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The replication initiator protein π of the plasmid R6K specifically interacts with the host-encoded helicase DnaB

(initiation of replication/protein–protein interaction)

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ABSTRACT **The replication initiator protein π of plasmid R6K is known to interact with the seven iterons of the γ origin/enhancer and activate distant replication origins α and β (ori α and ori β) by π-mediated DNA looping. Here we show that π protein specifically interacts *in vitro* with the host-encoded helicase DnaB. The site of interaction of π on DnaB has been localized to a 37-aa-long region located between amino acids 151 and 189 of DnaB. The surface of π that interacts with DnaB has been mapped to the N-terminal region of the initiator protein between residues 1 and 116. The results suggest that during initiation of replication, the replicative helicase DnaB is first recruited to the γ enhancer by the π protein. In a subsequent step, the helicase probably gets delivered from ori γ to ori α and ori β by π-mediated DNA looping.**

The plasmid R6K contains potentially three replication origins called α, β, and γ (ori α, ori β, and ori γ; refs. 1–9). ori γ remains silent but can be activated when both ori α and ori β, which are located ≈4000 and ≈1200 bp, respectively, from ori γ, are deleted (5, 10). ori γ acts as an enhancer of replication of ori α and ori β, i.e., both ori α and ori β have an obligatory requirement for the γ sequence, in *cis*, for initiation activity (4, 6, 8). The plasmid-encoded initiator protein π binds to seven 22-bp iterons at ori γ (11, 12) and contacts a single iteron at ori α and a half iteron at ori β by DNA looping (10, 13). π mutants defective in DNA looping have been isolated, and these mutants are also defective in activating ori α and ori β and, as predicted, derepress ori γ (10, 13, 14).

The π protein promotes pairing of intermolecular iteron sets between two ori γ sequences *in vitro* (15) and *in vivo* (16). The intermolecular iteron to iteron pairing, called handcuffing, shuts off the activities of both origins and is believed to be an important regulatory mechanism controlling initiation and copy control (16). Adjacent to the seven iterons, ori γ contains an A+T-rich region that is a potential site for loading of DnaB helicase. ori α contains a DnaG primase-binding site (ref. 17; see Fig. 1) that could potentially serve also as a DnaB loading site by DnaB–DnaG interaction. The interaction between DnaB and DnaG was initially suggested on the basis of indirect evidence (18), and direct evidence for such an interaction has been recently obtained (Y. Lu, P.V.A.L.R., B.K.M., and D.B., unpublished data).

ori β does not contain an A+T-rich region that could serve as a helicase loading site (7). We have postulated that DnaB helicase is first recruited to the enhancer γ sequence and then delivered to ori α and ori β by π-mediated γ–β or γ–α looping (13). Implicit in this hypothesis are two predictions. First, DnaB helicase interacts with π protein that is already bound to ori γ, and second, π-mediated DNA looping subsequently transfers the helicase to ori α and ori β.

In this paper, we present *in vitro* evidence that demonstrates specific interaction between the π initiator protein and DnaB helicase. The interaction domains of both DnaB and π have been mapped.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *Escherichia coli* strains JM109 (*supE44, relA1, recA, endA1, gyrA96, hsdR17D,* Δ [*lac-proAB*], [F′ *traD36, lacI*^{q}, Δ*lacZM15, proA*$^+$ *proB*$^+$], (rk$^-$, mk$^+$)) (19) and BL21 [DE3](F$^-$, *ompT, hsdS,* [r_B^-, m_B^-] *gal*) containing the plasmid pLysS (20) were used for all subcloning and protein expression, respectively. The protein coding sequences were expressed in the T7 promoter-based pET vectors (Novagen) or the *tac* promoter-based pGEX vectors (Pharmacia). Subclonings were performed in the Bluescript KS$^+$ phagemid (Stratagene).

ELISA. One member of an interacting pair of proteins was dissolved in 100 μl of coating buffer (0.1 M Na_2HCO_3, pH 9.2) at a concentration of 1 mg/ml and added to each well of a 96-well microtiter plate (Corning) and incubated at 37°C for 1 hr. The wells were rinsed three times with 200 μl of wash buffer [20 mM Tris, pH 7.4/0.15 M NaCl (Tris-buffered saline, TBS)/0.5% Tween 20]. Blocking buffer (5% BSA, in wash buffer/0.5% Tween 20), 200 μl, was added to each well and allowed to incubate at 37°C for 1 hr. The wells were filled with 10–500 ng of the second protein, dissolved in 100 μl of blocking buffer, and allowed to interact with the adsorbed first protein at 37°C for 1 hr. The wells were each washed five times with 200 μl of wash buffer. Where indicated, rabbit antisera raised against DnaB and DnaC were diluted 2000-fold in blocking buffer, and 100 μl of the diluted antisera was added to the designated wells and incubated at 37°C for 1 hr. After five washes with wash buffer, 100 μl of alkaline phosphatase-conjugated goat anti-rabbit IgG antiserum (1:40,000 dilution in blocking buffer) was added to the wells and incubated for 1 hr at 37°C. After five washes with washing buffer, 100 μl of chromogenic substrate disodium *p*-nitrophenyl phosphate hexahydrate at 1 mg/ml was added and incubated at 21°C for 10–15 min, and the absorbance was read at 405 nm in an ELISA plate reader.

***In Vitro*-Coupled Transcription–Translation.** DNAs encoding full-length DnaB and its peptides were cloned downstream from a T7 promoter and transcribed and translated *in vitro* with [^{35}S]methionine [10 mCi/ml, (1 Ci = 37 GBq)], amino acid mixture (1 mM), T7 RNA polymerase (4 units), and RNase inhibitor (40 units) in a commercial rabbit reticulocyte lysate system (TNT, Promega) at 30°C for 2 hr. Helicase II and luciferase, used as controls, were similarly transcribed and translated. The labeled products were analyzed by SDS/PAGE and autoradiography.

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Abbreviations: GST, glutathione *S*-transferase; IHF, integration host factor.

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Glutathione *S*-Transferase (GST) Affinity Column Chromatography. GST–π fusion protein (or GST–DnaB or GST–π peptides) in 50 μl (5 μg) of π buffer (150 mM NaCl/20 mM sodium phosphate, pH 7.2) was adsorbed to 40 μl of glutathione–agarose beads at 4°C for 45 min. The beads were blocked with 100 μl of 5% BSA (in TBS/Nonidet P-40, 0.1%) for 1 hr on ice. The beads were allowed to pellet, and either protein or *in vitro*-translated, labeled peptides in blocking buffer containing 5 mM $MgCl_2$ and 1 mM ATP were allowed to bind to the immobilized fusion protein for 30 min at 4°C. The beads were washed with TBS/Nonidet P-40, 0.1%. The beads were extracted with 0.1% SDS, 10 mM β-mercaptoethanol, and 10 mM Tris (pH 7.4) and analyzed by an SDS/15% polyacrylamide gel. For nonradioactive proteins, the gels were blotted electrophoretically onto membranes (Hybond C extra; Amersham), and the protein bands were visualized by antibody staining (rabbit IgG, goat anti-rabbit IgG conjugated with alkaline phosphatase). The ^{35}S-labeled peptides were visualized by drying the gel without blotting and by autoradiography.

Protein Purification. Full-length π protein and GST–π peptide fusions were purified as described (10). DnaB was purified from an overproducer plasmid as described (21).

RESULTS

ELISA Showed π–DnaB Interaction. Which protein recruits DnaB helicase to the γ origin/enhancer? Three sequence-specific ori γ-binding proteins are known to date, namely host-encoded DnaA, integration host factor (IHF), and plasmid-encoded π (22–24). DnaA has been reported to bind to DnaB (25). However, the DnaA site at ori γ of R6K is located some distance away from the A+T-rich, putative DnaB loading site, which is present adjacent to the π-binding iterons (Fig. 1). Therefore, we believe that DnaA is less likely to be the primary recruiter of DnaB to γ, unless a looping mechanism involving π and DnaA brings the DnaA into contact with the A+T-rich region. We wished to investigate if π directly interacts with DnaB helicase. We immobilized π to the wells of plastic microtiter plates, blocked the remaining free plastic surface with BSA, and then challenged the immobilized π with various concentrations of DnaB. DnaC protein that is known to bind to DnaB (26) served as a positive control. BSA and PriA protein of *E. coli* served as negative controls. The DnaB, bound to the π-coated wells, was quantitated, after washing, with primary anti-DnaB rabbit IgG and alkaline phosphatase-conjugated mouse anti-rabbit IgG. The results showed that DnaB protein, as expected, bound to immobilized DnaC. DnaB also bound to immobilized π but not to immobilized PriA or BSA (Fig. 2). Thus, the results showed specific interaction of π protein with DnaB helicase.

Protein Affinity Column Chromatography Confirmed DnaB–π Interaction. We generated an in-frame fusion of full-length π, including the ATG codon, with GST and immobilized the purified hybrid protein on glutathione–agarose beads. Control beads containing only GST protein were also prepared. Full-length DnaB, helicase II, and luciferase were labeled with ^{35}S by coupled *in vitro* transcription and translation, mixed in equimolar amounts, and loaded onto π–GST and control GST beads. The flowthrough fractions and the protein bound to the affinity matrices were resolved in SDS/polyacrylamide gels and autoradiographed. In some cases, the radioactivity present in each band of the gels was quantitated with a PhosphorImager (Molecular Dynamics). The input proteins are shown in Fig. 3*A* (lanes 1–3). The DnaB preparation contains full-length and several truncated peptides generated *in vitro*. In the quantitations of binding data presented below, the full-length and the truncated peptides were measured separately. An autoradiogram of an SDS gel showed that the input proteins, as expected, did not bind to the control GST matrix (binding of helicase II, DnaB, and luciferase were 0.8%, 1.48%, and 3.7%, respectively; 74.7%, 71.1%, and 82.7%, respectively, of the same proteins were recovered in the supernatant) and were recovered in the flowthrough (Fig. 3*A*, lanes 4 and 5). In contrast, 78.6% of the input DnaB was retained on the π–GST matrix (Fig. 3*A*, lane 6). Only traces of the helicase II (7.8%) and luciferase (6.3%) present in the mixture were bound to the π–GST matrix and were recovered in the flowthrough (57.9%, 8.16%, and 84.8% of helicase II, DnaB, and luciferase, respectively, were present in the flowthrough; Fig. 3*A*, lane 7). Thus, there was selective binding of DnaB to the π–GST matrix from an input mixture of proteins that included helicase II and luciferase. The latter two proteins served as negative controls.

Where is the π-interacting surface located on DnaB? We addressed this question by constructing two truncated peptides of DnaB that included two different lengths of the N-terminal region of the protein (see Fig. 3*B*). The peptides were labeled with ^{35}S as described in *Materials and Methods*. Labeled, full-length DnaB and luciferase served as positive and negative controls, respectively. We separately loaded the proteins onto GST and π–GST columns and recovered the bound and flowthrough fractions and resolved the proteins by SDS-gel electrophoresis and autoradiography. The bound fractions are shown, and the data from the flowthrough are mentioned in the text. The intact DnaB, the *Age*I, and the BC3 peptides, but not the luciferase protein, selectively bound to the π–GST matrix. Only trace amounts of binding to the control matrix

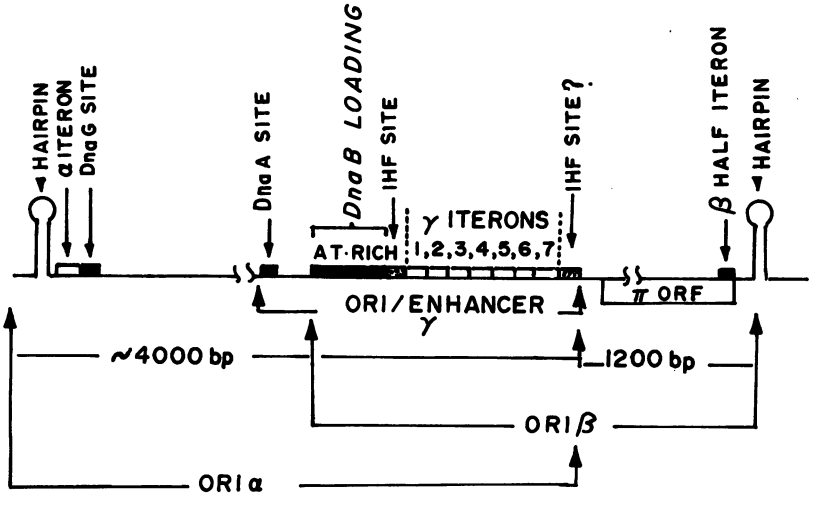

FIG. 1. Features of the replication control sequences of the plasmid R6K. ori α and ori β require the γ origin/enhancer sequence in *cis* for activity. ori α consists of, besides the γ enhancer, a single iteron and a DnaG-binding site. Near the ori α sequence is a hairpin sequence that is also present near ori β. ori β is located in the coding region of the C-terminal end of the π initiator protein and contains a half iteron. The ori γ sequence contains a DnaA-binding site; an A+T-rich, putative helicase loading site; an IHF-binding site; and seven tandem 22-bp-long iterons that bind to π protein followed by a sequence that may be a second IHF site. Activation of ori α and ori β requires π-mediated γ–α and γ–β looping.

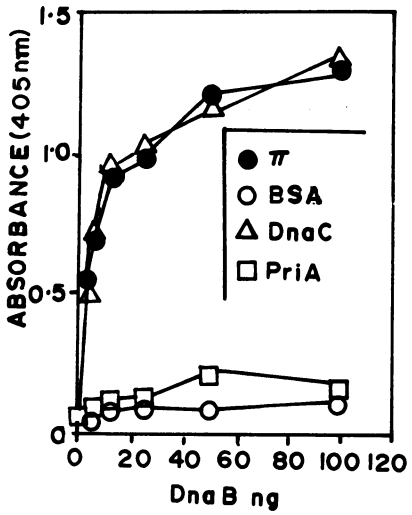

FIG. 2. ELISA of DnaB binding to π, DnaC, PriA, and BSA immobilized on a plastic surface. The bound DnaB was detected by interaction with rabbit anti-DnaB IgG followed by alkaline phosphatase-linked mouse anti-rabbit IgG. Note that DnaB binds equally well to π and DnaC but not to PriA and BSA.

were seen (compare lanes *Age*I/π, BC3/π, DnaB/π, and luc/π with lanes *Age*I/C, BC3/C, DnaB/C, and luc/C, respectively, in Fig. 3*B*). Full-length DnaB (88.0%) and its peptides *Age*I (76.9%) and BC3 (81.9%) bound to the π–GST matrix, the remainder being recovered in the flowthrough. The control beads bound only 1.79%, 2.49%, and 1.69% of the input *Age*I, DnaB, and BC3 peptides, respectively. In contrast, only 0.62% of the input luciferase was bound to the π–GST beads. Almost all of the input luciferase was found in the flowthrough of both the matrices.

We wished to confirm and further define the region of DnaB that interacted with π by generating tryptic peptides of DnaB and investigating the binding of the peptides to π–GST and control GST matrices. The controlled tryptic digestion of DnaB in the presence of ATP and Mg^{2+} is known to generate a 50-kDa peptide by the removal of 14 amino acids from the N terminus. Further digestion cleaves the 50-kDa peptide into an N-terminal 33-kDa piece and a C-terminal 12-kDa piece. Withholding ATP from the reaction mixture generates the 12-kDa piece in far excess over the 33-kDa piece (refs. 27 and 28; Fig. 4 *A* and *B*). We performed controlled tryptic digestion of DnaB in the presence of ATP and Mg^{2+}, and also in the absence of ATP, and stopped the digestion by adding soybean trypsin inhibitor. The digestion mixture (with 50-, 33-, and >12-kDa peptides) was loaded onto π–GST and GST matrices. The flowthrough and bound fractions were recovered and resolved in SDS/polyacrylamide gels, blotted onto nitrocellulose membranes, and developed with antibodies to DnaB as described. The input protein showed a prominent 50-kDa band and a smaller amount of the 33-kDa band. The 12-kDa peptide that was present in excess (by adding a separate tryptic digest that had a large amounts of 12-kDa peptide) appears as a diffused band (Fig. 4*B*, lane 1). The control GST beads retained only trace amounts of the input protein, most of which was recovered in the flowthrough (Fig. 4*B*, lanes 4 and 5). In contrast, the π–GST matrix selectively retained the 50- and 33-kDa peptides but not the 12-kDa peptide, which was recovered in the flowthrough (Fig. 4*B*, lanes 2 and 3). The N-terminal amino acid sequences of the 50-, 33-, and 12-kDa peptides are known (27, 28). Thus, by combining the binding data of the *Age*I, BC3, and tryptic peptides, the π-interaction domain of DnaB could be localized to the 37-aa-long region that overlapped the *Age*I and 33-kDa peptides. The sequence

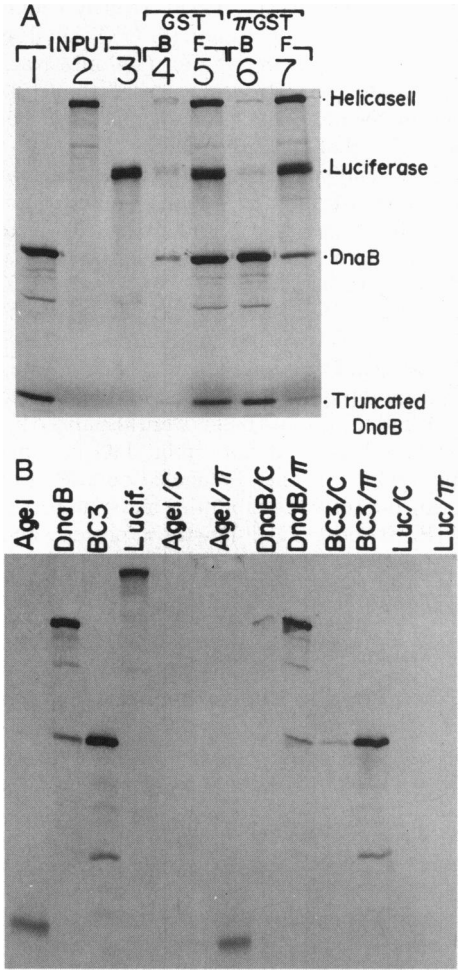

FIG. 3. Autoradiograms of SDS/15% polyacrylamide gels showing interaction between π and DnaB. (*A*) Autoradiogram showing the specific interaction of full-length DnaB with π–GST beads. Lane 1, input DnaB (note several minor and one major truncated product along with full-length DnaB); lanes 2 and 3, input helicase II and luciferase, respectively; lane 4, protein bound (**B**) to control GST matrix (note that only trace amounts of the proteins are bound to the control column); lane 5, flowthrough (**F**) from the control column (note that almost all of the applied proteins are present in the flowthrough); lane 6, proteins bound (**B**) to the π–GST matrix (note that DnaB and its peptides have been selectively retained on the column, whereas only trace amounts of helicase II and luciferase have bound to the matrix); lane 7, flowthrough (**F**) fractions from the π–GST matrix [note that most of the applied helicase II and luciferase, but a much smaller amount (8.16%) of the input DnaB, was present in the flowthrough]. (*B*) The lanes *Age*I, DnaB, BC3, and Lucif. show the respective input proteins; *Age*I/C, *Age*I retained by the control matrix; *Age*I/π, *Age*I peptide retained by the π–GST matrix; DnaB/C, DnaB retained on the control matrix; DnaB/π, DnaB retained on the π–GST matrix; BC3/C, BC3 retained on the control matrix; BC3/π, BC3 retained on the π–GST matrix; luc/C and luc/π, luciferase bound to the control matrix and the π–GST matrix, respectively. Note that the intact DnaB, the *Age*I, and the BC3 peptides but not luciferase are retained selectively on the π–GST matrix, whereas the binding of the same proteins to the control matrix is minimal.

of the peptide is ANKDEGPKNIADVLDATVARIEQLFQQPHDGVTGVNT (based on refs. 27 and 28).

Reciprocal Binding of π to a DnaB Affinity Matrix. To confirm further the specific interaction between π and DnaB, we prepared in-frame fusions of DnaB, including its ATG codon, with GST, purified the fusion protein, and immobilized the protein on glutathione–agarose beads. π protein was labeled with ^{35}S *in vitro* and loaded onto the DnaB–GST and the control GST columns. The π protein, under conditions of

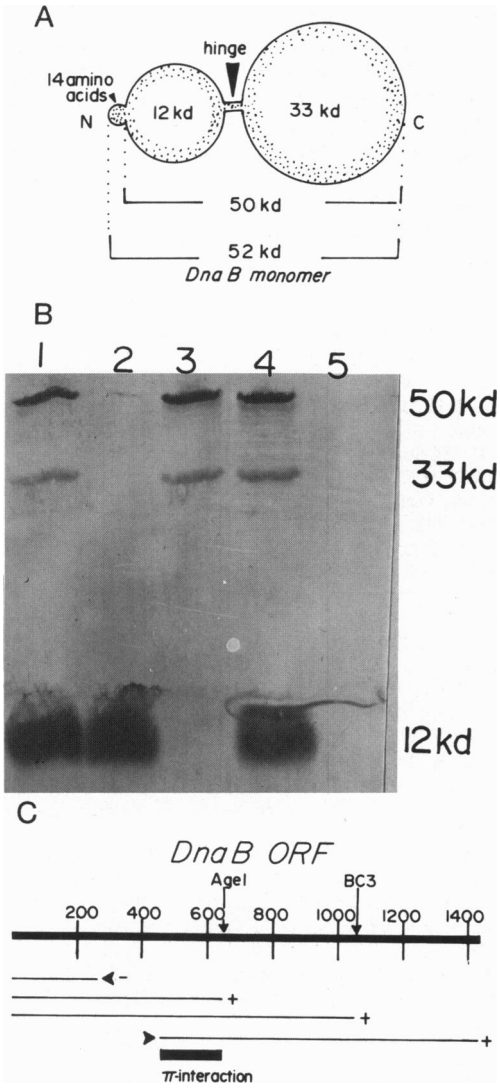

FIG. 4. Binding of tryptic peptides of DnaB to the π–GST affinity column. (*A*) Schematic representation of the sequence of trypsin cleavage of DnaB. Controlled cleavage first removed an N-terminal, 14-aa-long peptide, generating a 50-kDa peptide. Further cleavage at the hinge region generated an N-terminal, 12-kDa peptide and a C-terminal, 33-kDa peptide. This sequence of cleavage occurs in a controlled mode in the presence of ATP or ADP and Mg^{2+} (27, 28). (*B*) Western blot of peptides adsorbed to and eluted from π–GST and control beads. Lane 1, input tryptic peptides of DnaB (the 50-, 33-, and 12-kDa peptides). Note that the amount of 12-kDa DnaB peptide was deliberately increased by mixing DnaB digested with and without ATP to ensure that high amounts of 12-kDa peptide were present in the input but that it still did not show any binding to the π–GST column. Lanes 2 and 3, the peptide found in the π–GST supernatant and retained by the π–GST matrix, respectively, as revealed by interaction with polyclonal anti-DnaB antibodies; lanes 4 and 5, peptides found in the control GST matrix supernatant and that retained by control GST matrix. Note that the 50- and 33-kDa peptides but not the 12-kDa peptide bind to the π–GST beads (lane 3), and the 12-kDa peptide is found in the supernatant (lane 2).

lower salt concentrations used in the binding buffer, tends to precipitate out and thus shows some background binding to the GST matrices. In spite of this difficulty, the labeled π showed selective binding to the DnaB–GST column and a lower level of binding to the control GST column (Fig. 5; compare lanes *B* and *C* with lanes *D* and *E*). Up to 70% of the input π bound to the DnaB–GST column, whereas 10–15% bound to the control column.

Mapping the DnaB Interaction Domain of π. We cleaved the DNA encoding π with *Bgl*II, which resolved the coding

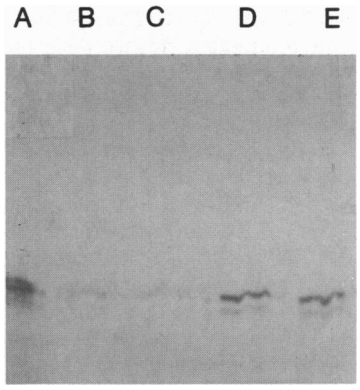

FIG. 5. Autoradiogram of an SDS/15% polyacrylamide gel showing the interaction of ^{35}S-labeled π protein with GST–DnaB fusion protein immobilized on glutathione–agarose beads. Lane A, input π; lanes B and C (duplicate lanes), π bound to control GST–agarose; lanes D and E (duplicate lanes), π bound to DnaB–GST–agarose. The π protein has a tendency to precipitate, giving slight background binding to the control beads. The faint bands below the full-length π protein in lanes D and E are truncated π protein generated during coupled *in vitro* transcription–translation. These peptides probably lack the C-terminal segment.

region into a 116-aa-long N-terminal fragment and a longer (amino acids 117–308) C-terminal fragment. The two DNA pieces encoding π were fused in-frame with GST, expressed, and purified. Strictly molar equivalent amounts of both proteins were separately immobilized on glutathione–agarose beads. We confirmed that equivalent amounts of both proteins were immobilized on the beads by boiling the beads and resolving the released fusion proteins by SDS-gel electrophoresis. Equal amounts of DnaB, labeled *in vitro*, were loaded onto the GST–N-terminal π, the GST–C-terminal π, and the control GST matrices. The flowthrough and bound fractions were recovered and analyzed by SDS/polyacrylamide gels. An autoradiogram of such a gel showed that the control GST beads retained only trace amounts (0.5–0.9%) of the labeled protein, most of which were recovered in the flowthrough (Fig. 6, lanes 2, 3, 8, and 9). The N-terminal π–GST beads selectively retained up to 57.9% of the input π protein, the remainder being recovered in the flowthrough (Fig. 6, lanes 4 and 5). In contrast, the C-terminal π–GST beads retained only 0.056% of the protein and most of the protein was recovered in the flowthrough (Fig. 6, lanes 6 and 7). Thus, the 116-aa-long, N-terminal peptide of π, but not the C-terminal part, had the

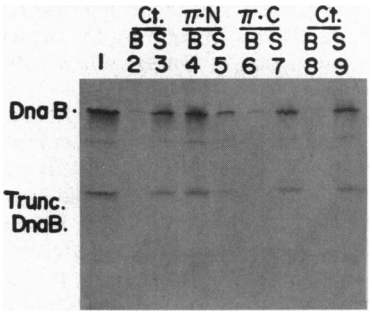

FIG. 6. Interaction of DnaB with the N-terminal region of π. Ct, control beads; π-N, N-terminal π–GST beads; π-C, C-terminal π–GST beads; B, bound fraction; S, supernatant fraction; Trunc. DnaB, truncated DnaB. Lane 1, input protein; lanes 2, 3, 8, and 9, protein bound to the control matrix and present in the flowthrough; lanes 4 and 5, protein bound to the N–π matrix and recovered in the flowthrough, respectively; lanes 6 and 7, protein bound to the C–π matrix and present in the flowthrough, respectively. Note that the DnaB protein selectively binds to the N–π matrix but binds poorly or not at all to the C–π matrix.

domain for interaction with DnaB. The results not only provide the location of the interaction surface on π but also provide further confirmation of the specificity of the protein–protein interaction.

DISCUSSION

The requirement for DnaB in R6K replication has not been specifically determined. However, the facts that the plasmid does not encode a helicase and that it replicates in a Cairns type mode (2, 5, 6) strongly suggest that the main replicative helicase, DnaB, encoded by the host is used for R6K replication. The results showing that the DnaB helicase specifically binds to the R6K plasmid-encoded π protein is significant because of its implications for the mechanism of initiation of R6K replication. The γ origin/enhancer of R6K contains a binding site for DnaA at a location that is $\approx$300 bp upstream from the π-binding iterons (Fig. 1). We have shown that replication of ori γ *in vitro* requires both DnaA and π proteins (22). Other investigators (25) have shown that DnaB interacts with DnaA. These observations, considered together, prompt the following question. Is it DnaA or π (or both) that recruits DnaB helicase to the γ origin/enhancer? This question can be approached by making mutants of π and DnaA that no longer interact with DnaB. However, the immediate proximity of π-binding iterons to the putative DnaB loading site (Fig. 1) at ori γ and the fact that γ–β and γ–α looping involves π-binding iterons would suggest that π most probably is the primary recruiter of DnaB helicase to the γ region. Subsequent DNA looping probably delivers DnaB to ori α and ori β. Future work will test this later prediction and map the entry site of DnaB at ori α and ori β. It is worth noting in this context that ori α has a DnaG primase-binding site (ref. 17; Fig. 1). We have recently observed direct interaction between DnaB helicase and DnaG primase *in vitro* (Y. Lu, P.V.A.L.R., B.K.M., and D.B., unpublished data). It would be interesting to investigate whether DnaB is delivered to ori α by DNA looping between γ and or α and whether the delivery occurs by interaction between π-bound DnaB with DnaG located at the G site of ori α. With the use of mutants of π that no longer interact with DnaB, it will be interesting to investigate if ori α and ori β are no longer activated by such mutants and if the known interaction of DnaB with DnaA (25) can partially bypass the defect in π and activate ori γ.

In bacteriophage λ, the phage-encoded P protein recruits one or more molecules of DnaB to the phage origin of replication. The delivery is mediated by the interaction of P protein with the origin-bound O protein. Thus, P serves as a linker between O protein and DnaB helicase (29) during the assembly of the replisome. In contrast, the present data would suggest direct interaction of DnaB with π without an intermediary protein.

Recently, we have discovered that the RepA initiator protein of the plasmid pSC101 also interacts with DnaB (P.V.A.L.R. and D.B., unpublished data). It would be interesting to determine whether other plasmid-encoded initiator proteins also interact with DnaB and whether the host-encoded initiator DnaA shares the same interaction surface on DnaB with the plasmid-encoded initiator proteins. Finally, it would also be interesting to determine whether the host range of a plasmid is determined by the ability of the plasmid-encoded initiator protein to interact with DnaB or DnaB homologs encoded by the host bacteria. The present work is significant because it reveals one of the early, protein–protein interaction steps leading to the initiation of plasmid DNA replication, by a looping mechanism.

Considering the observation that π protein is able to interact directly with DnaB, without the assistance of DnaA and DnaC proteins, one would like to answer in the future the following question. What roles do DnaA and DnaC proteins play in R6K replication?

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