Mechanism of recruitment of DnaB helicase to the replication origin of the plasmid pSC101
(preinitiation complex/reciplication initiation)

HIROCK J. DATTA*, GHAN SHYAM KHATRI†, AND DEEPAK BASTIA*‡

*Department of Microbiology, Duke University Medical Center, Durham, NC 27710; and †Institute of Biochemical Technology, Council of Scientific and Industrial Research, New Delhi, India

ABSTRACT Although many bacterial chromosomes require only one replication initiator protein, e.g., DnaA, most plasmid replicons depend on dual initiators: host-encoded DnaA and plasmid-encoded RepA initiator protein for replication initiation. Using the plasmid pSC101 as a model system, this work investigates the biological rationale for the requirement for dual initiators and shows that the plasmid-encoded RepA specifically interacts with the replicative helicase DnaB. Mutations in DnaB or RepA that disrupt RepA–DnaB interaction cause failure to load DnaB to the plasmid ori in vitro and to replicate the plasmid in vivo. Although, interaction of DnaA with DnaB could not substitute for RepA–DnaB interaction for helicase loading, DnaA along with integration host factor, DnaC, and RepA was essential for helicase loading. Therefore, DnaA is indirectly needed for helicase loading. Instead of a common surface of interaction with initiator proteins, interestingly, DnaB helicase appears to have at least a limited number of nonoverlapping surfaces, each of which interacts specifically with a different initiator protein.

Since its discovery by Cohen and Chang (1), pSC101, a tetracycline-resistant plasmid replicon, has become a favorite system for the analysis of the control of plasmid replication and stability (2–8). The plasmid replicon consists of a cis-acting ori sequence and a plasmid-encoded, trans-acting initiator protein called RepA. We have reported the purification of the RepA protein and its mode of interaction with the three iterons and the two inverted repeats of the plasmid ori (Fig. 1; refs. 3 and 4). Although initiation of replication of several prokaryotic replicons, e.g., Escherichia coli, phage λ, Bacillus subtilis, and the eukaryotic replicon simian virus 40 requires single initiator proteins (9), pSC101 (and several other plasmids) requires both the host-encoded DnaA protein and the plasmid-encoded RepA protein (10, 11).

An examination of the minimal ori of the plasmid reveals a single DnaA box (binding site dnaA S) that is separated from the RepA-binding iterons by a naturally bent AT-rich segment. Embedded in the AT-rich region is a binding site for the host-encoded DNA bending protein called IHF. The interaction of IHF with the cognate site further bends the DNA and the interaction is essential for plasmid replication (Fig. 1; refs. 12 and 13). IHF appears to enhance the binding of DnaA to the ori in vitro, and its principal role is believed to be binding of the DNA and thereby promoting contact between DnaA and RepA proteins that are bound to physically separated cognate sites and to weaker dnaA S sites in the iterons (11). Recently, we have discovered that RepA and DnaA specifically interact with each other in the absence of DNA and IHF and that the interaction is essential for initiation of pSC101 replication (H.J.D. and D.B., unpublished work). Cohen and Biek (14, 15) have shown that the requirement for IHF can be bypassed by either mutations in repA or in the host-encoded topA genes. Thus, suppressor mutations either cause alternative origin conformations that lead to DnaA–RepA interactions in the absence of IHF or else the mutations obviate the need for such an interaction.

This work was initiated to address two interrelated and interesting questions. First, besides origin binding, what other biochemical functions (e.g., protein–protein interactions) can be attributed to the plasmid initiator proteins? Second, because the recruitment of the replicative helicase is a key step of initiation, is it DnaA or RepA or both initiators that recruit the DnaB helicase to the ori and does this process involve pairwise interactions between DnaB and the two initiators? In this paper we present evidence showing specific protein–protein interaction between RepA and DnaB. We have identified the surfaces of both DnaB and RepA that participate in the interaction and have isolated noninteracting mutants that affect both surfaces. In vivo analysis showed that the interaction was essential for pSC101 replication. In vitro experiments showed that four purified proteins (IHF, DnaA, RepA, and DnaC) were needed to load DnaB helicase onto the plasmid replication origin and a mutant that disrupted DnaB–RepA, but not DnaA–DnaB, interaction, had a detrimental effect on helicase loading. Thus, without the critical protein–protein interaction between RepA and DnaB, DnaA by itself could not load the helicase at the plasmid ori.

MATERIALS AND METHODS

DNA Constructs, Plasmids, and Bacterial Strains. The plasmid pCV2 contains the minimal ori of pSC101 (in a HincII fragment), cloned into the vector pUC9 (4). The DnaB tagged with the kinase site was a gift from Mike O’Donnell (The Rockefeller University, New York; refs. 16 and 17). The bacterial strains DH5α [Arββ] supE44 lacU169 (680 lacZ Δ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1] and BL21 (DE3) [E. coli B: F– ompT hsdS (rK mK +) gal(ΔADE3)] were used for standard cloning and protein expression, respectively.

Protein Purification. The RepA, DnaA, IHF, and DnaB proteins were purified as described (12, 18–20). DnaC was purified from a overproducer strain that coexpressed DnaB and DnaC (a gift from N. Dixon, University of Sydney, Sydney, Australia). The strain yielded free DnaB and DnaC and a 1:1 complex of B-C that were separated on a monoQ column. The
RESULTS

Demonstration of Specific RepA–DnaB Interaction and Mapping of the Interaction Surface on DnaB. To detect possible interaction between DnaB and RepA, we constructed recombinant clones that expressed full-length and the following partial peptides of DnaB from a T7 promoter. The peptides were fused at the N-terminal end, in-frame with a hexahistidine moiety that made it convenient to purify the proteins on Ni-nitrioltriacetic acid-agarose affinity columns (Ni columns). The peptide AgeI was encoded in the DNA fragment that extended from the first ATG codon of DnaB to its 208th codon (that is marked by an AgeI restriction site; hence the name AgeI). The peptide BC2 extended from the first codon to the 78th codon (Fig. 2A). The full-length DnaB, the AgeI, and BC2 peptides were purified and immobilized on Ni-agarose. 32P-labeled RepA protein was generated by coupled in vitro transcription and translation and bound to the full-length DnaB and the AgeI and BC2 peptide columns and to control Ni-agarose columns without bound DnaB or its peptides. The bound protein was eluted and analyzed by SDS/PAGE. It should be noted that the coupled transcription translation of the complete RepA gene not only generated a full-length peptide but also a partial peptide from an internal methionine (Tr.RepA, Fig. 3, lane 1). Labeled luciferase and π protein of R6K (Fig. 3, lane 2) were used as negative and positive controls, respectively.

 Autoradiograms of the gels revealed that immobilized full-length DnaB, but not the control Ni-agarose matrix, readily retained RepA (Fig. 3, lanes 3 and 4, respectively). Luciferase did not bind to either the immobilized DnaB or to the control matrix (data not shown). While the immobilized BC2 peptide retained RepA (Fig. 3, lane 5), the shorter AgeI peptide failed to retain the labeled protein (Fig. 3, lane 6). However, as previously reported (19), the immobilized AgeI peptide bound to labeled π initiator protein of R6K (Fig. 3, lane 7). We have similarly mapped the binding site of DnaA to a region immediately downstream of the BC2 peptide (H.J.D. and D.B., unpublished work). Thus, interestingly, three different initiator proteins (RepA, π, and DnaA), instead of sharing a common binding site on DnaB, bound to three linearly con-
peptide. Lane 6, immobilized DnaB–DnaB. Lane 4, control Ni-Agarose. Lane 5, immobilized DnaB–c64-c142 peptide of RepA. N-terminal c1-c63. Lane 6, C-terminal c143-c316. Lane 7, N-terminal control GST matrix. Lane 4, immobilized peptide c1-c142. Lane 5, immobilized DnaB–AgeI peptide. Lane 7, π bound to immobilized DnaB–AgeI peptide.

**Fig. 2**

Coordinates 192 to 426. The binding results are summarized in long peptide segment encoded by the DNA from nucleotide RepA that interacted with DnaB was localized to the 78-aa-

**Fig. 3**

Autoradiograms of SDS-10% PAGE showing the specific binding of DnaB to RepA protein. Lane 1, input full-length RepA (and a truncated form Tr.RepA, produced by internal translation initiation). Lane 2, input π. Lane 3, RepA bound to full-length immobilized DnaB. Lane 4, control Ni-Agarose. Lane 5, immobilized DnaB-BC2 peptide. Lane 6, immobilized DnaB–AgeI peptide. Lane 7, π bound to immobilized DnaB–AgeI peptide.

**Fig. 4**

Autoradiograms of SDS-10% PAGE showing the mapping of the surface of RepA that interacts with DnaB. Lane 1, DnaB input. Lane 2, DnaB binding to full-length immobilized RepA. Lane 3, control GST matrix. Lane 4, immobilized peptide c1-c142. Lane 5, N-terminal c1-c63. Lane 6, C-terminal c143-c316. Lane 7, N-terminal c64-c142 peptide of RepA.
each transformation were picked at random and grown for multiple generations (>12 hr) in Luria broth at the nonpermissive temperature (42°C) without selection for ampicillin resistance. The cells then were plated on ampicillin-Luria agar plates at 42°C. Table 1 presents the average of three separate sets of experiments. The vector plasmid, as expected, was lost during growth in polA12 cells at 42°C. The chimeric pCV2 plasmid containing the wild-type RepA gene as expected replicated at 42°C in polA12 cells. The pCV2 m₃ plasmid that contained the m₃ mutant form of RepA was more readily lost from polA12 cells at 42°C in comparison with the pCV2 control (Table 1).

In Vitro Recruitment of DnaB to the ori of pSC101 Required DnaB–RepA Interaction. DnaB protein was tagged at the N-terminal end by fusion with a muscle kinase recognition sequence and labeled with muscle kinase and γ²P[ATP]. The fusion, as reported before (16, 17), and confirmed by us (data not shown), did not affect the helicase activity of DnaB. The strategy was to incubate the labeled DnaB with purified DnaC, IHF, DnaA, RepA, SSB proteins, and supercoiled pUC19 plasmid containing the minimal ori of pSC101 (or oriC, used as a control), in the molar proportions indicated in a preceding section and measure the retention of DnaB at the ori (Fig. 6A). The purified proteins mentioned above were chosen because of their known role in the preinitiation steps of oriC replication (9). After the incubation, the reaction mixtures were fractionated in 0.5 × 2.5 cm Biogel 1.5 M gel filtration columns to separate the protein–DNA complex from the free proteins. The label appearing in the excluded protein–DNA complex peak provided a measure of the amount of DnaB recruited to the plasmid or the host ori.

The loading of DnaB to the pSC101 ori required all four purified proteins (RepA, DnaA, DnaC, and IHF), and omission of any one of the four proteins from the reaction mixture almost completely abolished the helicase recruitment (Fig. 6A). Although SSB was used initially in the reaction mixture, subsequent work showed that its presence was unnecessary for helicase loading. As a control, pUC19 DNA without the pSC101 ori sequence was used in the reaction, and the small amount of background radioactivity was deducted from each experimental data point. The pUC19 control experiment showed that the recruitment of DnaB depended on the pSC101 ori sequence. The data in Fig. 6A represent the average of three independent sets of experiments. A quantitative estimate of helicase loading is shown in the histograms of Fig. 6B.

The m₃ mutant form of DnaB also was labeled with ³²P and by similar analysis showed a significant reduction in DnaB loading. The mₓ mutant form of RepA that showed reduced physical association with DnaB also was analyzed and it also showed a significant decrease in loading of DnaB to the ori (Fig. 6A and B). Thus, a reduction in physical interaction between DnaB and RepA was accompanied by a corresponding reduction in the ability of the mutant forms of the proteins to recruit DnaB helicase to the ori. We used the oriC plasmid in control experiments and performed similar helicase loading experiments (Fig. 6C), and the data (average of three experiments) are summarized in Fig. 6D. Wild-type DnaB and the mₓ protein loaded almost equally well onto oriC (30 fmol and 25 fmol of loading, respectively; Fig. 6D), whereas the mutant form consistently showed a significant reduction in loading to

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>No. of colonies after growth at 42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>2</td>
</tr>
<tr>
<td>pCV2</td>
<td>1,070</td>
</tr>
<tr>
<td>pCV2 (RepA mₓ mutation)</td>
<td>28</td>
</tr>
</tbody>
</table>

The data represent an average of three experiments.
the plasmid ori (27 fmol versus 8 fmol of loading promoted by
the wild-type and the m3 mutant form of RepA, respectively; Fig. 6D). Thus the m3 mutation selectively and significantly
diminished the loading of DnaB helicase to the plasmid ori
without significantly altering the loading to the host oriC.

DISCUSSION

Because the realization that initiation of replication of several
plasmid systems requires dual initiator proteins, namely DnaA
encoded by the host and the other encoded by the plasmid
replicon, the relative role of DnaA in plasmid replication as
contrasted with its role in the initiation of replication of oriC
of the host chromosome had remained obscure (10, 18, 24, 25).
We have endeavored to investigate the biological roles of the
twin initiators by systematically identifying the interactions
between two proteins (and other replisomal proteins) and
attempting to determine the physiological significance of such
interactions. We previously have reported the interaction
between proteins R6K and DnaB (19) but the physiological
role of the interaction remains to be determined.

The results described in this paper provide an important
insight into the relative roles of RepA and DnaA proteins in
pSC101 replication, i.e., RepA–DnaB interaction plays an
indispensable role in helicase recruitment to the plasmid ori,
and this requirement cannot be bypassed by DnaA–DnaB
interaction. Three other proteins (DnaA, DnaC, and IHF) also
are needed for the loading reaction. The following is a brief
consideration of the possible roles played by each of these
proteins.

What role does DnaA play in helicase recruitment? Despite
the known interaction between DnaA and DnaB (26), DnaA
by itself does not seem to be capable of sequestering the
helicase at the plasmid ori revealed by the inability of the m3
form of DnaB to be loaded onto the ori. It is worth recalling
in this context that the m3 helicase, although defective in its
interaction with RepA, retained normal interaction with
DnaA. Furthermore, both the wild-type and the m3 mutant
forms of DnaB were almost equally capable of carrying out
lagging strand replication of oriC in a DnaA-mediated ABC
primosome system, thereby suggesting that the mutation
specifically disrupted the plasmid but not host replication.

We previously have presented evidence that supported
interaction between RepA and DnaA at the replication origin
of pSC101 (11). Mutant forms of DnaA that support oriC
replication, but not pSC101, replication have been reported,
and these mutants also imply a functional interaction between
the two proteins (27). We recently have discovered a direct
physical interaction between DnaA and RepA (H.J.D. and
D.B., unpublished work) in the absence of DNA and hypoth-
ositize that this interaction, which is promoted by the DNA
bending action of IHF (see Fig. 1), is critical for the initial
unwinding at the plasmid ori. This initial unwinding probably
generates the single-stranded DNA that facilitates helicase
entry into the ori. Although this postulated two-step mecha-
nism for helicase loading awaits verification by the isolation
and analysis of mutants of RepA that no longer interact with
DnaA, we have isolated similar mutants in the p, initiator
protein of R6K. Recently, we have reported that DnaA protein
specifically interacts with the p initiator of R6K, and muta-
tional disruption of this interaction leads to loss of ori un-
winding in vitro and plasmid replication in vivo (23). The
preceding observations extrapolated from the R6K system
would suggest that DnaA–RepA interaction, which is facil-
tated by the DNA bending protein IHF, is needed for pSC101
ori unwinding. This initial unwinding probably generates the
single-stranded region needed for the entry of DnaB. The
collaboration between DnaA and a plasmid-encoded initiator
for ori unwinding also has been observed in another sys-
tem (28).

The role of DnaC in the helicase loading is not clear at the
present time. ATP alone has been reported to load the
toroidal, hexameric ring of DnaB onto naked single-stranded
DNA circles in the absence of RepA (27). DnaC is known to
form a 1:1 complex with DnaB (29) and perhaps this interac-
tion stabilizes the helicase complex and thus helps the loading
process. Isolation of mutants of DnaB and DnaC that prevent
the protein–protein interaction and biochemical analysis of the
mutants might shed light on the role of DnaC in preinitiation.

How general might be this mechanism of helicase recruit-
ment? We expect it to be common to other plasmid systems
where the plasmid initiator interacts with the host helicase
e.g., R6K, ref. 19). However, in the broad host range plasmid
RK2, the initiator protein TrfA does not seem to interact with
DnaB and the host-encoded DnaA by itself seems to recruit
the helicase to the ori (30). In phase lambda, helicase recruit-
ment involves protein–protein interaction between the phage-
encoded O and the P proteins and between P and DnaB. P
serves as a bridge between the O and DnaB proteins (31). Thus
several different pathways of helicase loading seem to operate
in nature.

The DNA-binding domain of RepA protein appears to be
located toward the C terminus (32), whereas most of the
specific protein–protein interaction seems to involve the N-
terminmal region (this paper and H.J.D. and D.B., unpublished
work). The investigations of protein–protein interactions be-
tween replisomal proteins continues to provided important
insights into the mechanism of DNA replication and its
control. Recent work shows that interaction with DnaB distinguishes the leading strand DNA polymerase III holoenzyme from the one that synthesizes the lagging strand and the interaction of DnaB with the \( \tau \) subunit of the DNA polymerase III holoenzyme heterodimer coordinates the rate of polymerization with that of DNA unwinding (17, 33). We recently have discovered that RepA protein (and DnaA and \( \pi \)) also interacts specifically with DnaG primase and with the \( \tau \) subunit of DNA polymerase III (H.J.D. and D.B., unpublished work). Do initiator proteins also catalyze chain elongation? This question and other interesting ones remain to be answered.

This work was supported by a Merit Award to D.B. from the National Institute of Allergy and Infectious Diseases and a grant from the National Institute of General Medical Sciences.