterized by the manufacturer as having average length 824. That the poly(dT) chain length was indeed much greater than 167 was confirmed by an independent technique in which (dA)₁₅, annealed at random to the poly(dT), was extended to the end of the template with Sequenase. This reaction produced a ladder of products, the longest of which was at least several hundred bases longer than 167.

Buffers. Buffer A was 50 mM Tris-HCl (pH 7.5). Buffer B contained 50 mM Tris-HCl (pH 7.5), 10% (wt/vol) sucrose, and 2 mM EDTA. Buffer C contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 30 mM KCl, 1 mM ATP, and 25% (wt/vol) glycerol. Buffer D (10×) contained 400 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-KOH (pH 7.5), 500 µg of bovine serum albumin (BSA) per ml, 110 mM magnesium acetate, and 34 mM ATP. Buffer E contained 20 mM potassium phosphate (pH 6.5), 1 mM dithiothreitol, and 0.5 mM EDTA. Buffer F contained 89 mM Tris-HCl (pH 8.0), 89 mM boric acid, and 2 mM EDTA.

Bacterial strains. MG1655 is an F⁻ prototroph of *E. coli* K-12 obtained from G. Weinstock (11). RM990 (*dnaB*⁺) and its *dnaB22*(Ts) derivative RM1031 have been described previously (21). RM2528 is a *recA56 srl-300::Tn10* derivative of RM990. BL21 is an F⁻ *ompT* r_B⁻ m_B⁻ derivative of *E. coli* B. BL21(DE3) carries a λ prophage bearing a copy of the T7 RNA polymerase gene under heterologous (*lac*) control. BL21 and BL21(DE3) were obtained from A. Rosenberg and F. W. Studier (29).

Cloning, subcloning, and mutagenesis of the *dnaB* gene. From a clone of the *E. coli dnaB* gene in λSE6 (9), a 3.6-kb *Bgl*II fragment containing the entire *dnaB* gene was subcloned into M13mp18 (32). By using oligonucleotide-directed mutagenesis (16), codon 231 of the *dnaB* gene in M13mp18 was changed to encode cysteine instead of the wild-type arginine, a change identical to the one in *dnaB129*, a strong dominant-lethal mutation in the *Salmonella typhimurium dnaB* gene (21). The mutant *E. coli* gene will be designated *dnaB720*, and its encoded protein will be referred to as RC231. The λSE6 and M13mp18 clones of *dnaB* each complemented the temperature-sensitive phenotype of *E. coli* RM1031, whereas *dnaB720* failed to show complementation.

Overexpression and purification. Using oligonucleotide-directed mutagenesis, we generated an *Nco*I site (5'-CCATGG-3') overlapping the initiator methionine codon. DNA fragments (from either the wild-type *dnaB* gene or *dnaB720*) starting from this *Nco*I site up to a *Bam*HI site 3' of the gene were subcloned into overexpression plasmid pET-3d (*dnaB*) or pET-11d (*dnaB720*) (29). The *dnaB* and *dnaB720* genes were thus placed under T7 transcriptional and translational signals. The wild-type DnaB protein was overexpressed from plasmid pET-3d in a BL21(DE3) host that also harbored plasmid pLysS (29). No overexpression of DnaB was seen in the absence of plasmid pLysS. The T7 lysozyme encoded by plasmid pLysS enhanced the stability of plasmid pET-3d with the *dnaB* insert, presumably by inhibiting background T7 RNA polymerase activity and the associated DnaB expression. Cell growth was even more sensitive to background expression of RC231. Even in the tightly regulated pET-11d vector, the *dnaB720* gene could be expressed in the cell only in the presence of plasmid pLysS.

Wild-type DnaB and RC231 were purified by the same procedure, throughout which the two proteins behaved identically. Purification was carried out by modifying the procedure of Khatri et al. (12). Briefly, cells were lysed by the action of the pLysS-encoded lysozyme, aided by a freeze-thaw step. The lysate supernatant was fractionated by precipitation with ammonium sulfate (ultrapure; Schwartz/Mann). The fraction that was soluble at 20% and insoluble at 40% ammonium sulfate saturation contained DnaB. This fraction was desalted and further fractionated over a DEAE-Sephacel (Pharmacia-LKB) ion-exchange column, which was washed with 150 mM KCl in buffer C and eluted with a linear gradient of 190 to 400 mM KCl in buffer C. In some preparations, alternatively, the column was washed at 190 mM KCl in buffer C, then washed at 240 mM KCl in buffer C, and eluted with a gradient of 240 to 400 mM KCl in buffer C. The peak fractions were pooled and were essentially pure. In some preparations, the DEAE-Sephacel chromatography was repeated or the pooled fractions were similarly chromatographed over a Mono-Q column (Pharmacia-LKB) to obtain sufficient purity. Although these preparations were free of detectable contaminating proteins, we recently found substantial amounts of RNA in some wild-type DnaB and RC231 preparations, including some of the preparations used for the experiments described here. Polyribonucleotides are known not to affect the ATPase activity of DnaB protein (2). We have found that this RNA can be removed from the purified protein by either hydroxyapatite chromatography, glycerol gradient centrifugation, or chromatography on Mono-Q.

ATPase assay. Assay of hydrolyzed ATP was carried out by a modification of the procedure described earlier (8, 10). A final reaction volume (180 µl) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 75 nmol (as nucleotide; =25 µg) of M13 ssDNA or poly(dT) (when included), 2.1 mM [γ -³²P] ATP at 700 cpm/nmol (obtained by mixing labeled ATP at 10 Ci/mmol [New England Nuclear] with unlabeled ATP [Sigma]), and 1.35 µg of either DnaB or RC231. The reaction mixtures, minus protein, were assembled on ice and warmed to 37°C for 5 min, and the reactions were initiated by the addition of protein and maintained at 37°C thereafter. At various times, a 25-µl aliquot was withdrawn from the reaction mixture and processed as follows. ATP hydrolysis in the aliquot was stopped by the addition of 100 µl of 20 mM silicotungstic acid in 20 mM sulfuric acid and 240 µl of 1 mM potassium phosphate (pH 7.0) at 0°C. To this were added sequentially 100 µl of 5% (wt/vol) ammonium molybdate in 4 M sulfuric acid, 60 µl of 1:1 acetone-5% (wt/vol) trichloroacetic acid, and 400 µl of 1:1 isobutanol-benzene. The tubes were vortexed at high speed for 30 s, phases were allowed to separate for 15 min, and the amount of P_i in the upper organic phase was estimated by counting a 100-µl aliquot of the upper phase. The total P_i released was adjusted for a total volume of 350 µl in the upper organic phase.

Helicase assay. The helicase substrate was prepared by hybridization of an end-labeled 24-mer oligonucleotide to a single-stranded M13 template and isolating the hybrid as described previously (12). The helicase reaction was carried out as follows. In a total volume of 15 µl were included 1.5 µl of 10× buffer D, 30 to 50 fmol of helicase substrate, and ~24 pmol of DnaB or ~14 to 56 pmol of RC231. After being assembled on ice, the mixture was incubated at 37°C for 10 min and then immediately loaded on a 20% polyacrylamide gel in buffer F and run at 4°C for 90 to 120 min at 150 V. The results were evaluated by autoradiography.

Partial trypsin digestion. Partial trypsin digestion was

carried out by a modification of a previously described procedure (24). A 12.5- μ g sample of DnaB or RC231 was digested with 13.5 μ g of trypsin (Sigma) in a total volume of 11 μ l. Incubation was at 0°C for 30 min. The reaction was stopped by adding 10 μ l of 3% sodium dodecyl sulfate (SDS)–40 mM dithiothreitol, and the digestion products were resolved by electrophoresis through an SDS-polyacrylamide gel (10% acrylamide) and stained with Coomassie stain. Identification of cleavage products was confirmed by amino-terminal microsequencing (20).

Glycerol gradient centrifugation. DnaB or RC231 was analyzed by centrifugation of 100 μ g of protein over a 20 to 45% (wt/vol) glycerol gradient in buffer E–200 mM KCl–5 mM ATP at 50,000 rpm for 20 h at 4°C in a Beckman SW50.1 rotor. Fractions (180 μ l) were collected from the bottom of the tube, and the protein concentration was assayed by using the Bradford reagent. Markers used were horse heart cytochrome c, BSA, and bovine liver catalase.

General priming assay. General priming was done essentially as described previously (1). Each reaction mixture contained 19 mM Tris (pH 7.5), 3.8% (wt/vol) sucrose, 7.7 mM dithiothreitol, 38 μ g of BSA per ml, 7.7 mM $MgCl_2$, 3.8 μ g of rifampin per ml, 3.8 μ g of primase per ml, 770 μ M (unlabeled) ATP or adenosine-5'-O-(3-thiotriphosphate (ATP γ S)), 10 μ Ci of [α - 32 P]ATP (>3,000 Ci/mmol; Amersham), 69 μ g of poly(dT) per ml, and 65 μ g of DnaB or RC231 per ml in a total volume of 26 μ l. Incubation was for 30 min at 37°C. From the original reaction tube, an aliquot of 8 μ l was denatured by heat and then fractionated over a 16% polyacrylamide–7 M urea gel and evaluated by autoradiography; duplicate 5- μ l aliquots were quantitated by binding to DE-81 filters (Whatman) (27).

RESULTS

Cloning and overexpression of the dominant-lethal mutant (*dnaB720*) of the *E. coli dnaB* gene. Dominant-lethal mutations in the *S. typhimurium dnaB* gene have been described (21). These mutant proteins have been proposed to interfere with DNA replication through their residual functionality and thus are promising candidates for structure-function analysis. For this study, we re-created one of the mutations in the *E. coli dnaB* gene to take advantage of a long history of biochemical work on the *E. coli* protein. The *S. typhimurium* and *E. coli* proteins are 93% identical, and the genes are functionally interchangeable *in vivo*, giving reason to think that any one mutation would exhibit a similar phenotype in either genetic background. The mutation that we chose had three distinct properties (as an *S. typhimurium* mutation) that dictated its selection for this study. First, the mutation had a strong phenotype *in vivo*, suggesting that its biochemical phenotype would be obvious. Second, its dominance *in vivo* was observed from 30 to 42°C, suggesting that the protein would be stable upon overexpression and purification. Finally, the mutation was located in a consensus nucleotide binding fold, suggesting explicit possibilities for the biochemical defect. All three of these suggestions were in fact borne out with the purified *E. coli* mutant protein.

To overexpress a strong dominant-lethal mutant gene such as *dnaB720*, it was essential to clone it downstream from a strong but very tightly repressed promoter because even minimal expression of RC231 was inhibitory to cell growth (see Materials and Methods). Figure 1 (lanes a and b) shows the level of overexpression both of the wild-type DnaB and of RC231, the mutant protein encoded by *dnaB720* having cysteine in place of arginine at residue 231. The wild-type

FIG. 1. Expression and purification of DnaB and RC231. Samples were fractionated over an SDS-polyacrylamide gel. Lanes: a, total cell lysate showing overproduction of DnaB protein 4 h after induction with isopropyl-1-thio- β -D-galactopyranoside; b, total cell lysate showing overproduction of RC231 7 h after induction; c, total cell lysate showing absence of overproduction of RC231 in the absence of plasmid pLysS 7 h after induction; d, purified wild-type DnaB (~30 μ g); e, purified RC231 (~30 μ g).

protein accumulated to be the most abundant cellular protein, whereas RC231 accumulated to a lesser but still significant extent, as judged from Coomassie-stained gels.

Purification. The wild-type DnaB and RC231 proteins were purified as described in Materials and Methods. The purification scheme did not rely on any functional properties of DnaB such as ATP binding or DNA binding. This allowed the purification of RC231 without regard to its functionality. Purification of both proteins was followed by gel electrophoresis of fractionated samples. The two proteins were purified to a high degree (>95% purity; Fig. 1, lanes d and e). The wild-type protein thus obtained was highly active in an *oriC*-dependent replication reaction reconstituted *in vitro* (data not shown) and in various partial assays as shown below.

Structural studies. The wild-type DnaB protein of *E. coli* exists as a stable hexamer in solution (6, 25). When RC231 protein was analyzed by sedimentation over a glycerol gradient, its rate of sedimentation was identical to that of wild-type DnaB, with a sedimentation coefficient of 11.3S in each case (Fig. 2). Using a Stokes radius value of 60 Å (1 Å = 0.1 nm) (25), we calculated the native molecular weight of each protein from the Svedberg equation to be 290,000 ($\pm$ 12,000). This molecular weight estimate, which might be low for reasons discussed elsewhere (25), is most consistent with a hexameric structure. The calculated molecular mass of a DnaB hexamer is 313,590 Da.

Partial trypsin digestion of wild-type DnaB protein yields two stable domains: an ~12,000-Da amino-terminal domain and an ~33,000-Da carboxyl-terminal domain (24). The carboxyl-terminal domain accumulates only in the presence of a nucleotide. Wild-type DnaB and RC231 from which ATP (included during purification) had been stripped by gel filtration were subjected to trypsin digestion with or without added nucleotides (Fig. 3). The major digestion products were similar for the two proteins, as would be expected if they were similarly folded. In the absence of added nucleotide, the carboxyl-terminal domains of both proteins were rapidly degraded. However, addition of either ATP or ADP

FIG. 2. Glycerol gradient centrifugation of DnaB and RC231 (see Materials and Methods). Each marker protein (beef liver catalase [232,000 Da], BSA [66,000 Da], and cytochrome *c* [12,400 Da]) was run in an independent tube at the same time, and its peak position in its gradient is indicated by an arrow.

dramatically stabilized both proteins against trypsin digestion, and the carboxyl-terminal domain transiently accumulated. Since residue 231 is in the carboxyl-terminal domain of the protein, these results show that the mutant protein is capable of binding ATP and ADP.

Thus, the mutant and wild-type proteins exhibited identical sedimentation behavior and were similar in their sensitivities to trypsin and in their interactions with ATP and ADP. All of these observations point to native folding of RC231.

Functional studies. (i) ATP hydrolysis. Wild-type DnaB has a potent ssDNA-dependent ATPase activity and a much weaker DNA-independent ATPase activity (2, 26). Either M13 viral DNA or poly(dT) will serve as effector (Fig. 4A). RC231 protein exhibited no ATP hydrolysis above the assay background even with M13 DNA or poly(dT) (Fig. 4B; note

FIG. 3. Partial trypsin digestion of DnaB and RC231. The products of each reaction were fractionated over SDS-polyacrylamide (10%) gels. The figure is a composite of two experiments, with the alignment between the two gels indicated. The DnaB proteins, prior to digestion, were stripped of ATP (present during purification) by gel filtration chromatography. The reaction mixtures were composed as indicated above the lanes. ATP or ADP, if indicated, was added at 5 mM. The N-terminal ~12-kDa fragment was not resolved in these particular gels but was observed in other gels.

FIG. 4. ATP hydrolysis. All sample data have been corrected for P_i detected at time zero. This assay background was generally between 100 and 200 pmol except in the DnaB-M13 assay, in which it was 515 pmol. (A) Activity of DnaB with M13 ssDNA cofactor (●), with poly(dT) cofactor (○), and without DNA (■); (B) activity of RC231 with M13 ssDNA cofactor (▲), with poly(dT) cofactor (□), and without DNA (△). For reference, the data for DnaB in the absence of DNA are repeated in panel B (■).

picomole scale). RC231 protein added to an M13-dependent ATPase reaction with wild-type DnaB had little effect on hydrolysis (data not shown). Therefore, the absence of hydrolytic activity in RC231 cannot be explained by the presence of an ATPase inhibitor; instead, RC231 must be inherently affected in ATP hydrolysis. Taken together with evidence indicating that RC231 can bind ATP (i.e., the trypsin digestion experiments in which the ATP concentration was only twice that used in the hydrolysis assay, and also the general priming assay described below, in which the ATP concentration was one-third of that in the hydrolysis assay), these results indicate that RC231 is unable to complete the hydrolysis of a bound ATP. This failure could be at the level of catalysis or at the level of binding to the allosteric effector, ssDNA. Marszalek and Kaguni (18) report that RC231 does not bind M13 DNA under conditions similar to those used in this study, but the strong activity of RC231 in supporting general priming on poly(dT) indicates that the mutant protein binds poly(dT) significantly. The concentration of poly(dT) in the priming assay is half of that in the ATPase assay. Thus, even when bound to effector ssDNA, RC231 is unable to hydrolyze ATP. We conclude that the defect in RC231 is at the level of catalysis, since there is no

FIG. 5. Helicase activity. The substrate was a partial duplex containing a 24-mer oligonucleotide hybridized to an ~9-kb M13 ssDNA molecule. DnaB or RC231 was included as indicated above each lane. ATP was present in all reaction mixtures at 3.4 mM.

hydrolysis under conditions in which both the ATP substrate and the poly(dT) effector can be bound to a substantial extent. This conclusion is also supported by the observation that ATP hydrolysis by RC231 is clearly less than that supported by wild-type DnaB in the absence of effector ssDNA (Fig. 4B).

(ii) **Unwinding.** The DNA-unwinding activities of wild-type DnaB and RC231 were compared by incubating the proteins with a partial duplex DNA substrate composed of a 24-mer oligonucleotide hybridized to M13 ssDNA. The wild-type DnaB protein showed considerable unwinding of this duplex, whereas under identical conditions, the RC231 protein possessed no detectable DNA-unwinding activity, even with up to fourfold-higher levels of RC231 than wild-type DnaB protein (Fig. 5 and additional data not shown). This result can be attributed to the inability of RC231 to interact with M13 ssDNA (18) and/or to the inability of the protein to hydrolyze ATP, the energy of which is required for DNA unwinding (17).

(iii) **Primase stimulation.** In general priming, wild-type DnaB protein stimulates primase to synthesize RNA primers on naked ssDNA (DNA not coated with *E. coli* ssDNA-binding protein) (1). This priming is known to be distributive; i.e., the DnaB protein does not have to migrate on the ssDNA molecule and does not have to hydrolyze ATP (4, 5, 30). Nevertheless, ATP binding and ATP-dependent ssDNA binding by DnaB protein are a prerequisite for this reaction, wherein a DnaB-ATP-ssDNA complex is thought to provide a recognition site for primase. Wild-type DnaB and RC231 proteins were analyzed for participation in general priming on a poly(dT) template, using [α - 32 P]ATP supplemented with either unlabeled ATP or unlabeled ATP γ S as a substrate. ATP γ S, an analog of ATP, is strongly bound but poorly hydrolyzed by wild-type DnaB. However, it remains a good substrate for polymerization by primase (4, 5). The products of the general priming reaction were fractionated over a 16% polyacrylamide-7 M urea gel, and the results were evaluated by autoradiography (Fig. 6). The total RNA synthesis in each of the reactions was quantitated by binding to DE-81 filters (Table 1).

The total amount of primer RNA synthesized and the range of lengths of the primers synthesized varied depending on the protein (DnaB or RC231) and the nucleotide (ATP or ATP γ S) used. All primer synthesis was dependent on a DnaB protein (Fig. 6, lane a) as well as on primase and template DNA (not shown). In the presence of DnaB plus

FIG. 6. General priming. Reaction mixtures were composed as indicated above the lanes. Each reaction mixture contained 10 μ Ci of [α - 32 P]ATP in addition to unlabeled nucleotides as indicated.

ATP, the primers synthesized ranged from 8 to 30 nucleotides (nt), with a bias toward the short end of this range (lane b). Total primer synthesis was 80 pmol (as nucleotide; Table 1). When ATP was replaced by the nonhydrolyzable ATP γ S, the lengths of primers ranged from 8 to ~60 nt (lane c), with a total synthesis of 309 pmol. By comparison, in the presence of RC231 plus ATP, the primers ranged from 8 to 36 nt (lane d), with a total synthesis of 186 pmol. The increase in primer synthesis relative to lane b was due to an increase in the number of long primers (lengths of 17 nt and up). Finally,

TABLE 1. Quantitation of general priming reactions^a

Lane in Fig. 6	Reaction	pmol of RNA synthesized (as nucleotides)
a	No DnaB; ATP	7
b	DnaB + ATP	80
c	DnaB + ATP γ S	309
d	RC231 + ATP	186
e	RC231 + ATP γ S	406

^a Aliquots (5 μ l) of the assays displayed in Fig. 6 were quantitated for incorporation of label into polynucleotide by adsorption onto DE-81 paper. The averages of duplicate filters are shown. The quantitation assumes that ATP and ATP γ S are equally efficient substrates for primase. The data have been normalized to the original reaction volume of 26 μ l.

in the presence of RC231 plus ATP γ S, the primers ranged from 11 to ~60 nt (lane e), with a total synthesis of 406 pmol. In the experiment represented in lane e, about 6.8% of the initial template was copied into RNA during the reaction. These results indicate that RC231 is able to interact with ATP, ATP γ S, and poly(dT) and is able to efficiently support the stimulation of primer synthesis by primase.

DISCUSSION

In this report, we have described the characterization of a mutant DnaB protein (RC231) containing cysteine in place of arginine at residue 231. Our results show that the mutant protein is severely defective in ATP hydrolysis and DNA-unwinding activity. On the other hand, the mutant protein is competent to participate in general priming on poly(dT), a reaction that requires that DnaB bind to ATP and poly(dT). Thus, these binding abilities are qualitatively intact in the mutant protein, as is the protein's ability to form a protein-DNA structure that can be recognized by primase. Our conclusion that the mutant protein retains many aspects of wild-type function is also supported and extended by the results of structural studies which show that, like the wild-type protein, the mutant protein can oligomerize, and its carboxyl-terminal domain becomes more resistant to digestion by trypsin in the presence of ATP or ADP than in the absence of all nucleotide. In addition, RC231 is capable of interaction with DnaC protein of *E. coli* (18).

Marszalek and Kaguni (18) show that the mutant protein is defective in interaction with M13 circular ssDNA, whereas our experiments indicate that RC231 interacts with poly(dT). The ATPase deficiency and/or defective interaction of RC231 with specific types of ssDNA molecules could result in the inability to unwind duplex DNA observed here and in an inability to participate in replication at the chromosomal origin *in vitro* (18) or *in vivo* (21).

Catalytic defect. Our data show that RC231 is able to bind ATP but cannot hydrolyze it; therefore, the defect is in catalysis *per se*. Although the catalytic mechanism of ATP hydrolysis by DnaB is unknown, we note that residue 231 corresponds to the first unspecified residue (X) in a motif, G/AXXXGKKT/S, commonly found in GTP- and ATP-binding (and hydrolyzing) proteins (28). This motif is often referred to as a phosphate-binding loop because that is the role it subserves in the case of the *H-ras* oncogene, whose three-dimensional structure is known to 1.35-Å resolution (24). It seems reasonable to expect that these amino acid residues play a similar role in DnaB. None of the unspecified side chains (X) in the phosphate-binding loop in *H-ras* has been implicated in the GTP hydrolysis mechanism, which has been proposed to involve "in-line" nucleophilic attack on the gamma phosphate by an oriented water molecule (15). If arginine 231 in DnaB plays some previously unrecognized role critical to hydrolysis, it would make DnaB an unusual hydrolase because arginines are rarely found in the unspecified positions of the phosphate-binding loop motif. Alternatively, interference with catalysis by cysteine, rather than promotion of catalysis by arginine, could lie at the heart of our observations.

How might cysteine interfere with catalysis? The side chain of cysteine is considerably smaller than that of arginine, making steric hindrance of hydrolysis unlikely. Cysteine is also more hydrophobic than arginine, a feature that would be significant if the space vacated by arginine has hydrophilic character or becomes occupied by solvent water. In this case, accommodation of the cysteine side chain might

result in adjustments in the conformations of other residues with direct involvement in catalysis (including those that ensure proper positioning of the nucleotide and the nucleophile). Such an explanation has been invoked to explain the GTPase deficiency of the glycine-to-valine substitution at the second unspecified loop position in *H-ras* oncogene (15).

Determination of primer length. A striking feature of the general priming experiments was the different length distributions obtained in different reactions. Primers with wild-type DnaB were short (8 to 30 nt) with ATP, whereas with ATP γ S, they were longer (up to ~60 nt). Primers made with RC231 were up to 36 nt with ATP and up to ~60 nt with ATP γ S. Many of the primers observed in this study were terminated prior to the end of the incubation period of the assay. Incubation of the reaction for an additional 30 min (total of 60 min) produced a slight shift to longer lengths in the pattern obtained with DnaB plus ATP γ S; i.e., fewer short primers and more long primers were observed at 60 min than at 30 min, indicating that some primers existing at 30 min were lengthened during the next 30 min (data not shown). Nonetheless, most incorporation took place during the first 20 min, suggesting that most of the primers obtained in the standard 30-min reaction either were terminated products or were close to their final lengths. In addition, we consider it unlikely that termination resulted from primase reaching the end of the template, because the primers were much shorter than the template (see Materials and Methods).

Thus, the difference in primer lengths in the DnaB-ATP reaction compared with those observed in the RC231-ATP reaction indicates that DnaB *per se* can influence the likelihood of primer termination. It has been suggested that DnaB stimulates the initiation of primers either by a direct protein interaction with the primase or by inducing a template structure recognized by primase (3). Our results may indicate that primer synthesis continues until decay of the initiating structure, whatever it is, leads to primer termination. The protein interaction model can be accommodated with little difficulty; since primase and DnaB move with opposite polarity, it suffices to postulate that template regions traversed by DnaB or primase enter a template loop anchored by the DnaB-primase complex. On the other hand, accommodation to the template structure model is more problematic because the template structure and its relationship to primase must be preserved as the primase moves along the template.

It is also a formal possibility that primase interacts with DnaB at the initiation of primer synthesis in a way that somehow sets its termination mode. Primase then moves away from DnaB and is not further influenced by it.

With either DnaB or RC231, longer primers are made when ATP γ S replaces ATP in general priming reactions. Our experiments do not distinguish whether this effect is mediated through DnaB or directly through primase. However, while an effect of ATP γ S on primase has not been explored, available information offers at least two ways in which ATP γ S could affect primer length through DnaB.

(i) **Dissociation of DnaB.** DnaB protein exhibits ~3- to 6-fold-lower affinity for ssDNA in the presence of ATP than in the presence of ATP γ S (3). Thus, in the presence of ATP, the DnaB protein may dissociate from the template more rapidly than in the presence of ATP γ S, leading to premature primase dissociation and shorter primers in the ATP reaction. The binding affinity of RC231 for poly(dT) has not been quantitated. Our data, if interpreted strictly according to this DnaB dissociation model, predicts that RC231 (with ATP or ATP γ S) would remain associated with the DNA template for

a longer time than does DnaB plus ATP, thus allowing the synthesis of longer primers.

(ii) **Effect of DnaB ATPase activity on primer lengths.** The cardinal feature of the DnaB dissociation model, namely, the obligatory dependence of primer termination on DnaB dissociation from the template, has not been proven. Therefore, we consider an alternative model in which DnaB, through its ATPase, actively ejects primase without leaving the DNA. This is an economical model because it explains both the protein effect (DnaB versus RC231) and the nucleotide effect (ATP versus ATP γ S). There are two variations on this idea. With respect to the first variation, a conformation effect, it is known that the conformation of DnaB changes with each cycle of ATP hydrolysis (24), and in one case it has been shown that primase binds more tightly to a DnaB-ssDNA-nucleotide complex that is not hydrolytically active than to an active complex (3). Thus, primase may interact strongly with DnaB-ATP-poly(dT) but weakly with DnaB-ADP-poly(dT). Each cycle of ATP hydrolysis would then be associated with a high probability of primase release and hence primer termination. The second variation postulates a mobility effect. In addition to cycling through conformational changes, a DnaB molecule undergoing rounds of ATP hydrolysis when bound to ssDNA is likely to migrate on the DNA (17). This mobility of the protein on DNA could destabilize a bound primase molecule that is in the process of RNA synthesis. Either of these effects would account for the synthesis of primers that are short in reactions with DnaB plus ATP. In contrast, the other three combinations, which are deficient in nucleotide hydrolysis, apparently do not cycle through conformational changes or migrate on ssDNA, thus providing a stable recognition structure for primase and allowing synthesis of longer primers.

Primer termination in the simplified general priming reactions may reflect a combination of mechanisms. ATP hydrolysis is unlikely to be the sole mechanism because blocking hydrolysis by using ATP γ S produces a different primer length distribution than does blocking hydrolysis by substituting RC231 for DnaB. However, at authentic replication forks, DnaB is unlikely to dissociate from the DNA because of its presumed processive action (7, 17). Therefore, we suggest that ATP hydrolysis by DnaB and its associated mobility on DNA could influence primer termination at authentic replication forks. This suggestion does not preclude the possibility that other replication proteins such as DNA polymerase III holoenzyme (33) could also affect primer length and could be the predominant effectors in vivo.

Genetic dominance of RC231. The inability of RC231 to hydrolyze ATP and to unwind DNA provide a sufficient explanation for the failure to support DNA replication in vitro, as reported previously (18). However, the observation in that study that RC231 competes with wild-type DnaB for DnaC protein, yet is delivered inefficiently to the *oriC* region, may be incomplete or misleading in explaining the characteristic genetic dominance of the mutant. In vivo, in strains that are heterodiploid for *dnaB*, monomers of both RC231 and the functional DnaB are synthesized simultaneously and, one could imagine, assemble into hetero-oligomers. These hetero-oligomers could be competent to be transferred to the *oriC* region through the action of DnaC and would be expected to stimulate primase, as judged from the results presented here. However, they may fail in a subsequent step of replication (primer termination, migration on ssDNA, or unwinding), leading to stalled replication forks. In this report, we have not described the properties of

such hetero-oligomers; even in experiments in which RC231 and wild-type DnaB were both present in a given reaction (18), they were added as separately purified proteins. There is no clear evidence that subunit exchange takes place under these conditions. While a detailed explanation of genetic dominance will have to await further experimentation, the results presented here and elsewhere (18) illuminate several ways in which ATP hydrolysis is central to the replication activity of DnaB.

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