Binding of DnaA Protein to a Replication Enhancer Counteracts the Inhibition of Plasmid R6K γ Origin Replication Mediated by Elevated Levels of R6K π Protein

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Replication of the γ origin of *Escherichia coli* plasmid R6K requires π protein, encoded by the R6K *pir* gene, and many host factors, including DnaA protein. π has dual roles, activating replication at low levels and inhibiting replication at high levels. The inhibitory function of π is counteracted by integration host factor and a specific sequence of the origin called the enhancer. This 106-bp DNA segment contains a binding site for DnaA protein (DnaA box 1). In this study, we mutated this site to determine if it was required for the enhancer's function. Using γ origin derivative plasmids with the DnaA box 1 altered or deleted, we show that this site is necessary to protect the origin against levels of wild-type π protein that would otherwise inhibit replication. To show that the base substitutions in DnaA box 1 weakened the binding of DnaA, we developed a new application of the agarose gel retardation assay. This quick and easy assay has broad applicability, as shown in binding studies with DNA fragments carrying a different segment of the R6K origin, the chromosomal origin (*oriC*), or the pUC origin. The gel retardation assay suggests a stoichiometry of DnaA binding different from that deduced from other assays.

Many replication origins have a bipartite organization, including an essential segment and an auxiliary segment which augments replication. Such enhancers have been discovered in organisms and systems as diverse as, for example, the bacteriophage ϕ 1 (8, 23), the *Staphylococcus aureus* plasmid pT181 (21), and the yeast autonomously replicating sequence ARS121 (49). In each of these cases, the enhancer carries, or is believed to carry, a protein-binding site (20, 37a, 49, 50).

The *Escherichia coli* plasmid R6K contains three origins of replication, α , γ , and β (Fig. 1), which all require the initiator protein π , encoded by the R6K *pir* gene. All three origins require the essential 277-bp core segment, which contains repeated binding sites (iterons) for π (14, 19, 33); each origin also needs a unique *cis*-acting DNA segment in addition to the core (28, 29, 36, 41, 42, 44). When the flanking α and β sequences are removed, the remaining γ origin can replicate autonomously (18, 43, 46) if the *pir* gene is provided in *cis* or in *trans* (22, 29). The γ origin responds to the intracellular concentration of π protein, and it contains the most critical information required for replication (12, 28). Therefore, we use the γ origin system (for the most recent review, see reference 10) as the simplest model derived from R6K to study the mechanisms regulating initiation of replication of an iteron-containing origin.

The γ origin consists of two modules, the essential 277-bp core segment and the adjoining 106-bp enhancer (51a) (Fig. 1). The enhancer is not required for the α or β origin (36, 41, 42, 44). The isolated core is able to replicate if the level of wild-type (wt) π is decreased (51a), a situation which reduces the stringency of replication control (12). At the normal level of wt π (4,000 to 10,000 π dimers per cell [12]), the isolated γ origin core cannot replicate. However, its function can be restored when the enhancer (51a) and DNA-binding protein

integration host factor (IHF) (4) are present. While the enhancer itself does not carry any IHF-binding sites (9, 11, 25), the presence of this DNA segment alters IHF binding in vitro, allowing IHF to fold γ origin fragments into a compact nucleoprotein structure (5, 11). These findings suggest that IHF and the enhancer together counteract the negative effect of π on replication.

This conclusion is also supported by studies with so-called "copy-up" variants of π protein. These mutant proteins allow an increase in the plasmid copy number over a wide range of π levels, suggesting that their ability to inhibit replication is weaker than their ability to initiate replication (13). The copy-up π proteins are able to drive R6K replication in the absence of the enhancer, even at the normal level at which the wt protein prevents replication (51a). In the absence of IHF, the copy-up proteins can also drive replication of the γ origin carrying the core and the enhancer, while the wt protein cannot (4).

These previous studies showed that the enhancer counteracts the negative effect of π on R6K replication. How does the enhancer do so? We had searched the enhancer sequence for sites for known DNA-binding proteins and discovered, as did workers from another laboratory, a DnaA-binding site, box 1 (26, 31, 51a). DnaA has been shown to bind to this site in a DNase I footprinting assay (51a). DnaA is essential for R6K γ origin replication in vitro (31) and in vivo (51a). In this study, we mutated the DnaA box 1 and correlated the presence of this site with the function of the enhancer. We also applied the technique of agarose gel retardations to DnaA to show that the altered DnaA box 1 was not bound in vitro.

MATERIALS AND METHODS

Plasmids and strains. Strain W3110 has been described previously (6). Strains carrying π -producing and γ origin plasmids were grown at 37°C in Luria broth (LB) or agar supplemented with 5 μ g of thymidine per ml and 125 or 250 μ g of penicillin G per ml. Plasmids used are shown in Table 1.

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FIG. 1. The three origins of replication of plasmid R6K, α , γ , and β , are shown at the top. Double-headed arrows indicate *cis*-acting DNA segments required for replication from each origin; the location of each Greek letter marks the approximate site of replication initiation for each origin. The dotted line separates the two *cis*-acting segments required for α origin replication (41). The core segment is required for all three origins; the *pir* gene encodes π protein. In the middle, the γ origin of R6K, consisting of 106-bp enhancer and 277-bp core segments, is shown. The numbers 1 and 2 indicate DnaA box 1 (TTGTCCACA, nt -24 to -16 [mismatch with the consensus underlined]) and box 2 (CTCTCAACA, nt 247 to 255); a, b, and c indicate the following DnaA box consensus sequences: a, TTATAAAAC (nt 32 to 40); b, TTATATTAA (nt 73 to 81); c, TTGTTCAAA (nt 87 to 95). Roman numerals I and II represent IHF binding sites *ihf1* (nt 61 to 73) and *ihf2* (nt 251 to 263) (4, 9), respectively. Tandem black arrows designate seven 22-bp iterons that bind π protein (nt 93 to 247). The open arrow indicates the P2 promoter (nt 221 to 226 [-35 hexamer] and nt 245 to 250 [-10 hexamer]) (35). Coordinates are according to Stalker et al. (45). H, *HindIII* site (R6K nt 0) and an artificial *HindIII* site to the right of the core; E, artificial *EcoRI* site to the left of the enhancer. At the bottom, thick lines indicate portions of the γ origin carried by various R6K derivative plasmids. X indicates mutation in DnaA box 1.

Restriction enzymes and T4 DNA ligase used in plasmid construction were obtained from New England Biolabs or Promega Corp.

Site-directed mutagenesis. The unique site elimination mutagenesis technique (7) was employed to introduce two changes into pMF34, which carries the R6K γ origin cloned into pUC9 (14). The oligonucleotide 5'CCTGGCTTGTAG TACTCAACCGTTAAACC3' was used to alter DnaA box 1 (bold, with all mismatches to the DnaA consensus underlined) and introduce a *ScaI* site to screen for mutants. Simultaneously, the oligonucleotide 5'GCAGGAAAGAACCGGTGA GCAAAAGG3' was used to change the unique *AflIII* site near the pUC origin (nucleotide [nt] 806) to an *AgeI* site (bold) to aid in selecting for mutants. This procedure resulted in the double-mutant plasmid pFW17. The *HaeII* pUC origin fragment (nt 680 to 1050) was then deleted, creating pFW18, which was used in the transformation assays. DNA sequencing by the dideoxy method (39) was performed to verify the DnaA box 1 mutation and ensure that no other mutations in the enhancer were introduced.

Protein purification. DnaA protein was purified from strain HMS174 by using the DnaA-overproducing plasmid pKC596. Both strain and plasmid were kindly provided by Jon Kaguni. The reagents, buffers, and procedures were as described by Fuller and Kornberg (17), except that instead of the Bio-Rex 70 and high-pressure liquid chromatography, a heparin-Sepharose column was used and developed with 0.1 to 1.0 M KCl. DnaA protein eluted at approximately 0.7 M KCl.

Gel retardation assay. Purified DNA fragments (60 to 100 ng, 0.3 to 1.4 pmol) were incubated with DnaA in a 7- to 11- μ l reaction volume (see figure legends for details of each experiment). Each sample contained the binding buffer previously used in footprinting assays by Braun et al. (2). Reaction mixtures were incubated at room temperature for 5 min before addition of 1 μ l of native dye (20% sucrose, 1 mM EDTA, 20 mM Tris-HCl [pH 7.5], 0.01% bromophenol blue, 0.01% xylene cyanol) and loaded onto 1.2% agarose gels already running at room temperature at 125 V in TB (90 mM Tris base, 90 mM boric acid [pH 8.2]). Gels were stained with ethidium bromide; photographically reversed images are shown in the figures. The following DNA fragments were used: *EcoRI*-*HindIII* fragment from pMF38 (wt enhancer) (see Fig. 2); the *EcoRI*-*HindIII* fragment from pFW23 (enhancer with DnaA

TABLE 1. Plasmids used in this study

Plasmid(s)	Origin(s)	Marker	Source or reference
$\Delta 14$ - π wt, $\pi 113$, $\pi 116$, $\pi 200$	RK2	Tet ^r	12, 51a
$\Delta 22$ - π wt, $\pi 113$, $\pi 116$, $\pi 200$	RK2	Tet ^r	12, 51a
pFW12	R6K γ	Pen ^r	This study; the <i>HaeII</i> pUC origin fragment was deleted from pMF32
pFW17	R6K γ , pUC	Pen ^r	This study; DnaA box 1 in pMF34 was mutated (see Materials and Methods)
pFW18	R6K γ	Pen ^r	This study; the <i>HaeII</i> pUC origin fragment was deleted from pFW17
pFW23	pUC	Pen ^r	This study; the <i>HindIII</i> core segment of pFW17 was deleted, leaving the mutated enhancer
pLAR7	<i>oriC</i> , pBR322	Pen ^r	38
pMF31	R6K γ , pUC	Pen ^r	This study; the R6K core was inserted as a <i>HindIII</i> - <i>BglII</i> fragment into pUC8 digested with <i>HindIII</i> and <i>BamHI</i>
pMF33	R6K γ , pUC	Pen ^r	51a
pMF34	R6K γ , pUC	Pen ^r	14
pMF36	R6K γ	Pen ^r	12
pMF38	pUC	Pen ^r	This study; the <i>HindIII</i> core segment of pMF34 was deleted, leaving the wt enhancer
pUC9	pUC	Pen ^r	52

box 1 altered) (see Fig. 2); the *Hae*II fragment from pUC9, nt 680 to 1050 (pUC origin) (see Fig. 4); the *Hind*III fragment from pLAR7 (*oriC*) (see Fig. 5); and the *Hind*III fragment from pMF34 (R6K core) (see Fig. 6). Addition of ATP (1 mM) did not alter the formation of complexes (data not shown).

Copy number analysis. W3110 cells carrying π -producing plasmids and γ origin plasmids were grown overnight in LB supplemented with 125 μ g of penicillin G per ml. DNA was purified by the alkaline lysis procedure (39) and linearized by digestion with *Sna*BI. DNA samples were electrophoresed at 150 V on 0.8% agarose gels in 0.5 $\times$ TBE (45 mM Tris base, 45 mM boric acid, 1.35 mM EDTA). Gels were stained with ethidium bromide; photographically reversed images are shown in the figures. Relative quantities of γ origin plasmid DNA were determined from dilutions.

RESULTS AND DISCUSSION

Mutating the DnaA box in the enhancer. The DNase I footprint assay had previously shown that DnaA protein binds to several sites in the R6K γ origin (51a). These sites include DnaA box 1, which lies in the enhancer, a 106-bp segment necessary to counteract the negative effect of π protein on R6K replication. To determine if we could correlate the presence of the DnaA box 1 to the role of the enhancer, we performed site-directed mutagenesis to alter box 1 from TTGTCCACA (nt -24 to -16) to TAGTACTCA. The wt sequence contains one mismatch (underlined), and the mutant site contains three mismatches with either the DnaA box consensus sequence originally proposed, TTAT(C/A)CA(C/A)A (15), or the revised DnaA box consensus proposed by Schaefer and Messer (40), (T/C)(T/C)(A/T/C)T(A/C)C(A/G)(A/C/T)(A/C).

Introduced mutations prevent binding of the DnaA protein to the enhancer. To show that the substitutions in the DnaA box 1 decreased or abolished DnaA binding in vitro, we purified DnaA protein (see Materials and Methods) and used it in an agarose gel retardation assay. As described below in the last section of Results and Discussion, this assay was established as a valid test of DnaA binding in vitro by using a variety of templates.

The isolated wt enhancer fragment carries a single DnaA box; upon addition of purified DnaA, the enhancer forms one major shifted complex, though a faint band is sometimes detectable above this complex (Fig. 2). The mutant enhancer, carrying the altered DnaA box, does not form any detectable complexes with DnaA under the conditions we employed. Therefore, we could use this altered site to determine whether DnaA box 1 affected the enhancer's function in vivo.

The DnaA-binding site in the enhancer is required to counteract the negative effect of π protein. We had previously shown that the enhancer was required for replication at the normal level of wt π but dispensable at a decreased level (51a). To determine if DnaA box 1 was needed for this function of the enhancer, we employed three isogenic R6K γ origin plasmids. Plasmid pFW12 carries only the core segment; pMF36 carries the core and the wt enhancer; and plasmid pFW18 is identical to pMF36, except that pFW18 contains the DnaA box 1 substitutions. These three γ origin plasmids (diagrammed in Fig. 1) were transformed into the W3110 host strain carrying compatible helper plasmids producing wt π protein, either at the normal level ($\Delta 22$ -*pir*WT) or at a low level (approximately 10%) ($\Delta 14$ -*pir*WT). Because all three plasmids carry the core segment, they were all expected to replicate at the low level of π . Furthermore, pMF36 but not pFW12 was expected to replicate at the normal level of π

FIG. 2. Agarose gel retardation of wt and mutant enhancer fragments with DnaA protein. A schematic diagram of the enhancer, with DnaA box 1 indicated, is shown at the top. Naked DNA and complexes are marked by arrows, and DnaA quantities used (0.08 μ g = 1.8 pmol) are indicated. For each sample, 100 ng of DNA fragment (1.4 pmol) was used and the final volume was 7 μ l.

protein. If DnaA box 1 were needed to counteract the negative effect of π , then pFW18 should not replicate at the normal level of wt π .

As a control, we used chimeric plasmids pMF33, pFW17, and pMF31, which carry the γ origins of pMF36, pFW18, and pFW12, respectively, and also the pUC origin in the vector. These chimeric plasmids transformed all the host strains (Table 2 and data not shown), indicating that any lack of transformants with pMF36, pFW18, or pFW12 was not caused by lack of transformation competency or runaway replication.

Table 2 shows that, as expected, γ origin plasmid pMF36 replicated at the normal level of wt π , while core plasmid pFW12 did not. The test plasmid pFW18, which carries the altered DnaA box 1, replicated at the low level of wt π but not at the normal level. Thus, these transformations indicate that the DnaA box 1 was needed to counteract the negative effect of π , expressed at the normal level of wt π .

The only other known protein-binding site in the enhancer is a recently discovered π binding site (30a). We have found that, unlike DnaA box 1, this π site is not needed for replication at the normal level of wt π (30a).

The DnaA box in the enhancer does not influence plasmid copy number at the low level of copy-up π mutants but does at the normal level. Interestingly, in the transformation experiment, transformants of W3110 ($\Delta 14$ -*pir*WT) were obtained with pFW12 only at the low concentration of penicillin G (125 μ g/ml) and not at the high concentration (250 μ g/ml). In contrast, pFW18 and pMF36 transformed this host at both concentrations of antibiotic. Because the level of resistance to penicillin is gene dosage dependent, the transformation assay suggested that pFW12 replicated at a lower plasmid copy number than did pFW18. Plasmid copy number reflects the relative strengths of factors activating and inhibiting replica-

TABLE 2. Effect of altering or deleting DnaA box 1 on transformation capability of γ origin plasmids^a

Transforming γ origin plasmid	Penicillin G concn (μ g/ml)	No. of colonies obtained with host cells of W3110 host cells already carrying the π -producing strain:				
		$\Delta 14$ - <i>pir</i> WT	$\Delta 22$ - <i>pir</i> WT	$\Delta 22$ - <i>pir</i> 113	$\Delta 22$ - <i>pir</i> 116	$\Delta 22$ - <i>pir</i> 200
pMF36	125	9.6×10^3	4.3×10^2	7.1×10^3	2.1×10^3	5.6×10^3
	250	5.8×10^2	40	4.7×10^3	5.7×10^3	6.3×10^3
pFW18	125	3.8×10^3	0	2.5×10^4	9.8×10^3	2.0×10^4
	250	3.1×10^2	0	2.5×10^4	8.4×10^3	1.5×10^4
pFW12	125	5.8×10^3	0	7.1×10^3	5.4×10^3	4.2×10^3
	250	0	0	8.7×10^2	9.2×10^3	4.2×10^3
pMF33	125	2.9×10^4	3.5×10^4	7.7×10^3	7.7×10^3	6.5×10^3
	250	3.5×10^4	2.9×10^4	1.1×10^4	6.7×10^3	1.3×10^4

^a W3110 was transformed with helper plasmids producing low levels of wt π protein ($\Delta 14$) or normal levels ($\Delta 22$) of wt π or copy-up π variant 113, 116, or 200. These strains were made competent, and 1.5×10^8 cells of each were transformed with 0.25 μ g of closed circular γ origin plasmid DNA. Numbers indicate colonies obtained per microgram of DNA per 4×10^7 cells plated on antibiotic-containing LB plates supplemented with 5 μ g of thymidine per ml.

tion. Thus, if pFW18 had a copy number higher than that of pFW12, then a portion of the enhancer other than the DnaA box 1 (or the π site) might also be contributing to counteract the negative effect of π .

Direct copy number analysis was also performed with the three γ origin plasmids in strains producing copy-up π variants $\pi 113$, $\pi 116$, and $\pi 200$. These π mutant proteins, at the low ($\Delta 14$) or normal ($\Delta 22$) level, had been shown to allow replication of γ origin core plasmids, though none had been selected for this function (51a). Copy number analysis had not previously been performed with these strains.

Table 2 (and data not shown) indicates that all three γ origin plasmids transformed all the hosts producing low or normal levels of copy-up π mutants. Transformants were obtained at high frequencies, even at the high penicillin concentration. Cells carrying the various π -producing and γ origin plasmids were grown overnight in LB supplemented with penicillin. Plasmid DNA was isolated, linearized, and electrophoresed on an agarose gel. As shown in Fig. 3A, all three γ origin plasmids replicate at approximately the same copy number at the low level ($\Delta 14$) of wt π . Thus, the absence of transformants of W3110 ($\Delta 14$ -*pir*WT) with pFW12 at the high concentration of penicillin might be due not to an effect on R6K replication, but rather to an effect on the expression of the β -lactamase gene via an unknown mechanism. The low level of the $\pi 113$ protein uniformly raised the copy number of all three γ origin plasmids twofold over that obtained with the wt protein. Both the $\pi 116$ and $\pi 200$ proteins uniformly raised the plasmid copy number of all three γ origin plasmids fourfold over that obtained with wt π . Thus, at the low level of π , the plasmid copy number is greatly influenced by the genetic variant of π but not by the DnaA box 1 or any other potential binding site in the enhancer.

The situation at the normal level ($\Delta 22$) of the mutant π proteins (Fig. 3B) is quite different. At the normal level of $\pi 113$, pFW18 and pMF36 replicate at similar copy numbers but the core plasmid pFW12 is almost absent (an eightfold decrease in copy number in comparison with pFW18 or pMF36). This decrease may occur because pFW12, unlike the other two γ origin plasmids, is highly unstable in this strain (51). At the normal level of $\pi 116$ or $\pi 200$ protein, the plasmids with the DnaA box 1 altered (pFW18) or deleted (pFW12) both replicate at a copy number twofold lower than that of the pMF36 plasmid, although both pFW18 and pFW12 are stable in these strains (51). Thus, the origin composition is very important in determining plasmid copy number at the normal level of copy-up π mutants in wt cells, although, as shown in Fig. 3A, origin composition is not important at the low level of copy-up π mutants. Such a phenomenon has not previously been reported for R6K.

Like pMF36, another γ origin plasmid, pRK526, carries both the core and the enhancer. Also like pMF36, pRK526 replicates at a high copy number at both low and normal levels of copy-up π variants in a wt host (4). However, when tested in a strain lacking IHF protein, the copy number of pRK526 is greater at the low level of $\pi 113$ than at the normal level. Thus, copy-up mutants overreplicate in comparison with wt π , but at the normal level, some of these mutants also retain a residual ability to inhibit R6K replication. IHF and DnaA box 1 must counteract this negative activity of π .

The role of the enhancer and IHF in counteracting the negative effect of π . The primary conclusion from these experiments is that the DnaA box 1 in the enhancer is

FIG. 3. Copy number analysis of R6K γ origin plasmids pMF36, pFW18, and pFW12 at low ($\Delta 14$) (A) and normal ($\Delta 22$) (B) levels of wt π , $\pi 113$, $\pi 116$, and $\pi 200$. Arrows indicate plasmids linearized by digestion with *Sna*BI.

necessary to counteract the negative effect on R6K replication mediated by the normal level of wt or mutant π protein. It was suggested that high levels of wt π might inhibit replication by simultaneously binding to clusters of iterons on two plasmid molecules, thereby promoting coupling of the plasmids ("handcuffing" [32]); evidence exists for a similar scenario in another system (27). IHF is necessary to prevent excessive wt π from inhibiting R6K replication (4); IHF also folds γ origin fragments (5, 11). It has been proposed that IHF-mediated binding and folding might prevent handcuffing simply by blocking access of π to some iterons (4, 11). This simple model must be incomplete, because it does not take into account our present finding that the DnaA box 1 is also important. Perhaps the negative effect of π is counteracted by a multicomponent nucleoprotein structure which the isolated core cannot form. For example, IHF binding in the middle of the γ origin and folding of the DNA might allow protein-protein interactions between DnaA bound to DnaA box 1 in the enhancer and DnaA bound to several DnaA boxes present in the core (51a) (Fig. 1). Interactions between DnaA bound to the enhancer and π bound to the core might also be possible. This general model is similar to IHF-dependent site-specific recombination of phage λ (34) or nucleoprotein structures in the pSC101 origin (47).

Ultimately, whether an (hypothetical) IHF-DnaA-enhancer complex would counteract the negative effect of π by stabilizing a replication complex already formed at the core or by constructing an entirely different type of nucleoprotein complex remains unknown. In either case, the enhancer serves to increase the range of π levels which allow R6K γ origin replication.

Broad applicability of agarose gel retardation assay in binding studies involving DnaA protein. To show that altering the DnaA box 1 weakened or abolished binding to the enhancer, we applied the agarose gel retardation assay to DnaA (Fig. 2). Although DnaA binding to specific sites has been demonstrated thus far by a variety of *in vitro* techniques (reviewed in reference 40), to the best of our knowledge, a successful gel retardation has not been reported for this protein. The gel retardation assay requires proteins continuously and stably bound to DNA (whereas some other assays do not) and thus may complement these other assays by being a valuable tool to determine the stoichiometry of binding.

The technique of agarose gel retardation was originally developed by Chelm and Geiduschek (3) and has been subsequently used in a number of laboratories, including our own, in the analysis of complexes of DNA with IHF (9) and π (14). Figure 2 shows that DnaA forms one major complex with the wt enhancer carrying one DnaA box but no detectable complexes with the mutant R6K enhancer, which carries no sequences with a match to the DnaA consensus of greater than six of nine. To show the general applicability of the agarose gel retardation assay and examine the type of complexes formed, we used purified DNA fragments carrying sites previously shown to bind DnaA protein *in vitro*: the pUC origin (15), *oriC* (15), and the γ origin core (51a). The involvement of DnaA in replication of pBR322 (40a) and *oriC* (16, 17, 24, 48) has been described previously.

As shown in Fig. 4, upon addition of low levels of DnaA (0.04 to 0.16 μ g), the pUC origin fragment forms a single complex. This complex most likely contains DnaA protein bound to the single site (TTATCCACA, a perfect match to the consensus [15]) present in this fragment. At the highest concentration of DnaA shown in Fig. 4 (0.24 μ g), the nucleoprotein complexes form a ladder, not a smear. The ladder at the high concentration of DnaA may represent specifically

FIG. 4. Agarose gel retardation of pUC origin fragment with DnaA. A 100-ng amount of DNA (0.4 pmol) was used for each sample, and the final volume was 8 μ l. At the top is a schematic of the pUC origin, with the DnaA box (open box) indicated. Arrows mark naked DNA and DNA complexed with DnaA, and DnaA quantities are indicated.

bound DnaA monomers recruiting other DnaA molecules from the solution (15). Alternatively, this ladder may represent DnaA binding as a multimer. DnaA is known to aggregate when purified by a technique different from the one we used (17); as revealed by cross-linking studies with DnaA and bis[2-succinimidooxycarbonyloxy]ethyl sulfone (BSOCOES), our preparation of DnaA also contains some multimeric forms (data not shown).

We next tested the nature of nucleoprotein complexes formed between DnaA and fragments containing multiple sites; we used *oriC* and the R6K core. The *Hind*III *oriC* fragment from plasmid pLAR7 carries three of the four *oriC* DnaA boxes (R1, R2, and R3 [38]) plus three 13-mers in the AT-rich segment believed to bind DnaA (53). At the highest concentration of DnaA at which some fragments remained unbound (0.16 μ g), at least four nucleoprotein complexes were observed (Fig. 5). Interestingly, the complexes are not of equal intensity; one "rung" in the ladder of complexes is reproducibly absent. This may reflect different binding affinities for different sites (39a) or the effect of the different orientation of the DnaA boxes on DnaA-DnaA interactions, though these possibilities have not been rigorously tested.

The γ origin core fragment carries DnaA box 2 and three other sites with a good match to the consensus DnaA box (51a) (Fig. 6). Enhancements of DNase I cleavage were seen in the area of the three consensus sequences, which lie in an AT-rich region (51a); all the sites are in the same orientation. In contrast to the *oriC* fragment, the core fragment produces at least six distinct complexes (Fig. 6, lanes marked 0.16 and 0.24 μ g), all of similar intensity.

Surprisingly, with all the fragments tested, the number of complexes seen with low levels of DnaA differs from that suggested by other assays. Electron microscopic data suggested that *oriC* fragments were wrapped around a mass of 20 to 30 DnaA monomers (15). DNase I footprints of *oriC* (15), the pUC origin (15), and the R6K core and enhancer (51a), even at low concentrations of DnaA, all showed a large area of protection punctuated by periodic cleavage enhancements,

FIG. 5. Agarose gel retardation of *oriC* fragment with DnaA. A 60-ng amount of DNA (0.3 pmol) was used for each sample, and the final volume was 11 μ l. At the top is a schematic of the *oriC* fragment used, with 13-mers (short black rectangles) and three DnaA boxes (open boxes) indicated. Arrows indicate naked DNA and DNA-DnaA complexes.

again suggesting a large number of DnaA molecules interacting at each consensus sequence. Interestingly, in our gel retardation assay, the stoichiometry of DnaA binding appears to be much closer to one protein molecule per site, e.g., the mutant enhancer with no consensus sequences forms no detectable complexes (Fig. 2); the pUC origin, carrying one consensus sequence, produces one specific complex (Fig. 4); and the enhancer, also carrying a single site with a weaker match to the consensus, forms one major complex (Fig. 2). The *oriC* fragment used, carrying up to six sites, forms at least four complexes, although at least one rung in the ladder is skipped (Fig. 5). The core, with four DnaA boxes, forms six complexes (Fig. 6). It is not known why these different techniques suggest different stoichiometries of binding, although gel retardations may require tighter and more continuous binding than some

FIG. 6. Agarose gel retardation of R6K core origin fragments with DnaA protein. At the top is a schematic diagram of the R6K γ origin core, with consensus sequences a, b, and c and DnaA box 2 indicated. Naked DNA and complexes are indicated by arrows, and DnaA quantities used are marked. For each sample, 100 ng (0.5 pmol) of DNA fragment was used and the final volume was 7.5 μ l.

other assays. Another possibility is that the binding properties of DnaA may be altered by different techniques.

While attempting to apply gel retardations to DnaA, we investigated several electrolytes and different agarose and acrylamide concentrations. The conditions described in this paper were optimal for retardation of DNA fragments by purified DnaA protein. For example, DnaA did not bind DNA fragments at all in agarose gels when TA buffer (50 mM Tris base, 30 mM acetate [pH 8.3]) was used as an electrolyte instead of TB (data not shown). The binding of several proteins *in vitro* is similarly influenced by buffer composition; Mu repressor, for example, binds its operators in Tris-glycine buffer but not TBE (1). Complexes with DnaA were not seen when polyacrylamide was substituted for agarose (data not shown); this may be due to differences in pore sizes in these gels (reviewed in reference 30). Another laboratory was also unable to demonstrate reliable gel retardations with DnaA when polyacrylamide was used (37). In addition, complexes with DnaA were detectable only at room temperature; running the retardation gels at 4°C produced only smears (data not shown). The gel retardation technique has several advantages over the other assays (reviewed in reference 30). This assay is technically easy and simple, and only small quantities of material are necessary. Detailed information about the nature of multiple protein molecules bound to a DNA fragment can be gained.

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