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DnaA Protein Is Required for Replication of the Minimal Replicon of the Broad-Host-Range Plasmid RK2 in *Escherichia coli*

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The minimal origin of replication of the broad-host-range plasmid RK2 has two potential recognition sequences for the DnaA protein of *Escherichia coli*. DNA transfer by transformation into a *dnaA*-null mutant of *E. coli* showed that DnaA protein is needed for replication or maintenance of mini-RK2. We isolated and purified DnaA protein as a chimeric protein, covalently attached to a piece of collagen and β-galactosidase. The hybrid protein specifically bound to restriction fragments from the *oriV* region of RK2, which contained the two *dnaA* boxes. Deletion of the second *dnaA* box inactivated the origin and abolished the binding of the hybrid protein to the DNA fragment that had suffered the deletion. When the second *dnaA* box was replaced with an *Eco*RI linker of identical length, origin activity was restored. Binding experiments showed that the linker provided a weak *dnaA* box. An alternative explanation was that the linker restored proper spacing between sequences on either side of the deleted box, thus restoring origin activity.

The Inc-P plasmid RK2 (similar and probably identical to RP1, RP4, R68, and R18) (4) is a broad-host-range plasmid, capable of maintenance in all gram-negative bacteria tested with the exception of *Bacteroides* spp. (14). Therefore a study of the mechanism of initiation of replication of RK2 should provide insights into general features of replication control in gram-negative bacterial species.

Previous work has resolved the replicon of RK2 into a *cis*-acting origin sequence, designated *oriV*, which maps to coordinates 12.3 to 13.0 kilobases on the 60-kilobase RK2 map, as well as a *trans*-acting initiator protein encoded by the *trfA* gene (16.0 to 17.3 kilobases) (30–32, 34). These two determinants constitute the minimal origin which is sufficient for replication in many bacterial species including *Escherichia coli*. A second class of determinants containing the *kil* and *kor* genes is required to maintain RK2 in other gram-negative bacteria (8, 9, 25, 26, 29, 30). Additionally the *trfA* gene exists as two species, one of 43 kilodaltons (kDa) and the other of 32 kDa, which arises from a second initiation event within the *trfA* coding sequence. Only the 32-kDa *trfA* protein is required for replication in *E. coli*. However, the promoter for the operon containing *trfA* is normally very dependent for activity upon the presence of *kor* genes (*korA* and *korB*) (9).

We wished to determine, systematically, which gene products of *E. coli* are required for the replication of RK2 both by DNA transformation in vivo and by in vitro biochemical analyses. In this paper we show that the mini-RK2 plasmid is not maintained in a strain of *E. coli* that lacks a functional DnaA protein.

Concurrent with the in vivo observations, we performed in vitro studies that showed the binding of a DnaA hybrid protein to at least two sites in the replication origin of RK2. Deletion of the second *dnaA* box not only abolished the binding of the protein to the mutant site but also abolished origin activity.

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MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1.

Nomenclature. The map positions for determinants on the RK2 genome are in kilobases from the unique *Eco*RI site. The coordinate positions are those of Lanka et al. (18). The coordinates for features of the *oriV* region are those of Cross et al. (6).

DNA methodology. Preparation of plasmid DNA and agarose gel electrophoresis were done as previously described (19). Restriction endonucleases, DNA polymerase I, Klenow fragment, and T4 DNA ligase were purchased from commercial suppliers and were used as specified by the suppliers. The 8-base-pair (bp) *Eco*RI linkers were purchased from New England BioLabs, Inc. Transformations of *E. coli* were done by the standard calcium chloride method (19).

β-Galactosidase assay. The β-galactosidase activity of the protein solution was assayed by the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside as described by Miller (21). Protein concentrations were determined by the Coomassie dye-binding technique of Bradford (3).

Purification of DnaA hybrid protein. The cells containing pNT77 were grown in Luria broth at 30°C to a cell density of 4×10^8 cells per ml and then induced by an increase of the temperature of the culture to 42°C, thus inactivating the temperature-sensitive *c*I857 repressor. The DnaA–collagen–β-galactosidase hybrid protein was purified through a 40% ammonium sulfate cut followed by *N*-propylamine hydrophobic interaction chromatography. The details of the purification are to be described elsewhere.

The hybrid protein can be cleaved by collagenase to generate a DnaA moiety (13). The hybrid protein in the 40% ammonium sulfate cut and its cleavage by collagenase are shown in Fig. 1. The structure of pNT77 is also shown in Fig. 1.

Immunoprecipation of antigen-DNA complex. The DNA-protein complexes were immunoprecipitated essentially as described previously (12, 20). For each reaction, 10 μg of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
CM1793	*asnB32 relA1 spoT1 thi-1 fuc-1 lysA ilv-192 zia*:pKN500 (mini-R1)	36
EH3827	CM1793 Δ*dnaA mad-1*	15
EC558	F⁻ *argE3 his-4 leu-6 proA2 thr-1 ara-14 galK2 lacY1 thi-1 supE44 rpsL31*	16
polA12	K-12 MM383 *polA12* [*polA*(Ts)]	22
Plasmids		
pJG200	Expression vector, *lacZ* fusion with collagen linker, P_R promoter, Ap^r	13
pCM007	E2 ORF[a] of bovine papillomavirus inserted into pJG200	23
pBR322	Ap^r Tc^r	2
pNT77	*dnaA* in pJG200 (details of construction to be described elsewhere) Ap^r	This work
pBF110	*dnaA* overproducer, Ap^r	11
pRK2328	Minimal RK2 replicon, *trfA*$^+$ *ori*V$^+$, *trfA* promoter, uses gentamicin resistance promoter for *trfA kilB1*^{r}, Tc^r	27
pSC101	*dnaA*-dependent replicon, Tc^r	5
pCV21	pUC9 plus pSC101 origin region[b], Ap^r	
pRK2128	750-bp origin of RK2 (*Hae*II-C) linked to Cm^r	27
pPG1	Minimal *oriV Hpa*II fragment (coordinates 416 to 809) cloned into pHW1 at *Hin*dIII and *Eco*RI sites	This work
pHW1	Derivative of pBR322, Ap^r	37
pPG10	*Hae*II-C cloned into *Hin*cII site of pUC19	This work
pPG11	pPG10 in which the second *dnaA* box has been replaced with an *Eco*RI linker	This work
pPG12	pPG10 in which the second *dnaA* box has been deleted	This work
pUC19	ColE1 replicon, Ap^r	38
pJZ101	*oriC* construct in pBR322, Ap^r	39

[a] ORF, Open reading frame.
[b] This is a deletion derivative of the *cis* required region of the pSC101 origin. It extends from coordinate 125 to 577 (35).

unlabeled sonicated salmon sperm DNA and 100,000 cpm of the appropriate end-labeled DNA (prepared by filling in the 3′ recessed ends of the DNA with the Klenow fragment of DNA polymerase I and either [α-^{32}P]dATP or [α-^{32}P-dCTP]) were mixed with 30 μl of DnaA protein-binding buffer (40 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.6], 5 mM magnesium acetate, 50 mM potassium chloride, 0.1 mM EDTA). To the appropriate reaction mixtures was added 250 U (of β-galactosidase activity) of the chimeric protein. Each mixture was incubated at 37°C for 20 min. Then 1 μg of monoclonal anti-β-galactosidase antibody (Promega Biotec) was added to the appropriate reaction mixtures, which were then incubated for 20 min on ice. Finally 50 μl of a 10% suspension of *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) in NET buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100) was added to each reaction mixture, which was then incubated on ice for 20 min. The complex containing the *Staphylococcus*-protein A-antibody-chimeric protein-DNA was spun down in an Eppendorf centrifuge and washed twice with 200 μl of NET buffer. The pellet was suspended in 100 μl of a 0.1% sodium dodecyl sulfate solution and phenol-chloroform extracted, and the DNA was then alcohol precipitated and loaded onto polyacrylamide gels, dried, and autoradiographed. The input lane contained 10,000 cpm of DNA.

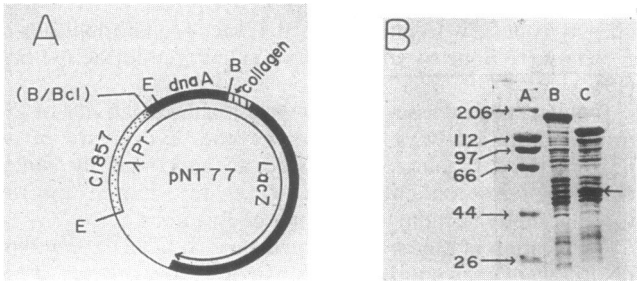

FIG. 1. Structure of the DnaA overproducer pNT77 and polyacrylamide gel profiles of proteins from *E. coli* containing pNT77. (A) Restriction map of pNT77 (details to be described elsewhere). Abbreviations and symbols: E, recognition sites for *Eco*RI; B, restriction sites for *Bam*HI; Bcl, sites for *Bcl*I; cI857, temperature-sensitive lambda repressor; Pr, rightward promoter from bacteriophage lambda; ▨ region of DNA which encodes 60 amino acids of a chicken collagen gene; LacZ, open reading frame for β-galactosidase. The arrow indicates the direction of transcription. (B) Protein profile of the 40% ammonium sulfate precipitate from strain TG1 harboring pNT77 after thermal induction. Lanes: A, molecular weight markers (sizes in kilodaltons are shown); B, 40% ammonium sulfate precipitate of pNT77; C, the 40% ammonium sulfate precipitate after cleavage with collagenase, which specifically cleaves the collagen sequence between the DnaA reading frame and that of β-galactosidase. The arrow indicates the position of the released DnaA protein.

RESULTS

The RK2 miniplasmid pRK2328 is not maintained in an *E. coli* strain deleted for *dnaA*. We examined the ability of the *dnaA*-null strain EH3827 (15) to support the replication of pRK2328 (27). This plasmid contains the *oriV* and *trfA* determinants of RK2; however, because of a spontaneous deletion, the 32-kDa *trfA* gene is transcribed, not by its own promoter, but by the promoter for the gene encoding gentamicin resistance. In this plasmid only the coding sequence for the 32-kDa *trfA* gene product is present, the amino terminus of the 43-kDa *trfA* protein having been deleted. Since it uses the constitutive gentamicin resistance promoter, it is no longer dependent upon the presence of *korA* and *korB* (9).

Both the *dnaA*-null strain, EH3827, and the isogenic *dnaA*-positive strain, CM1793 (36), were rendered compe-

TABLE 2. Failure of RK2 replicons to be established in *dnaA* deletion strains

Plasmid	No. of colonies[a]		Ratio of transformants (EH3827/CM1793)
	EH3827	CM1793	
pBR322	5,229	6,121	0.85
pSC101	0	1,107	<0.001
pRK2328	0	1,478	<0.001

[a] Total number from nine independent transformations.

tent for transformation by treatment with calcium chloride and transformed with equal molar amounts of the three plasmids pRK2328, pBR322, and pSC101. The data shown in Table 2 reveal that in several experiments the positive control pBR322, which does not require DnaA (10, 15), was consistently maintained in the *dnaA*-null and *dnaA*$^+$ strains of *E. coli*, whereas the test plasmid pRK2328 and the negative control pSC101, which has been shown to require DnaA (10), were maintained only in the *dnaA*$^+$ strain. Thus pRK2328 requires DnaA protein in vivo for its maintenance in *E. coli*.

Biological activity of the DnaA hybrid protein and its complementation of a *dnaA* defect in *E. coli*. DnaA protein was supplied in *trans* with plasmids which overexpress the protein. Since the DnaA protein encoded by the plasmid pNT77 (Fig. 1) is a translational fusion protein, being fused in frame with β-galactosidase via a collagen linker (the details of the construction of pNT77 will be described elsewhere), we wished to determine whether the fusion protein had authentic biological activity by in vivo complementation of *dnaA* defects. Plasmid pNT77 showed complementation of *dnaA* defects in both the *dnaA*(Ts) strain EC558 (16) at the nonpermissive temperature (data not shown) and the *dnaA*-null strain. The *dnaA*-null strain was first transformed with one of three plasmids: the positive control plasmid pBF110, a DnaA overproducer constructed by Fuller and Kornberg (11); the test plasmid pNT77, in which the chimeric protein transcription is under the control of the rightward promoter of bacteriophage lambda; or a negative control plasmid, pCM007, which encodes the bovine papillomavirus E2 open reading frame–collagen–β-galactosidase fusion protein (23).

Three test plasmids were introduced into host cells containing the three resident plasmids mentioned above. The test plasmids were pBR322 (DnaA independent), pSC101 (DnaA dependent), and pRK2328 (presumed to be DnaA dependent). The results shown in Table 3 support the conclusion that the DnaA hybrid protein produced by pNT77 complemented the *dnaA* defect in the mutant *E. coli*. This interpretation is subject to the caveat that the hybrid protein could be randomly processed in vivo, separating the DnaA-collagen moiety from β-galactosidase. However, it is highly unlikely that precise cleavage of the collagen moiety from DnaA protein could occur in vivo, since this would invoke the presence of a specific processing protease in *E. coli*.

TABLE 3. Complementation of *dnaA* requirement with pNT77[a]

Resident plasmid	Test plasmid	No. of colonies[b]	
		EH3827	CM1793
PCM007	pBR322	1,117	1,451
pCM007	pRK2328	0	1,106
pCM007	pSC101	0	1,685
pNT77	pBR322	918	1,209
pNT77	pRK2328	859	1,138
pNT77	pSC101	780	914
pBF110	pBR322	874	1,071
pBF110	pRK2328	837	1,116
pBF110	pSC101	731	766

[a] The resident plasmid was first introduced into the host cell by selecting for resistance to ampicillin (50 μg/ml). The test plasmid was subsequently put in by transformation. Transformations were selected using tetracycline (15 μg/ml).

[b] Values represent the sum of six independent transformations.

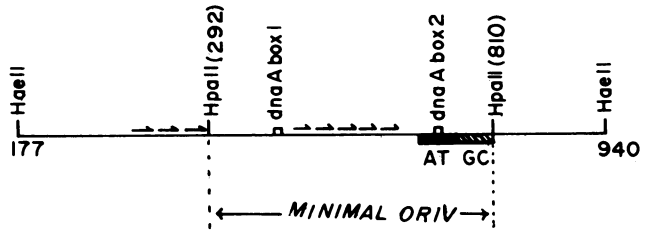

FIG. 2. Topographical features of the replication origin (*oriV*) of RK2. The 750-bp *Hae*II C fragment of RK2 is depicted with coordinates as described by Cross et. al. (6). The arrows depict the two sets of 17-bp direct repeats; the two *dnaA* boxes are shown by the squares. The region marked AT is a 55-bp sequence which is 74% A+T rich; GC depicts a 60-bp sequence which is 80% G+C rich.

Deletion analysis of the *oriV* of RK2. The 750-bp *Hae*II C fragment of RK2 contains all of the *cis* elements that are required for replication (7, 33). This stretch of DNA contains several interesting features (Fig. 2). There are two sets of 17-bp direct repeats, one from coordinates 330 to 410 and the other from coordinates 525 to 630 (6, 30), the former being dispensable for replication. There is also a putative *dnaA* box immediately preceding the second set of repeats, and an A+T-rich region following the repeats in which is embedded a second sequence with partial homology to a *dnaA* box. Although the first such *dnaA* box is an exact match of the consensus TTAT(C/A)CA(C/A)A (11, 39), the second box contains two mismatches. The A+T-rich region is followed by a G+C-rich sequence. The sequence required for replication has been further limited to a 393-bp *Hpa*II fragment which extends from coordinates 416 to 808 (33).

The second *dnaA* box is flanked by recognition sites for two blunt-end-cutting restriction endonucleases, *Dra*I and *Ssp*I. We digested the 750-bp *Hae*II C fragment of pPG10 with *Dra*I and *Ssp*I and eliminated the *dnaA* box by blunt-end ligation of the *Dra*I site with that of the *Ssp*I site to create the plasmid pPG12. We also introduced the synthetic *Eco*RI linker GGAATTCC by blunt-end ligation between the *Dra*I and *Ssp*I ends, thus replacing the deleted *dnaA* box with precisely the same number of base pairs containing an *Eco*RI recognition site, to create the plasmid pPG11 (see Fig. 5). The *Eco*RI linker was chosen in an attempt to minimize changes in the overall base composition of the region. Both mutants were verified by restriction analysis (data not shown).

We then tested the ability of the mutant origins to replicate in a host strain that has a temperature-sensitive mutation in the DNA polymerase I gene [*polA12*(Ts)] (22). This strain was used to prevent any replication from the ColE1-type origin of the vector plasmid. The *polA*(Ts) strain was first transformed with pRK2328, a plasmid that could supply the *trfA* protein in *trans*. We observed that pPG12, which had the deletion of the second *dnaA* box, failed to replicate at the nonpermissive temperature, whereas plasmid pPG11, which

TABLE 4. Replication competence of alterations of the second *dnaA* binding site of the *oriV* region

Plasmid	Sequence of second site[a]	Ratio of transformants in a *polA*(Ts) host (30°C/42°C)
pPG10	GCTT**TTAAACCAAT**ATTTATA CGAA**AATTTGGTTA**TAAATAT * *	0.65
pPG11	GCTT**TTGGAATT**CCATTTATA CGAA**AACCTTAA**GGTAAATAT * * *	0.46
pPG12	GCTTTT ATTTATA CGAAAA TAAATAT	<0.001

[a] Asterisks indicate mismatches from the consensus sequence TTAT(C/A)CA(C/A)A as described previously (11, 39). Boldface type indicates *dnaA* box.

had the *Eco*RI linker substitution and the wild-type plasmid pPG10 (Table 1), replicated in the absence of DNA polymerase I. Table 4 shows the sequence flanking the second *dnaA* box in these three plasmids, as well as their ability to support replication. The failure of pPG12 to replicate as contrasted with pPG11, which did replicate, suggests that the spacing around the second *dnaA* box may be important for a functional origin. Alternatively, the substitution of the second *dnaA* box by the *Eco*RI linker created a weak *dnaA* box, with three mismatches from the consensus sequence. The sequence of pPG11 around the second *dnaA* box not only contained three mismatches (the wild-type sequence has two mismatches from the consensus), but also this putative *dnaA* box of pPG11 was on the opposite strand from that of the wild-type pPG10 (Table 4).

The hybrid dnaA protein has normal DNA-binding activity. To examine the biological activity of the hybrid protein in vitro we performed immunoprecipitations of protein-DNA complexes (12, 20). The availability of the hybrid protein allowed us to perform the highly sensitive immunoprecipitation assay with monoclonal antibody directed against the β-galactosidase moiety. We therefore tested the ability of the tripartite hybrid protein encoded in pNT77 to bind to both *oriC* (11) and the origin of pSC101. The *oriC*-containing plasmid pJZ101 (16) and the pSC101 origin plasmid pCV21 (Table 1) were digested with *Hin*fI and a combination of *Hin*fI, *Hin*dIII, and *Bam*HI, respectively (Fig. 3). The fragments were 3′ end labeled by filling in with α-^{32}P-labeled deoxynucleoside triphosphates and the Klenow fragment of DNA polymerase I. The labeled fragments were incubated with the tripartite DnaA protein, immunoprecipitated with anti-β-galactosidase antibodies, and then adsorbed to protein A-*S. aureus* cells. The precipitated DNA was recovered and analyzed by gel electrophoresis. The autoradiograms of the gels (Fig. 4) show that both the *oriC* fragment and the pSC101 *ori* fragment were immunoprecipitated specifically with the chimeric DnaA protein. In control experiments in which the antibody, the protein A-*S. aureus* cells, or the hybrid protein was excluded or a heterologous DNA-binding fusion protein (e.g., the E2 open reading frame of bovine papillomavirus fused to β-galactosidase via the collagen linker) was substituted, none of the origin fragments were immunoprecipitated. From these experiments we concluded that the chimeric DnaA protein recognizes the same sequences as the nonhybrid DnaA protein.

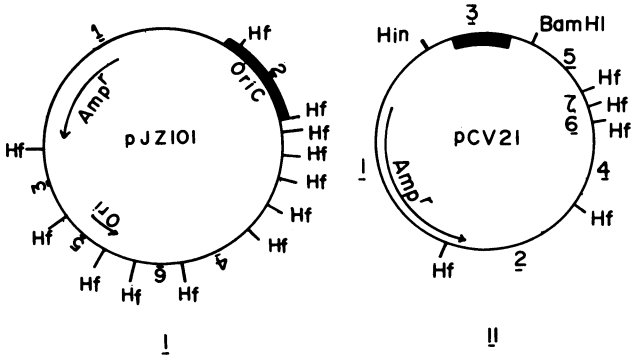

FIG. 3. Restriction maps of the *oriC* plasmid (pJZ101) and the pSC101 *ori* plasmid (pCV21). (I) Restriction map of plasmid pJZ101, used for the immunoprecipitation of *oriC*. ▬, Position of the *oriC* segment with respect to the gene for ampicillin resistance and the origin of replication of the pBR322-based plasmid. The positions of cleavage for the restriction enzyme *Hin*fI (Hf) are shown, as well as the ordinal ranking of the restriction fragments. (II) Restriction map of the plasmid used for the immunoprecipitation of the pSC101 origin region, pCV21. ▬, pSC101 origin region with respect to the ampicillin resistance gene of the pUC9-based plasmid. The restriction sites for the enzymes *Hin*dIII (Hin), *Bam*HI, and *Hin*fI (Hf) are shown, as well as the fragments which would arise from such a triple digest.

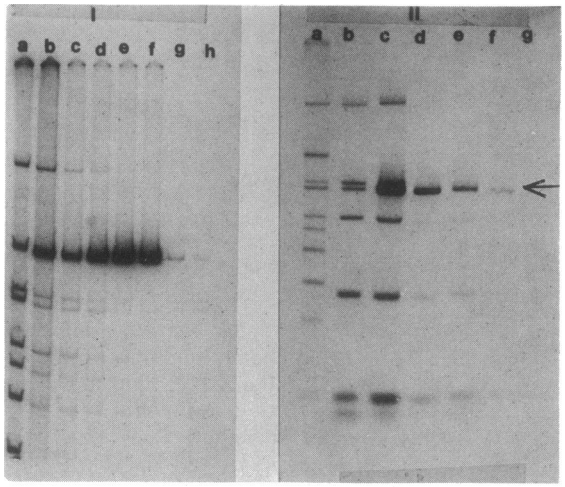

FIG. 4. Immunoprecipitation–DNA-binding assays, showing specific binding of the DnaA hybrid protein to known sites. (I) Autoradiogram of a 5% polyacrylamide gel showing the binding of DnaA protein to *oriC*. Lane a, input DNA (pJZ101 digested with *Hin*fI); lanes b to f, immunoprecipitations of pJZ101 with DnaA hybrid protein with various amounts of carrier: 0.1 μg (lane b), 0.5 μg (lane c), 1.0 μg (lane d), 5 μg (lane e), and 10 μg (lane f); lane g, immunoprecipitation control in which no antibody was added; lane h, control in which no DnaA hybrid protein was added. (II) Autoradiogram of a 5% polyacrylamide gel showing the specific binding of DnaA hybrid protein to the pSC101 origin. Lane a, molecular weight markers (pJZ101 digested with *Hin*fI); lane b, input DNA (pCV21 digested with *Hin*dIII, *Bam*HI, and *Hin*fI); lanes c to f, immunoprecipitations performed with various amounts of carrier DNA: 0.1 μg (lane c), 1 μg (lane d), 10 μg (lane e), and 20 μg (lane f); lane g, control in which no antibody was added.

Interaction of the *oriV* of RK2 with the chimeric DnaA protein. To study the DnaA interactions with the origin of RK2, we performed immunoprecipitations to determine whether DnaA binds to *oriV*. For this we used plasmid

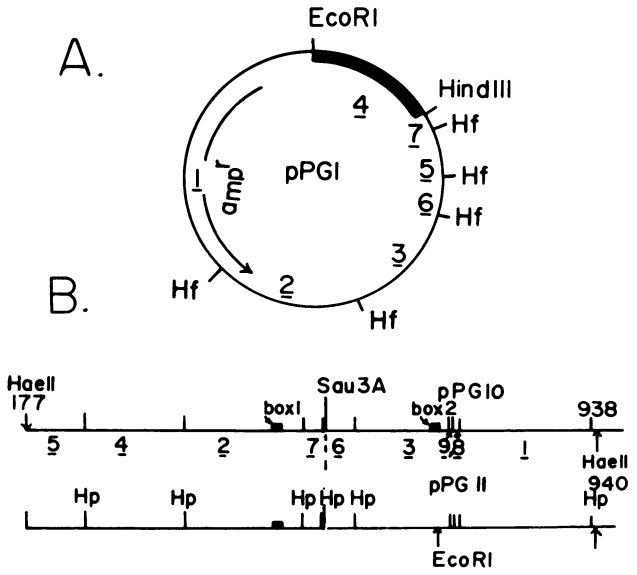

FIG. 5. Restriction maps of the *oriV* plasmids of RK2 used in this study. (A) Restriction map of pPG1. ▬, minimal *oriV* fragment cloned into the *Hin*dIII and *Eco*RI sites of pHW1 by using the pUC19 polylinker region; Hf, *Hin*fI sites. The plasmid regions which correspond to the various bands of the immunoprecipitations are indicated by the numbers (Fig. 6). (B) Restriction maps of pPG11 and pPG12. ■, The two *dnaA* boxes of *oriV*. Hp depicts the *Hin*PI sites. The numbers indicate the ordinal ranking of the fragments of a *Hin*PI digest. The coordinate position for the downstream *Hae*II site is approximate, being deduced from the fragment size in the gel. The deletion in pPG12 is at the same site as the *Eco*RI linker insertion in pPG11.

pPG1, which contains the 393-bp *Hpa*II fragment of *oriV* inserted into the *Eco*RI and *Hin*dIII sites of pHW1 (37), a mini-pBR322 vector. This plasmid (Fig. 5) was digested with *Eco*RI, *Hin*dIII, and *Hin*fI, and the fragments were 3′ end labeled. Immunoprecipitations with the DnaA hybrid protein showed that *oriV* was specifically bound by the tripartite hybrid DnaA protein (Fig. 6).

To further delineate the binding sites of the protein, the *Hae*II C fragments of pPG10, pPG11, and pPG12 were digested with *Hin*PI and 3′ end labeled. The immunoprecipitations of these fragments showed that both the fragment containing the first *dnaA* box (spanning coordinates 501 to 509) and the fragment containing the second *dnaA* box (coordinates 711 to 719) were specifically immunoprecipitated by the hybrid DnaA protein, thus confirming the presence of at least two DnaA recognition sites (Fig. 7). In the mutant pPG11 *oriV* fragment, the fragment containing the first *dnaA* box was strongly immunoprecipitated, whereas the *Eco*RI linker-containing fragment showed only weak binding. In pPG12, there was no detectable binding of the fragment which suffered the deletion of the second *dnaA* box (Fig. 7). These results demonstrate that the second *dnaA* box is indeed the only binding site for DnaA protein in this fragment. Thus the in vitro binding studies were consistent with the in vivo transformation data which show the requirement of DnaA protein for the maintenance of the RK2 minimal replicon.

DISCUSSION

The in vivo and in vitro studies reported in this paper show that the DnaA protein is needed for the establishment and presumably the replication of the minimal replicon of the broad-host-range plasmid RK2 in *E. coli* and that the DnaA protein binds to at least two sites within the minimal origin sequence. The relative roles of DnaA protein and the RK2-encoded *trfA* protein in RK2 replication are unknown. The role of DnaA in *oriC* replication appears to be in the assembly of a replisome, specifically at the origin sequence, and perhaps in the unwinding of the *ori* region by the combined action of DnaA, the DnaB helicase, DnaC, and Ssb proteins (1).

Recently Seufert and Messer (28) have shown that DnaA protein can substitute for protein i in the in vitro replication of pBR322. Thus DnaA protein apparently can participate in the assembly of the primosome. DnaA protein could play similar roles in RK2 replication. However, the function of the plasmid-encoded initiator TrfA protein in initiation remains unclear.

The sensitive immunoprecipitation experiments reported here confirm the presence of at least two sites in the minimal *oriV* sequence which serve as binding sites for the DnaA protein. Furthermore, the substitution of the second *dnaA* box with the *Eco*RI linker shows that either the spacing around that site is important and the site itself is dispensable for replication to occur or a much weaker binding site for the

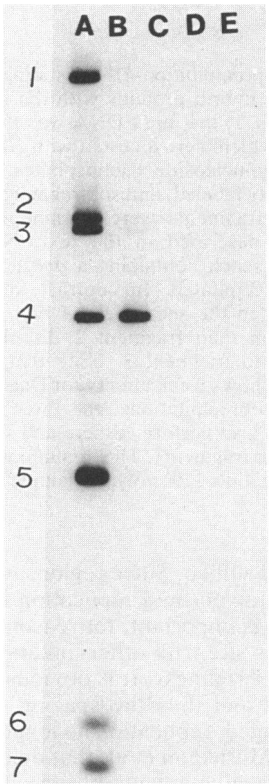

FIG. 6. Immunoprecipitation–DNA-binding assay, showing specific binding of DnaA hybrid protein to the *oriV* of RK2. The figure shows an Autoradiogram of a 5% polyacrylamide gel, with the binding of DnaA protein to *oriV*. Lanes: A, input DNA (pPG1) digested with *Hin*dIII, *Eco*RI, and *Hin*fI; B, immunoprecipitation with DnaA-galactosidase fusion protein; C, control in which no protein was added; D, control in which no antibody was added; E, control in which a heterologous fusion protein (bovine papillomavirus E2 open reading frame–collagen–β-galactosidase) was added. Note the specific immunoprecipitation of fragment 4 in lane B (Fig. 5A).

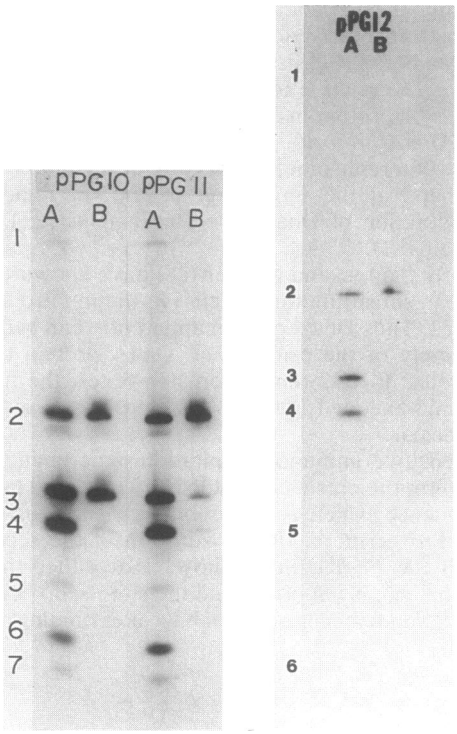

FIG. 7. Immunoprecipitation–DNA-binding assays showing interactions of DnaA hybrid proteins with the mutant *oriV* of RK2. For pPG10 and pPG11, the input DNA was the *Hae*II *oriV* inserts shown in Fig. 5B, which were digested with *Hin*PI and labeled with α-^{32}P-labeled deoxynucleoside triphosphates. Subfragments 1, 5, and 7 are more lightly labeled, and subfragment 8 has run off the gel. For pPG12, DNA fragments were immunoprecipitated by DnaA hybrid protein as described in the text. Note that in pPG10, fragments 2 and 3 (each containing a *dnaA* box [Fig. 5B]) were strongly immunoprecipitated. In contrast, in pPG11, the *Eco*RI linker that substituted the second *dnaA* box in fragment 3 shows weaker precipitation than fragment 2. In pPG12, there was no detectable binding to fragment 3. Note that in both pPG10 and pPG11, fragment 4 shows weak affinity for DnaA hybrid protein. For each set of immunoprecipitations, the three control experiments used in Fig. 4 were also performed here and showed no detectable binding to any of the fragments. The immunoprecipitation of pPG12 was electrophoresed on a 12% polyacrylamide gel to better separate fragments 2 and 3.

DnaA protein will suffice. Since regions which are A+T rich seem to be vital for plasmid replication initiation to occur, we believe that it is important, for reasons of interpretation, not to replace this site with other linkers which would alter the degree of A+T richness. It is obvious that whenever the site is eliminated and thus the spacing of the neighboring sequences is changed, replication no longer occurs. Recently it was shown by Murakami et al. that deletions of one of the origins of mini-F which extend into the *dnaA* boxes abolish replication of that plasmid in vivo (24).

In vitro replisome assembly experiments are needed to unravel the relative roles of the DnaA and TrfA proteins in the initiation of RK2 replication. Since RK2 replicates in a wide range of gram-negative bacteria, it is possible that other gram-negative bacterial species have DnaA analogs which are recognized by the RK2 origin or that there are alternative DnaA-independent pathways by which replication initiation of RK2 can occur. Systematic mutagenesis of the *dnaA* boxes and transfer of the mutant origins to widely separated species of gram-negative bacteria (e.g., *Pseudomonas* spp.) should help to distinguish between these possibilities.

In addition to *oriC* of *E. coli*, several other replicons have been shown to be dependent upon the DnaA protein. However, the replication of these replicons was either restricted mostly to *E. coli* (15, 17) or was from very closely related species (e.g., pSC101: a plasmid isolated from *Salmonella* spp.). The strategies used by the broad-host-range plasmid RK2 to initiate its replication should give valuable insights into mechanisms of initiation in other gram-negative species.

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