Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA bending protein at the replication origin of pSC101

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We have investigated the interaction of the host-encoded DNA bending protein IHF, the host-encoded initiator DnaA, and the plasmid-encoded initiator RepA with the replication origin of pSC101. We have discovered that DNA bending induced by IHF in vitro promoted the interaction of DnaA protein with two physically separated binding sites called *dnaA*$_s$ and *dnaA*$_w$. This cooperative interaction at a distance, most probably, caused looping out of the *ihf* site. We have also discovered that RepA protein binding to its cognate sites promoted enhanced binding of DnaA protein to the physically distant *dnaA*$_s$ site, probably also by DNA looping. The addition of RepA to a binding reaction containing IHF and DnaA further enhanced the binding of DnaA protein to the *dnaA*$_s$ site. Thus, the three DNA-binding proteins interacted with the origin, generating a higher order structure in vitro. On the basis of the results of the known requirement of all three proteins for replication initiation, we have proposed a model for the structure of a preinitiation complex at the replication origin.

[*Key Words:* Initiation of replication; DNA–protein interaction; integration host factor]

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In addition to several host-encoded replisomal proteins, three sequence-specific origin-binding proteins are necessary for the initiation of replication of the plasmid pSC101 (Cohen and Chang 1977). These are the host-encoded initiator called DnaA (Hasunuma and Sekiguchi 1977), the plasmid-encoded initiator RepA (Vocke and Bastia 1983a,b), and the host-encoded DNA bending protein called integration host factor (IHF; Gamas et al. 1986; Prentki et al. 1987; Stenzel et al. 1987).

IHF is a ~20-kD heterodimeric, histone-like protein that binds to specific sequences in DNA and is involved in replication (Gamas et al. 1986; Stenzel et al. 1987), site-specific recombination (Craig and Nash 1984; Friedman 1988; Robertson and Nash 1988; Thompson and Landy 1988; Nash 1990), and transcription (Hoover et al. 1990).

Interaction of IHF with its binding site at the origin of replication (ori) causes a large bend in the DNA (Stenzel et al. 1987). The observation that IHF often binds to DNA at or near sites that interact with a variety of other sequence-specific DNA-binding proteins prompted the suggestion that IHF may promote protein–DNA and protein–protein interaction by bending the DNA rather than by direct interaction with a variety of other proteins in the neighborhood of its binding site (Goodman and Nash 1989). This suggestion has been supported by the observed role of IHF in λ integration (Moitoso de Vargas et al. 1989).

DnaA protein is the key initiator protein of the host chromosome that is also required for pSC101 replication (Hasunuma and Sekiguchi 1977) in a way that is, hitherto, unclear. Plasmid pSC101 replication is also totally dependent on the plasmid-encoded RepA initiator protein (Vocke and Bastia 1983a,b).

We have reported that a mutant *ihf* site that shows impaired binding to IHF in vitro fails to replicate in vivo (Stenzel et al. 1987). Previously, we have proposed a model suggesting that IHF-mediated DNA bending caused looping of the DNA at the ori, allowing DnaA protein bound at the *dnaA*$_s$ site to interact with RepA protein bound to *repA* sites and perhaps with other associated proteins (Stenzel et al. 1987).

In this paper, we have attempted to test certain predictions of the looping model by in vitro protein–DNA interaction studies and by site-directed mutagenesis. Our results revealed cooperativity at a distance between

This paper is dedicated to Dr. Sherman Weissman on the occasion of his 60th birthday.

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the primary $dnaA_s$ site (Gaylo et al. 1987) and the newly discovered $dnaA_w$ sites reported here. We have also discovered that IHF promoted the interaction between the two sites and that RepA protein bound to its cognate site also promoted binding of DnaA protein to the physically distinct $dnaA_s$ site. Although other models cannot be strictly excluded at this time, the available evidence strongly supports distant site–site interaction by DNA looping at the ori region of pSC101 in vitro.

Results

IHF enhances the binding of the DnaA protein to the ori of pSC101

Does IHF promote interaction between distant or physically noncontiguous sites (Fig. 1) at the ori presumably by DNA looping? Does this promotion of cooperativity at a distance require specific binding of IHF to the *ihf* site? We approached these questions by performing DNase I footprinting experiments to measure occupation of the cognate binding site(s) by DnaA protein in the presence and absence of IHF on a normal DNA substrate and on a mutant substrate that had three point mutations in the *ihf* site. Previous work has shown that the triple mutant does not bind to IHF at low concentrations of the protein, but some binding does occur at high concentrations of IHF (Stenzel et al. 1987).

The DNase I footprints of the normal DNA substrate showed that the footprints of IHF and DnaA did not overlap, which suggested a lack of direct side-by-side contact between these two proteins on a linear array. The footprints also showed that binding of IHF to its cognate site enhanced the binding of DnaA significantly, not only to its known consensus site called $dnaA_s$ (strong site), but also to a set of previously unknown weak sites ($dnaA_w$). The magnitude of the enhanced binding has been quantified by densitometry and showed an enhancement of binding of approximately sixfold and twofold to the $dnaA_w$ and $dnaA_s$ sites, respectively (see legend to Fig. 2). Using the *ihf* mutant DNA, the enhancement of DnaA binding to the $dnaA_s$ site was essentially abolished and the enhancement of DnaA binding to the $dnaA_w$ site was greatly reduced (Fig. 2, left and right; cf. lanes C, D, and E with J, K, and L, respectively). Whereas IHF binding to the normal *ihf* site promoted an enhancement of binding of approximately sixfold to the $dnaA_w$ site, the mutant *ihf* site allowed an enhancement of only approximately twofold binding to the $dnaA_w$ site (see legend to Fig. 2). The enhancement of binding seen on the mutant *ihf* DNA at the $dnaA_w$ sites can be explained by our previous observation (Stenzel et al. 1987) that IHF does bind to the mutant site at high concentrations of the protein.

The most striking manifestation of DnaA binding to the $dnaA_w$ sites in the normal DNA substrate was the appearance of four enhanced bands at the $dnaA_w$ sites as well as partial clearing of adjacent bands. Because the $dnaA_w$ and $dnaA_s$ sites were separated by ~150 bp of DNA that contained the *ihf* site and because the footprints of the two proteins were nonoverlapping, the results suggested cooperativity at a distance between the $dnaA_s$ and $dnaA_w$ sites, probably by IHF-induced enhanced bending of the already bent intervening AT-rich DNA (see Fig. 1). We proceeded to test the looping model by site-directed mutagenesis and DNase I footprints of the mutant DNAs as described below.

Deletion of the $dnaA_s$ *and the* $dnaA_w$ *sites indicates that each site promotes occupation of the other site by DnaA protein*

Using the synthetic oligonucleotide 1 (see Materials and methods), we specifically deleted the $dnaA_s$ site, thus generating the 9-bp deletion called $\Delta dnaA_s$, and measured the occupation of the $dnaA_w$ sites by DnaA protein in the presence and absence of IHF on the mutant DNA by DNase I footprinting. The results showed a dramatic reduction in the occupation of the $dnaA_w$ sites, as indicated by the absence of enhanced cleavage at the residues (marked by asterisks, Fig. 3), under conditions that promoted clear occupation of the same sites in the normal DNA (Fig. 3, cf. left and right panels). The locations and sequences of the $dnaA_s$ and $dnaA_w$ sites are shown (Fig. 4).

Does the $dnaA_s$ site promote binding of DnaA to the $dnaA_w$ site by simply increasing the local concentration of the protein at the tethered $dnaA_s$ site, or is the interaction truly cooperative? We approached this question by deleting the $dnaA_w$ sites and comparing the occupation of the $dnaA_s$ site in the presence and absence of IHF in the normal and $dnaA_w$-deleted substrate DNAs. In

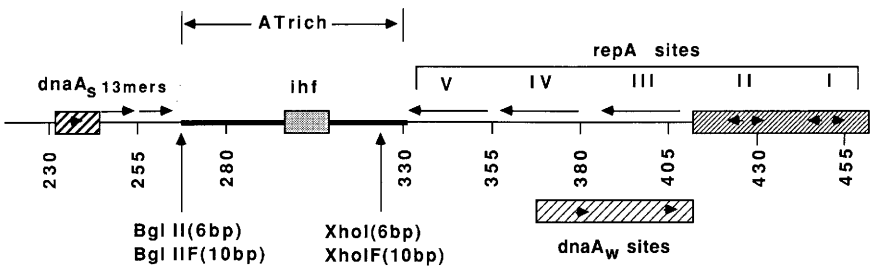

Figure 1. Physical map of the ori region of pSC101 showing location of the various binding sites of proteins. The map coordinates are according to Vocke and Bastia (1983a). The following features should be noted: $dnaA_s$ (strong binding site) has the consensus binding site for DnaA protein; (arrowhead) polarity of the sequence; (right arrow) the two 13-mer repeats; (heavy bar) AT-rich region in which the IHF binding site (*ihf*) is embedded; (left arrow) the weak RepA-binding sites (*repA* sites) III, IV, and V; (double arrows) strong sites I and II; the $dnaA_w$ (weak sites) for DnaA, described in this paper are indicated. Insertion mutants of 6 and 10 bp were generated by site-directed oligomutagenesis at the sites indicated (upward arrow). Note that the *repA* sites overlap the $dnaA_w$ sites.

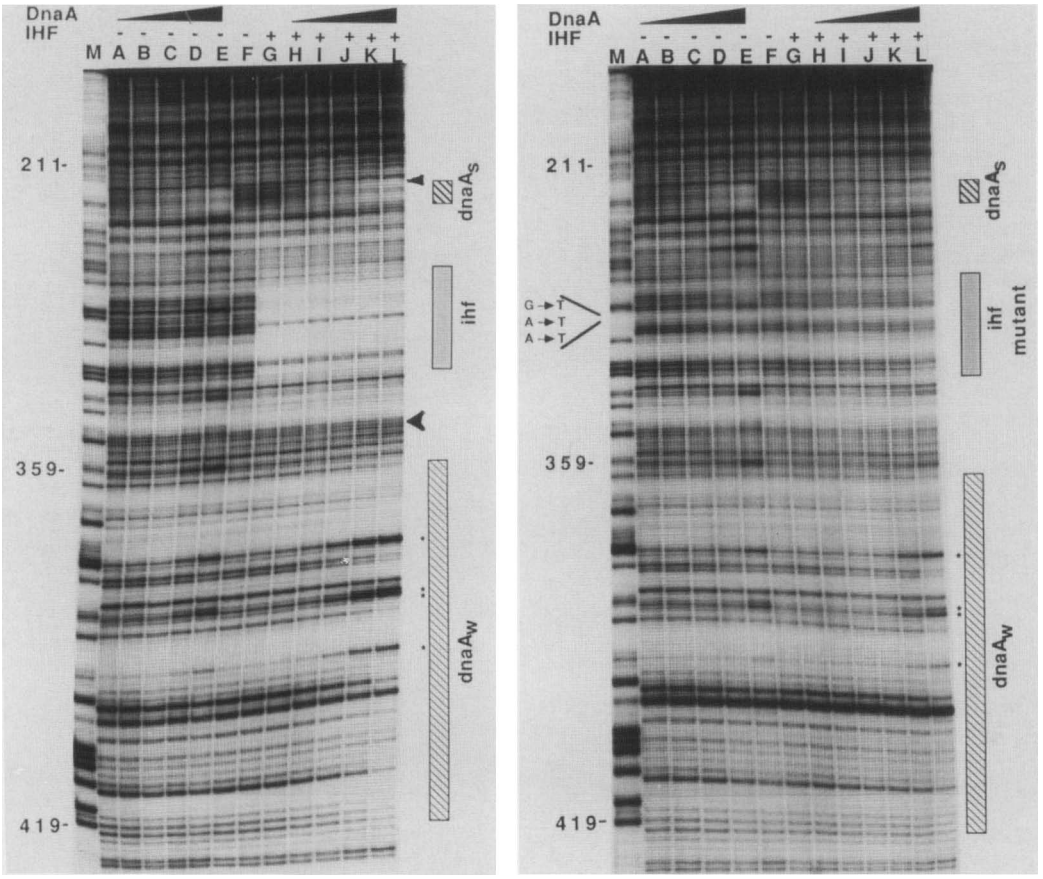

Figure 2. DNase I footprint showing that binding of IHF to its specific binding site (*ihf*) promotes enhanced binding of DnaA protein to its cognate binding sites, and a mutant *ihf* site that fails to bind IHF shows reduced enhancement of DnaA binding. (*Left*) *M* = G > A reaction showing the coordinates. (*A–E*) Protection of the *dnaA*$_s$ site by 20, 40, 80, 160, and 320 ng of DnaA protein, respectively, in the absence of IHF; (*F*) no protein control; (*G*) protection by 14 ng of IHF by itself; (*H–L*) protection of *dnaA*$_s$ and *dnaA*$_w$ sites in the presence of 14 ng of IHF and 20, 40, 80, 160, and 320 ng of DnaA, respectively. (*) Enhanced cleavage. Densitometry scans comparing lanes *C*, *D*, and *E* with lanes *J*, *K*, and *L* showed a 2.6-, 6.3-, and 5.9-fold enhancement of DnaA binding, in the presence of IHF, to the *dnaA*$_w$ sites and a 1.8-, 2.1-, and 2-fold enhancement of DnaA binding, in the presence of IHF, to the *dnaA*$_s$ sites, respectively. The band indicated by the bottom asterisk was used to quantify binding of DnaA to the *dnaA*$_w$ sites. The band indicated by the small arrowhead within the *dnaA*$_s$ site was used to quantify the binding of DnaA to the *dnaA*$_s$ site. The band indicated by the large arrowhead, between the *ihf* site and the *dnaA*$_w$ site, was used to normalize binding. (*Right*) (*M* and *A–L* are exactly as at *left*, except that a triple *ihf* site mutant was used as the DNA substrate. Note that when IHF fails to bind to the *ihf* site, enhanced binding of DnaA to the *dnaA*$_s$ site is essentially abolished and enhanced binding of DnaA to the *dnaA*$_w$ sites is greatly reduced. Densitometry scans comparing lanes *C*, *D*, and *E* with lanes *J*, *K*, and *L* showed a 1.8-, 2.1-, and 2.8-fold enhancement of DnaA binding, in the presence of IHF, to the *dnaA*$_w$ sites and a 1.7-, 1.1-, and 1-fold (or no) enhancement of DnaA binding, in the presence of IHF, to the *dnaA*$_s$ site, respectively. Binding was quantified and normalized using the same bands as described at *left*.

attempting this experiment, one must keep in mind that the *dnaA*$_s$ site is a strong site and by definition is expected to bind some DnaA protein even in the absence of any other interacting site.

We labeled the strand of ori DNA that is complementary to the one shown in Figure 3, with [γ-^{32}P]ATP and again performed DNase I footprints. The intensity of a protected band from the *dnaA*$_s$ region was determined by scanning densitometry and normalized with respect to an unprotected band from the same lane of an appropriately exposed autoradiogram. The results clearly showed that the *dnaA*$_w$ site promoted up to an approximately fourfold enhancement of the occupation of the *dnaA*$_s$ site in the presence of IHF (Figs. 5 and 6).

It should be noted that the lack of overlap of the IHF and DnaA footprints and the observed cooperativity at a distance between the *dnaA*$_s$ and *dnaA*$_w$ sites are consistent with the formation of a DNA loop between the two sites directly mediated by DnaA protein and indirectly by the DNA bending protein IHF.

The cooperative interaction of DnaA protein with the dnaA$_s$ *and* dnaA$_w$ *sites is dependent on both sites being on the same face of the DNA double helix*

A clear prediction of the looping model is that both the *dnaA*$_s$ and the *dnaA*$_w$ sites should be on the same face of

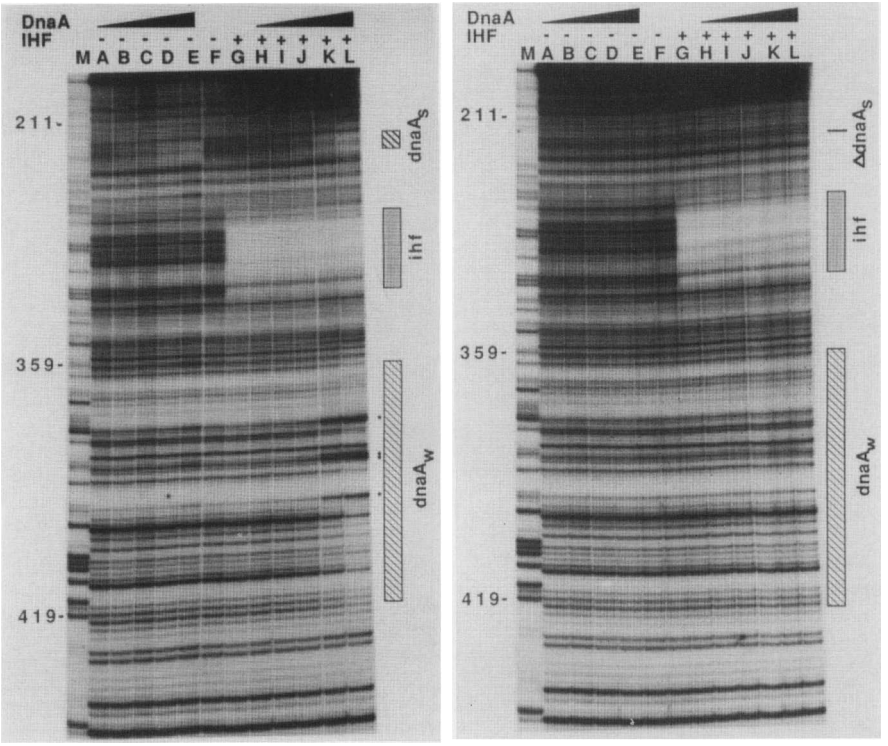

Figure 3. DNase I footprints showing cooperativity at a distance between the *dnaA*$_s$ and *dnaA*$_w$ sites and demonstration that deletion of the *dnaA*$_s$ site eliminates binding of DnaA protein to the *dnaA*$_w$ sites even in the presence of IHF. (*Left*) *M* = G > A reaction showing the ori coordinates. (*A–E*) Protection of the wild-type *dnaA*$_s$ site by 20, 40, 80, 160, and 320 ng of DnaA protein, respectively, in the absence of IHF; (*F*) no protein control; (*G*) protection by 14 ng of IHF alone; (*H–L*) protection of both the *dnaA*$_s$ and *dnaA*$_w$ sites by 14 ng of IHF and 20, 40, 80, 160, and 320 ng of DnaA protein, respectively. (*) Enhanced DNase cleavage. Densitometry scans comparing lanes *C*, *D*, and *E* with lanes *J*, *K*, and *L* showed a 1.1-, 1.8-, and 2-fold enhancement of DnaA binding, in the presence of IHF, to the *dnaA*$_s$ site, respectively. Binding was quantified and normalized with the same bands as described in Fig. 2. (*Right*) *M* and *A–L* are exactly as at *left*, except that Δ*dnaA*$_s$ ori DNA was used. Note that in the Δ*dnaA*$_s$ mutant, IHF failed to stimulate binding of DnaA protein to the *dnaA*$_w$ sites.

the DNA double helix, that is, there has to be an integral number of full turns of the DNA double helix between the two sites to effect cooperative interaction. It follows that the introduction of a subintegral number of turns (e.g., 5–6 bp) of DNA between the two sites should abrogate cooperativity and introduction of a net full turn should restore it. We tested this clear prediction of the looping model as described below.

We introduced a 6-bp *Bgl*II recognition sequence between the *dnaA*$_s$ and *ihf* sites by site-directed mutagenesis using oligo 2 (see Materials and methods). We then generated a 10-bp insertion mutant called *Bgl*IIF by cleaving with *Bgl*II and repairing the sticky ends and blunt ligation of the filled-in *Bgl*II site (see Fig. 1). We measured the occupation of the *dnaA*$_w$ sites in the two mutant DNAs, in the presence and absence of IHF, by DNase I footprinting. The results showed that IHF enhanced the occupation of the *dnaA*$_w$ sites, as indicated by the appearance of enhanced bands in the *Bgl*IIF mutant but not in the *Bgl*II mutant DNA (Fig. 7, cf. lanes

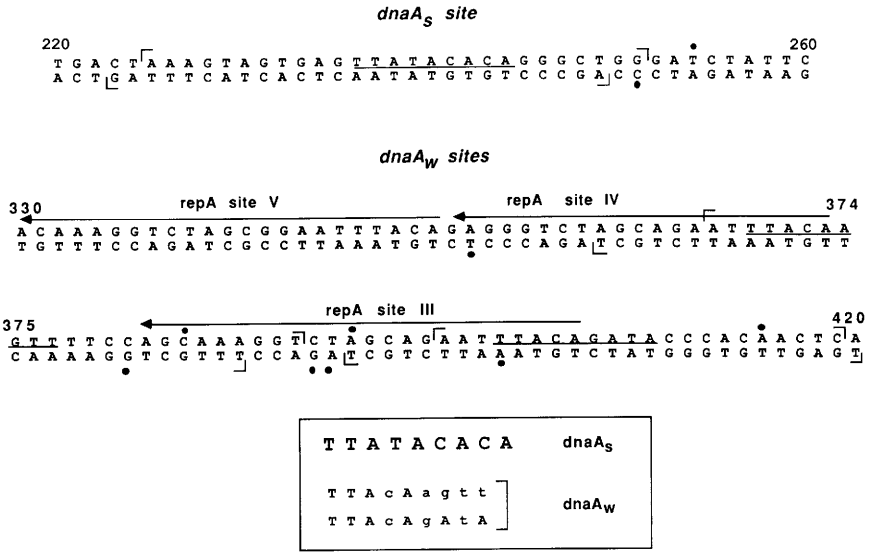

Figure 4. Nucleotide sequence of the ori region of pSC101 (Vocke and Bastia 1983a) showing the location of the *dnaA*$_s$- and *dnaA*$_w$-binding sites. Protection by DNase I of the *dnaA*$_s$ and *dnaA*$_w$ sites is shown in brackets. (●) Enhanced DNase I cleavage; (overhead arrows) *repA* sites. The potential *dnaA*$_w$ site and the known *dnaA*$_s$ site are underlined. The regions of homology between the strong *dnaA*$_s$ and the weak *dnaA*$_w$ sites are also indicated. Nucleotides of *dnaA*$_w$ that deviate from the consensus *dnaA*$_s$ (strong site) are shown in lowercase letters.

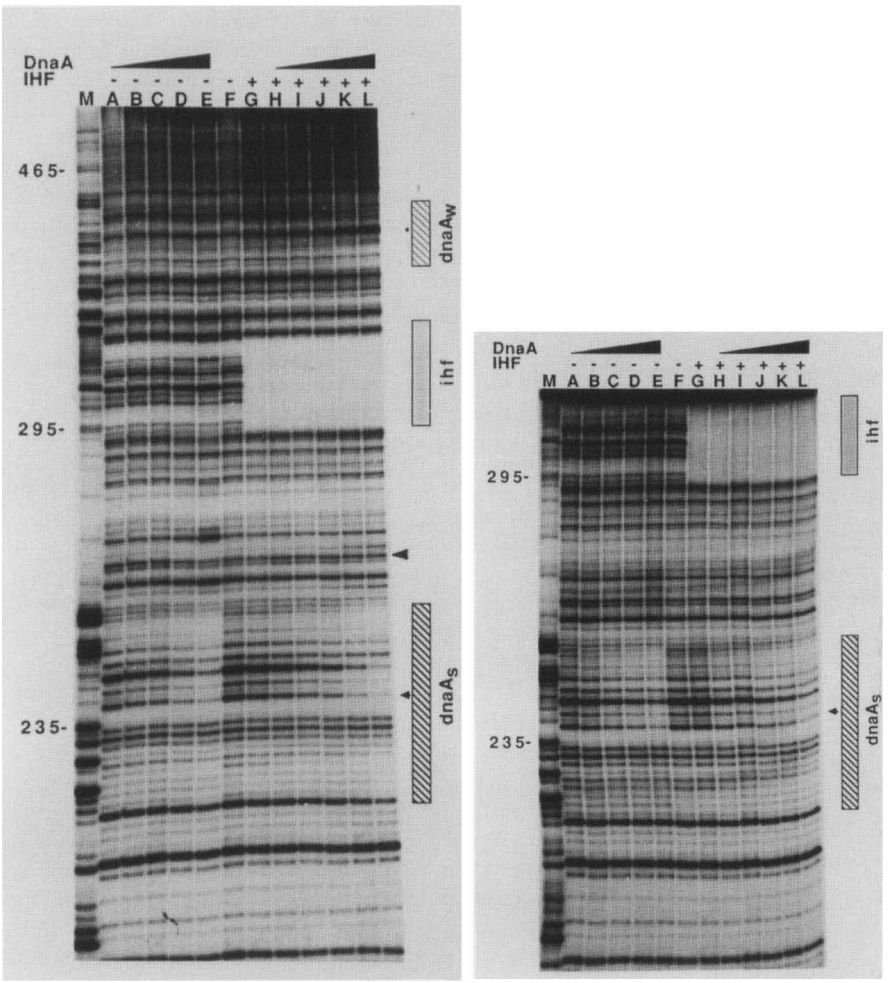

Figure 5. DNase I footprints showing cooperativity at a distance between the $dnaA_w$ and $dnaA_s$ sites as revealed by reduced binding of DnaA protein to the $dnaA_s$ site when the $dnaA_w$ sites are deleted. (*Left*) *M* = G > A reaction showing the coordinates. (*A–E*) Protection of the $dnaA_s$ site by 20, 40, 80, 160, and 320 ng of DnaA protein, respectively, in the absence of IHF; (*F*) no protein control; (*G*) protection by 14 ng of IHF by itself; (*H–L*) protection of both the $dnaA_s$ and $dnaA_w$ sites by 14 ng of IHF and 20, 40, 80, 160, and 320 ng of DnaA protein, respectively. (*) Enhanced cleavage. Densitometry scans comparing lanes *C*, *D*, and *E* with lanes *J*, *K*, and *L* showed a 1.3-, 2.1-, and 4.2-fold enhancement of DnaA binding, in the presence of IHF, to the $dnaA_s$ site. The band indicated by the small arrowhead was used to quantify binding of DnaA to the $dnaA_s$ site. The band indicated by the large arrowhead was used to normalize binding. (*Right*) *M* and *A–L* are exactly as at *left*, except a mutant ori DNA lacking the $dnaA_w$ sites was used. Densitometry scans comparing lanes *C*, *D*, and *E* with lanes *J*, *K*, and *L* showed a 1.2-, 1.2-, and 0.88-fold (or no) enhancement of DnaA binding, in the presence of IHF, to the $dnaA_s$ site. Binding was quantified and normalized with the same bands as at *left*. Note the reduced binding to the $dnaA_s$ site and abrogation of IHF-mediated enhanced binding to the $dnaA_s$ site when the $dnaA_w$ sites were deleted.

D,E with K,L, left and right). The relative occupation of the $dnaA_w$ sites as indicated by the relative intensity of the enhanced residues (*) at the $dnaA_w$ sites was close to that of the wild-type ori DNA (Figs. 2 and 3, left). We did not expect the restoration of the enhancements to be quantitatively identical to that of normal ori DNA because, although the angular rotation between the $dnaA_w$ and $dnaA_s$ sites had been restored by a 10-bp insertion, the translational relationship of other contact points in the higher order structure at the ori would be altered by a 10-bp insertion.

In addition, we changed the relative angular rotation between the *ihf* site and the $dnaA_w$ sites by insertion of a 6-bp *Xho*I and a 10-bp repaired *Xho*I site (*Xho*IF; see Fig. 1). DNase I footprints, performed as described above, showed that the enhanced residues were present at the $dnaA_w$ sites in the *Xho*IF mutant in the presence of DnaA and IHF but were not detected in the *Xho*I mutant (cf. lanes D,E with K,L in Fig. 8, left and right). Thus, there was cooperativity between the two sites in the presence of IHF and DnaA only when the $dnaA_s$ and $dnaA_w$ sites were located on the same face of the DNA. This result was consistent with the predictions of the looping model.

Site-directed mutagenesis shows that the $dnaA_s$ *site is essential for in vivo replication and all insertion mutations tested cause origin inactivation*

Is the observed dependence of the cooperativity between the $dnaA_s$ and $dnaA_w$ sites on the helical phase, as revealed by the loss and restoration of enhanced binding at $dnaA_w$, also reflected in the loss of ori function by 6-bp insertions and the restoration of activity by 10-bp insertions? In approaching this hypothesis, one has to keep in mind that the ori region almost certainly associates with several other proteins of the replisome in addition to DnaA, IHF, and RepA. Insertion of 10 bp at the indicated sites could restore the correct angular rotation between the two sites, but at the same time is likely to disturb the contact between other pairs of proteins and/or sites that may require side-by-side contact.

In spite of this potential complication posed by multiple interactions inherent in a multiprotein system, we measured the ability of the *Bgl*II, *Bgl*IIF, *Xho*I, and *Xho*IF mutants to replicate in vivo and observed that all of the insertions studied inactivated the origin (Table 1).

The in vitro data presented here suggested that cooperativity between the $dnaA_w$ and $dnaA_s$ sites was en-

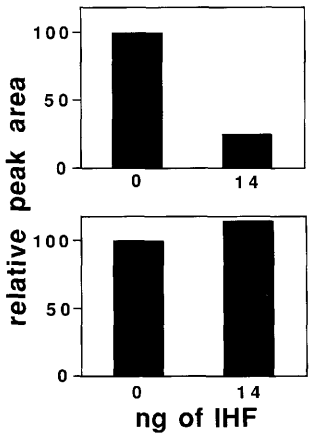

Figure 6. Quantitation of the relative occupation of the *dnaA*$_s$ site by DnaA protein in the presence and absence of IHF in the normal ori DNA and the mutant that lacks the *dnaA*$_w$ sites. Appropriately exposed autoradiograms were used so that the quantitated bands were not overexposed and were within the linear portion of the dose-response curve of the film. The autoradiograms (e.g., Fig. 5) were traced with a densitometer, and the peak area of the protected band (arrowhead, Fig. 5) was normalized with respect to a band in the same lane, but outside the protected region. The normalized peak areas are shown as solid bars. (*Top*) In wild-type ori DNA, the addition of 14 ng of IHF (320 ng of DnaA) increased the protection (decreased bar height) of the *dnaA*$_s$ site by a factor of 4.2. (*Bottom*) In contrast with the *top*, in the Δ*dnaA*$_w$ mutant DNA, addition of 14 ng of IHF (320 ng of DnaA) caused no enhanced protection of the *dnaA*$_s$ site (note bar heights are similar).

hancing the binding of DnaA to the *dnaA*$_s$ site. It was important to know, then, whether or not the *dnaA*$_s$ site was an essential element of the ori. In vivo replication experiments showed that the deletion of the *dnaA*$_s$ site inactivated the origin (Table 1). Therefore, the *dnaA*$_s$ site appeared to be critical for ori function. Because the presumptive protein–protein interactions and the observed protein–DNA interactions promoted occupation of the essential *dnaA*$_s$ site, such interactions observed in vitro were also likely to be significant in vivo. The inclusion of the *dnaA*$_w$ sites within the *repA* sites made it difficult at this time to assess the physiological role of the *dnaA*$_w$ sites by mutagenesis.

RepA protein binding to the repA/dnaA$_w$ *sites promotes DnaA binding to the* dnaA$_s$ *site*

Because the *dnaA*$_w$ sites overlap the binding sites for the RepA protein (*repA* sites; see Figs. 1 and 4; Stenzel et al. 1987), we wished to find out whether RepA protein interacts with DnaA protein.

We measured by performing DNase I footprinting the occupation of the *dnaA*$_s$ site by DnaA protein in the presence and absence of RepA protein, bound to the *repA* sites on a normal linear ori DNA substrate. Our comparison of lanes D and E with K and L, respectively, of Figure 9 showed that the binding of RepA to its cognate sites, which were physically separated from the *dnaA*$_s$ site, promoted enhanced binding of DnaA protein to the *dnaA*$_s$ site. Quantitative measurements (Fig. 10) indicated up to an approximately sixfold enhancement of DnaA binding to the *dnaA*$_s$ site promoted by RepA protein in vitro. The results strongly suggested protein–protein interactions between RepA and DnaA. Because RepA protein binds to both faces of the *repA* sites (Vocke and Bastia 1983b), it was not possible to measure the phase dependence of both sites by changing the relative angular rotation.

In the presence of saturating amounts of IHF and limiting amounts of DnaA, RepA enhances the binding of DnaA protein to the dnaA$_s$ *site*

The replication in vivo of pSC101 (in the presence of the normally limiting amounts of RepA and DnaA proteins in the cell milieu) requires IHF in addition to other replisomal proteins. If RepA can promote DnaA binding to its cognate sites in vitro, why is IHF needed in vivo? Do all three proteins cooperate to promote DnaA binding to ori when one or more is present in limiting concentration in vivo? Can the postulated in vivo interactions be duplicated in vitro?

We compared the DNase I footprints of normal linear ori DNA in the presence of saturating amounts of IHF and increasing amounts of DnaA protein, either in the absence or presence of saturating amounts of RepA. By comparing lanes C,D, and E with lanes L,M, and N, respectively, in Figure 11, we estimated that RepA enhanced the occupation of the *dnaA*$_s$ site by DnaA protein by at least a factor of 2. Therefore, under these conditions, the cooperative enhancing effects of RepA and IHF on binding of DnaA to the *dnaA*$_s$ site was not inconsistent with the need for IHF, RepA, and DnaA in pSC101 replication in vivo. Inspection of Figure 11 suggests that both RepA and DnaA proteins were most likely bound to the overlapping *dnaA*$_w$ and *repA* sites and that one protein did not appear to displace the other from the overlapping binding sites. For example, the enhanced cluster of bands at the *dnaA*$_w$ sites (arrowhead) were observed when both IHF and DnaA were added (Fig. 11, lanes D,E) or when all three proteins were present (lanes L,M,N) but not when only IHF and RepA were present and DnaA was missing (lane I). Our observation was consistent with the prediction that all three proteins were cooperating to promote DnaA binding to the *dnaA*$_w$ sites.

Methylation protection experiments (data not shown) also suggested that RepA and DnaA were unlikely to be competing for binding to the *dnaA*$_w$ sites but, instead, were coexisting at the overlapping sites.

Discussion

The main objective of this study was to investigate the role of IHF-induced DNA bending on protein–DNA and protein–protein interactions at the ori of plasmid pSC101 and to test a model proposed earlier by us stating that IHF-induced DNA bending promoted cooperative

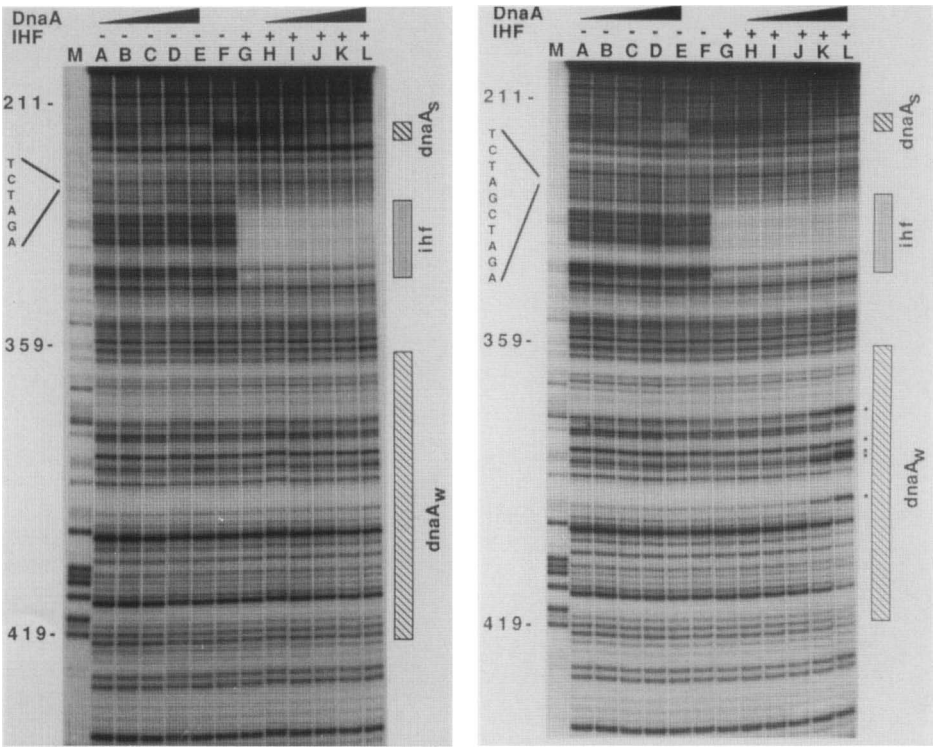

Figure 7. DNase I footprints showing the effect of relative angular rotation of the *dnaA*$_s$ site with respect to the *ihf* site, on cooperativity between the *dnaA*$_s$ and the *dnaA*$_w$ sites. *M* = G > A reaction showing the coordinates. (*A–E*) Amounts of 20, 40, 80, 160, and 320 ng of DnaA protein were added, respectively, in the absence of IHF; (*F*) no protein control; (*G*) 14 ng of IHF added; (*H–L*) 14 ng of IHF and 20, 40, 80, 160, and 320 ng of DnaA protein were added, respectively; (*) enhanced cleavage. (*Left*) Note the *Bgl*II (6-bp) insert between the *dnaA*$_s$ and the *ihf* site abolished enhanced binding of DnaA protein to the *dnaA*$_w$ sites (note disappearance of the enhanced bands) and reduced binding to the *dnaA*$_s$ site. (*Right*) The *Bgl*IIF (10-bp) insert restored enhanced binding to the *dnaA*$_w$ and the *dnaA*$_s$ sites. Note that the enhanced cleavage at the *dnaA*$_w$ sites was restored close to that of wild-type levels.

interaction at a distance between DnaA- and RepA-binding sites by DNA looping (Stenzel et al. 1987). Although looping has not been demonstrated visually, mainly because a ~150-bp loop would have been difficult to resolve by standard procedures of electron microscopy, three biochemical considerations were totally consistent with a DNA loop formed by the interaction of the *dnaA*$_s$ and *dnaA*$_w$/*repA* sites as shown in Figure 12. First, DNase I footprints showed that the binding of DnaA to the *dnaA*$_s$ site did not overlap the IHF footprint, which, in turn, did not overlap the footprint of DnaA at the *dnaA*$_w$ sites. The RepA footprint also did not overlap the *dnaA*$_s$ site (Vocke and Bastia 1983b; Stenzel et al. 1987). Thus, there was no evidence of side-by-side contact between proteins covering the *dnaA*$_s$ and *dnaA*$_w$/*repA* sites. Second, the observed cooperativity at a distance between the *dnaA*$_s$ and *dnaA*$_w$ sites was lost when the *dnaA*$_s$ site was deleted. Deletion of the *dnaA*$_w$ sites likewise reduced the IHF-mediated enhanced binding of DnaA to the *dnaA*$_s$ site. Third, the observed cooperativity between the *dnaA*$_s$ and *dnaA*$_w$ sites was lost when the two sites were placed on opposite faces of the DNA double helix and was restored, at least partially, when the sites were returned to the same face of the double helix by 10-bp insertions. These results were all consistent with a DNA looping model (Fig. 12).

Why did the 10-bp insertions restore the cooperativity nearly, but not completely, to that of the wild-type levels? This was not surprising because the translational relationship of the sites probably also played some role in cooperativity, suggesting a higher order structure in addition to that suggested by the simplified model shown in Figure 12. Phase changes caused by base-pair insertions in other systems have also been reported to restore only a fraction of the wild-type level of activity (Dunn et al. 1984). It should be noted that the 6-bp *Xho*I insertion was especially effective in destroying cooperativity. Although we did not know the reasons for this effect, we speculated that the *Xho*I insertion not only changed the relative angular rotation between the interacting sites but also reduced the natural bend.

Why was the loss of cooperativity by 6-bp insertions and the (at least) partial restoration of the same by 10-bp insertions not reflected in a corresponding loss and restoration of origin function? Why did all insertions inactivate the origin? The results were not difficult to understand, keeping in mind that the higher order structure at the replication origin almost certainly involves several

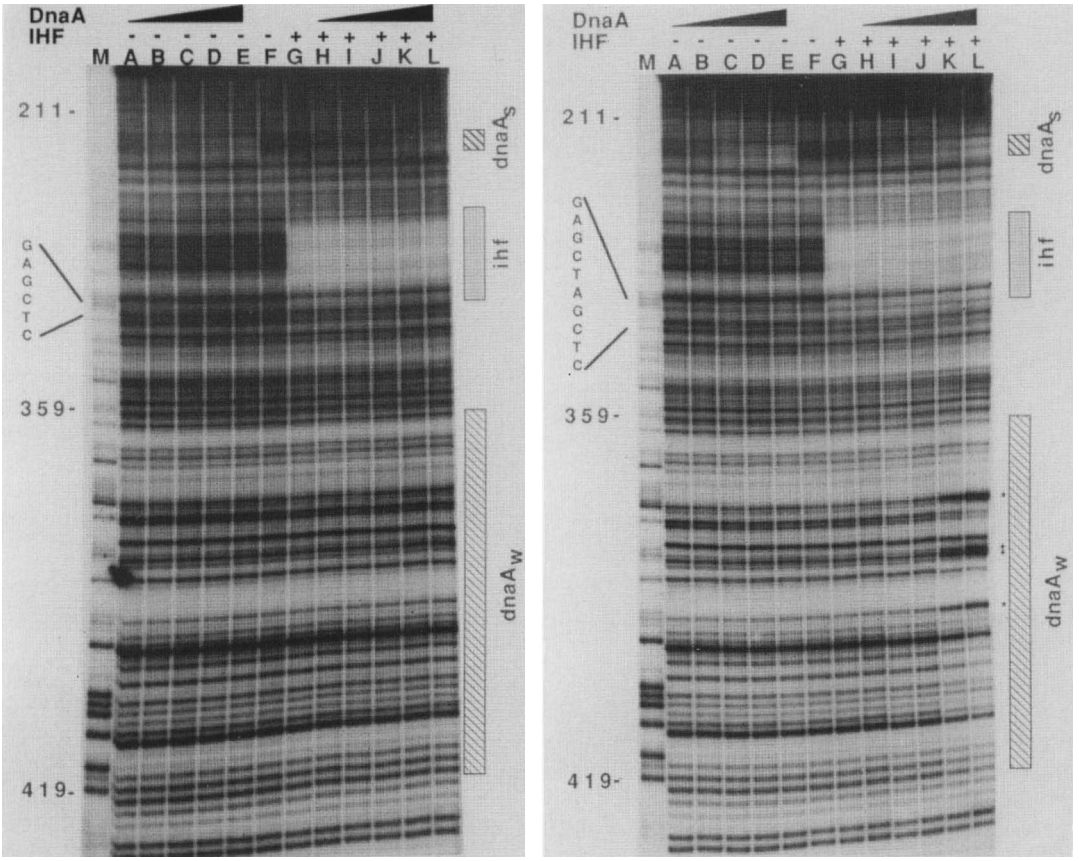

Figure 8. Effect of relative angular rotation of the *ihf* site with respect to the $dnaA_w$ sites on the cooperative interaction between the $dnaA_s$ and the $dnaA_w$ sites. (*Left*) Lanes *M* and *A–L* are as in Fig. 7, except that the *Xho*I (6-bp) insertion mutant ori DNA was used. Note that the 6-bp insertion caused the abolition of enhanced binding to the $dnaA_w$ site in the presence of IHF, as indicated by the disappearance of enhanced cleavage at the $dnaA_w$ sites. Note that the 6-bp insertion greatly reduced binding to the $dnaA_s$ site even in the presence of IHF (lanes *E* and *L*). (*Right*) Lanes *M* and *A–L* are as indicated in Fig. 7, except that the *Xho*IF (10-bp insertion) mutant DNA was used. By comparing lanes *K* and *L* with the same lanes at *left*, it was clear that the 10-bp insert caused the reappearance of the enhanced cleavage (*) at the $dnaA_w$ sites and increased occupation of the $dnaA_s$ site (cf. lanes *E* and *L* at *left* and *right*).

other proteins in addition to RepA, DnaA, and IHF. It should not be surprising, then, that insertions of 6 and 10 bp between known binding sites might disrupt other protein–protein and protein–DNA contacts and therefore inactivate the origin.

It should be pointed out that the in vitro experiments reported here used high molar ratios of DnaA to IHF. The reason for using higher levels of DnaA was the propensity of this protein to aggregate and precipitate out of solution in the absence of a chaperon protein such as DnaK (D. Chattoraj et al., pers. comm.). The DnaA protein used was fully active in supporting *oriC* and R6K replication in vitro (MacAllister et al. 1991). We used higher levels of DnaA protein merely to obtain a clear footprint.

The data presented in this paper show that RepA protein by itself, when added at a high enough concentration in vitro, can bypass the need for IHF protein in promoting enhanced binding of DnaA protein to the $dnaA_s$ site. Why, then, is IHF protein needed for pSC101 replication in vivo? In addressing this question, one should keep in mind that the *ihf* site has a natural bend and IHF merely enhances the degree of bending (Stenzel et al. 1987). IHF is therefore an efficiency factor that can be bypassed by a change in the superhelicity of the DNA caused by host *topA* mutations or by a point mutation causing an amino acid substitution in the third codon of the RepA protein (Biek and Cohen 1989a,b). Perhaps activation of the origin requires a higher order structure similar to the one proposed in this paper. IHF probably aids the formation of this structure but can be bypassed by other mutational changes that lead to a functionally quasi-equivalent higher order structure. With this scenario in mind, one should take into account the observation that RepA protein is present in the cell in limiting amounts (C. Vocke and D. Bastia, unpubl.), and the increased bending by IHF, presumably, is needed to promote DnaA–RepA and possibly other protein–protein interactions. However, in the presence of an excess of RepA, the need for additional bending by IHF is bypassed. It should be pointed out that in the presence of subsaturating amounts of DnaA and saturating amounts

Table 1. *In vivo replication of wild-type and mutant origins of pSC101*

Recombinant plasmids[a,b]	Km^R colonies
Wild type	4424[c]
$\Delta dnaA_s$	2[d]
*Bgl*II	0
*Bgl*IIF	0
*Xho*I	2[c]
*Xho*IF	1[c]

[a]Ligation products were checked in 1% agarose gels.
[b]The wild-type and mutant origins were all ligated to a nonreplicating Km^R marker.
[c]Plasmid DNAs were made from four of these colonies and determined to be the correct products.
[d]DNAs were made from these colonies and determined to be the incorrect products.

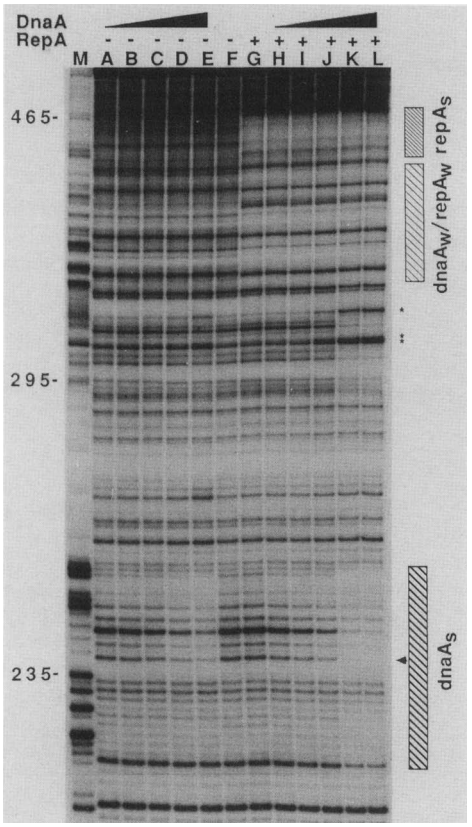

Figure 9. DNase I footprints showing the enhancement of binding of DnaA protein to the $dnaA_s$ site by RepA protein. M = G > A markers with coordinates indicated. (*A–E*) Protection of the $dnaA_s$ site by 20, 40, 80, 160, and 320 ng of DnaA protein, respectively, in the absence of RepA; (*F*) no protein control; (*G*) protection by 140 ng of RepA by itself; (*H–L*) increased protection of the $dnaA_s$ site by 140 ng of RepA and 20, 40, 80, 160, and 320 ng of DnaA protein, respectively; (*) enhanced DNase cleavage. The arrowhead indicates the DNase I-protected band utilized in densitometry scanning of the autoradiogram. Binding was normalized with the same band as described in Fig. 5 (*left*). For simplicity of presentation, the overlapping $dnaA_w$ and $repA_w$ sites are indicated by only one box in the right margin. (For a more detailed presentation of the overlapping sites, see Fig. 1.)

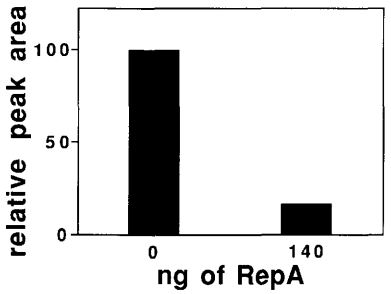

Figure 10. Quantitation of the autoradiogram shown in Fig. 9 reveals that RepA protein binding to the *repA* sites promotes enhanced binding of DnaA protein to the $dnaA_s$ site. The protected band (arrowhead in Fig. 9, lanes *E* and *L*) was quantitated by densitometry and normalized with respect to a band outside the protected area in the same lane (see legend to Fig. 9). Note an approximately sixfold reduction of DNase I cleavage (enhanced protection) at the $dnaA_s$ site in the presence of 140 ng of RepA protein (and 320 ng of DnaA).

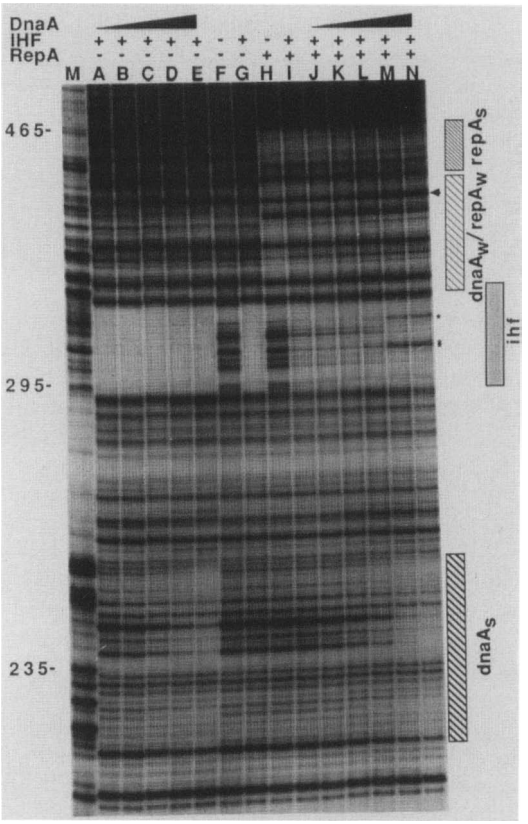

Figure 11. DNase I footprints showing that addition of RepA protein further stimulates binding of DnaA protein to its binding sites even in the presence of saturating amounts of IHF protein (all three proteins, DnaA, RepA and IHF, are present in vitro). M = G > A markers. (*A–E*) Protection of wild-type ori sites by 14 ng of IHF and 20, 40, 80, 160, and 320 ng of DnaA protein, respectively; (*F*) no protein control; (*G*) 14 ng of IHF added; (*H*) 140 ng of RepA added; (*I*) 14 ng of IHF and 140 ng of RepA added; (*J–N*) increased protection of the $dnaA_s$ site in the presence of 140 ng RepA, 14 ng of IHF, and 20, 40, 80, 160, and 320 ng of DnaA protein, respectively (cf. lanes *J–N* with *A–E*). For simplicity of presentation, the overlapping $dnaA_w$ and $repA_w$ sites are indicated by only one box in the right margin.

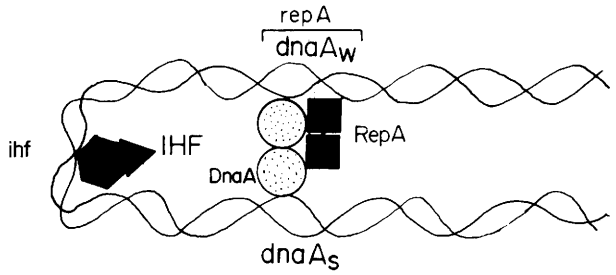

Figure 12. A simplified model showing the relative locations of the *dnaA*$_s$, *dnaA*$_w$, and *repA* sites at the origin and the observed interaction by DNA looping between the *dnaA*$_s$ and *dnaA*$_w$/*repA* sites in the presence of IHF, RepA, and DnaA. Note that the model shows looping out of the *ihf* site, implying that IHF-induced DNA bending promotes protein–protein interaction without direct contact between IHF and DnaA.

of IHF, further addition of RepA protein still enhances the binding of DnaA protein to the essential *dnaA*$_s$ site, perhaps mimicking the interactions that take place in vivo.

What is the physiological role of the *dnaA*$_w$ sites that have partial homology to the *dnaA*$_s$ site? The complete overlap of the *dnaA*$_w$ and *repA* sites made it difficult to assess the role of the *dnaA*$_w$ sites by mutagenesis. It is, however, tempting to speculate that the *dnaA*$_w$ sites mainly promote DnaA and RepA interaction by DNA looping with the *dnaA*$_s$ site.

Do RepA and DnaA proteins bind simultaneously to the *dnaA*$_w$/*repA* sites or does binding of one displace the other protein? Preliminary methylation protection analyses (data not shown) indicate that both proteins remain bound to the *dnaA*$_w$/*repA* overlapping sites in vitro.

Why does pSC101 need two initiator proteins for replication? Do DnaA and RepA catalyze different substeps of the initiation mechanism? One attractive hypothesis to consider in this regard is that RepA may control plasmid copy number and also cooperate with DnaA for ori unwinding. We postulate that RepA probably interacts with and uses DnaA as an adapter protein to assemble other primosomal proteins. Work is currently in progress to test this hypothesis in vitro.

Finally, the present work reveals a new property of DnaA protein, namely, to catalyze action at a distance, probably by promoting DNA looping. Whereas other alternatives cannot be strictly ruled out at this time, the helical phase dependence of cooperativity at a distance between the sites is most consistent with DNA looping. Previously, we have shown that Pi protein of R6K promotes long-range looping in vitro between a replication origin and an enhancer site (Mukherjee et al. 1988). Chattoraj et al. (1988) have also shown DNA looping by the initiator protein of plasmid P1.

Materials and methods

Bacterial recombinant plasmid strains

The bacterial strain used for vivo replication experiments was *Escherichia coli, DH1* [F^-, *gyr A96, recA1, relA1, endA1, thi1, hsdR17* (r_k^- m_k^-), *supE44*, λ^-].

Plasmid pTS89 was constructed by isolating the 323-bp *Fok*I–*Bgl*II ori fragment from pPP901B (see Stenzel et al. 1987), filling in the ends with DNA polymerase (Klenow) and dNTPs, and ligating the flush-ended fragment to the *Sma*I site of pUC19. The cloned ori fragment was excised from pTS89 by *Eco*RI–*Bam*HI digestion, blunt-ended by repair reaction, and cloned into the *Sma*I site of M13 mp18 to yield pTS150. pTS151–pTS155 were similarly constructed, except these plasmids had either deletions of the *dnaA*$_s$ site or 6- and 10-bp insertions.

DNase I footprinting

Binding of proteins to ori DNA was carried out in 40 mM HEPES (pH 7.6), 50 mM potassium glutamate, 10 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 50 μg/ml of BSA, and 40 μg/ml of sonicated calf thymus DNA. The other details have been published previously (Vocke and Bastia 1983b; Stenzel et al. 1987).

Site-directed mutagenesis

The mutagenesis procedure has been described (Stenzel et al. 1987). Oligonucleotides 1, 2, and 3 had the sequences 5′-ATAGATCCCAGCCCCTCACTATTTAGT, 5′-TTTATAGAATAAAGAAGATCTAAGAATAAAAAAAGA, and 5′-GACCTTTGCTCGAGTGTGTTTT, respectively. The underlined residues show the sequences that were inserted. Oligo 1 was used to specifically delete 9 bp of the *dnaA*$_s$ sequence generating the Δ*dnaA*$_s$ mutation. Oligos 2 and 3 were used to insert a 6-bp *Bgl*II recognition and a 6-bp *Xho*I site, respectively. The insertion mutants were cut with either *Bgl*II or *Xho*I at the unique sites and repaired, and the blunt ends were religated to generate the *Bgl*IIF and *Xho*IF mutants.

Protein purification

IHF was purified from overproducer clones as described (Robertson and Nash 1988). Details of the purification of DnaA will be published elsewhere. Briefly, an overproducer clone in a T_7 promoter vector was constructed and the protein was purified as described (Sekimizu et al. 1988). RepA protein was purified from an overproducer clone in a T_7 promoter vector and purified through an ammonium sulfate, a Mono S, and a Superose gel filtration step to homogeneity. Details will be published elsewhere; the procedure will be supplied on request (T.T. Stenzel and D. Bastia, in prep.).

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Cooperativity at a distance promoted by the combined action of two replication initiator proteins and a DNA bending protein at the replication origin of pSC101.

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