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Purified dnaA protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication

(*oriC*/site-specific DNA binding/RNA polymerase/DNA gyrase)

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ABSTRACT **Soluble protein fractions from *Escherichia coli* *dnaA*$^+$ cells but not *dnaA* temperature-sensitive cells replicate plasmids containing the *E. coli* chromosomal origin of replication (*oriC*). Complementation of these mutant fractions provided an assay for dnaA protein activity in initiation of replication at *oriC*. From a strain (constructed *in vitro*) that overproduces the dnaA protein more than 200-fold, the 52,000-dalton polypeptide was purified to near homogeneity. Although the protein tends to aggregate, monomer-sized protein purified by high-performance liquid chromatography is fully active for replication. It binds specifically and tightly to *oriC* in a supercoiled plasmid as judged by a Millipore filter-binding assay and by protection of the unique *Hin*dIII site within the *oriC* sequence. In the *oriC* replication reaction, dnaA protein acts at an early step preceding DNA synthesis.**

The *dnaA* gene was identified by conditional lethal mutations near 82 min (on the revised *Escherichia coli* map) that were defective at an elevated temperature in initiation of a cycle of chromosome replication (1, 2). The *dnaA* gene product is thought to act early in initiation, at about the same time as RNA polymerase (3). Several second-site suppressors of *dnaA* mutations that map within *rpoB* suggest a direct interaction between RNA polymerase and the *dnaA* gene product (4, 5). Besides the *E. coli* chromosomal origin of replication (*oriC*), only the plasmid pSC101 requires *dnaA* function for sustained replication *in vivo* (6, 7). This replicon specificity, early action, and functional interaction with RNA polymerase suggest that *dnaA* action is targeted directly to *oriC*.

The *dnaA* gene has been cloned in λ transducing phages by exploiting its close linkage to *tna* (8). The reported molecular weight of the *dnaA* polypeptide has ranged from 48,000 to 54,000 (8–11); a molecular weight of 52,574 has been calculated from the complete sequence of the gene (12).

Replication of *oriC*-containing plasmids *in vitro* provides a novel way to study the *dnaA* product (13). Replication of *oriC* plasmids in this system resembles authentic *in vivo* replication by several criteria, including absolute dependence on *dnaA* function (13, 14). This system has provided an assay for purification of dnaA protein from strains constructed *in vitro* that overproduce the protein more than 200-fold. The purified protein binds *oriC* and participates at an early stage in initiating bidirectional replication from *oriC*.

MATERIALS AND METHODS

Strains and Phages. Strains were: WM433 (*dnaA*204) from W. Messer (13), N4830 (*cI*857) from H. Echols (15), and K37 (Hfr) from J. M. Kaguni (16); phages M13*oriC*26 and M13*oriC*26Δ221 were from J. M. Kaguni (16).

Reagents and Buffers. Hepes, polyvinyl alcohol (type II), creatine kinase (type I), creatine phosphate, ribonucleoside triphosphates, and uridine were from Sigma; dNTPs were from P-L Biochemicals. [^{3}H]Thymidine triphosphate (30–40 Ci/mmol) and [^{3}H]thymidine (75 Ci/mmol) were from New England Nuclear (1 Ci = 3.7×10^{10} Bq). Buffer C is 25 mM Hepes·KOH, pH 7.6/0.1 mM EDTA/2 mM dithiothreitol/20% sucrose; buffer C′ differs only in that Hepes·KOH is 50 mM; buffer D is buffer C′ with addition of $Mg(OAc)_2$ to 10 mM, KCl to 0.1 M, and $(NH_4)_2SO_4$ to 0.2 M.

Enzymes and Proteins. T4 lysozyme was prepared as cited (13); *Eco*RI was a gift of P. Modrich; *Hin*dIII was from New England BioLabs. Protein was assayed by the method of Bradford, with bovine serum albumin (Miles) as a standard (17).

Preparation of DNAs. Unlabeled phage M13*oriC*26 replicative form (RF) I DNA was prepared as described (13). For ^{3}H-labeled RF I, 1.5-liter cultures of strain K37 were grown in M9 medium (18) containing 0.1% Casamino acids (Difco), 0.2% glucose, and thiamine (1 μg/ml). At an OD_{595} of 0.3, uridine was added to a final concentration of 200 μg/ml along with 5 mCi of [^{3}H]thymidine. At an OD_{595} of 0.4, phage M13*oriC*26 or M13*oriC*26Δ221 was added at a multiplicity of infection of 80; the cells were harvested after 2 hr, and RF I was purified by the same procedure used for unlabeled DNA; specific activities were 96,000 cpm/μg. To prepare DNAs linearized by *Eco*RI or *Hin*dIII, 5 μg of ^{3}H-labeled M13*oriC*26 RF I or ^{3}H-labeled M13*oriC*26Δ221 RF I in 50 μl of medium salt buffer (19) was treated for 30 min at 37°C with 10 units of restriction enzyme.

Assay of Replication Activity of dnaA Protein. Complementation of *oriC* replication in fraction II from a *dnaA* mutant strain WM433, prepared as previously described (13), was in a volume of 25 μl containing Hepes·KOH (pH 7.6), 40 mM; GTP, CTP, and UTP, each at 0.5 mM; ATP, 2 mM; dGTP, dCTP, TTP (^{3}H at 48 cpm/pmol), and dATP, each at 100 μM; magnesium acetate, 11 mM; polyvinyl alcohol, 7% (wt/vol) creatine phosphate, 40 mM; creatine kinase, 100 μg/ml; WM433 fraction II protein, 300 μg; and M13*oriC*26 RF I, 200 ng (600 pmol as nucleotide). Components, except for creatine kinase and DNA, were assembled on ice and centrifuged for 10 sec in an Eppendorf microfuge. Creatine kinase, template DNA, and the dnaA protein fraction to be assayed were added to the supernatant. After 20 min at 30°C, the extent of DNA synthesis was determined as described (13). One unit of dnaA protein activity (in the linear range of 300–800 pmol of dNMP incorporation) is 1 pmol of dNMP incorporated per min.

Assay of *oriC* Binding Activity of dnaA Protein. Reaction mixtures (25 μl), assembled on ice, contained Hepes·KOH (pH 7.6), 40 mM; KCl, 150 mM; $Mg(OAc)_2$, 10 mM; dithiothreitol, 2 mM; bovine serum albumin, 100 μg/ml; and ^{3}H-labeled DNA, 200 ng. After addition of dnaA protein, reactions were incu-

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Abbreviation: RF I, replicative form I DNA.

bated at 30°C and then spotted onto 2.4-cm Millipore HA filters (pore size, 0.45 μm). Filters, prepared by boiling in four changes of quartz-distilled water and stored at 4°C in water, were soaked in wash buffer (reaction buffer without bovine serum albumin and DNA) at room temperature for 30 min before use. Filtration by gentle suction was followed by washing with 0.5 ml of wash buffer at 30°C and drying under an infrared lamp. Radioactivity was determined by liquid scintillation counting. Maximal efficiency of retention of the complex was 80–90%; this correction has not been applied to the results.

RESULTS

Purification of dnaA Protein. Table 1 summarizes the procedure. The protein was overproduced by inserting the *dnaA* gene into vector pAD329 (20) to generate plasmid pBF110 (unpublished data; this plasmid will be furnished on request) (Fig. 1). Amplification by 200-fold of the dnaA protein level resulted from enhanced transcription of the *dnaA* gene from the λ phage p_L promoter induced by temperature inactivation of the product of the *cI857* gene in strain N4830.

A heat-produced lysate in 20 mM spermidine·HCl (21) could be clarified by low-speed centrifugation and yielded the same amount of dnaA protein activity with only half as much protein as in a freeze–thaw lysate (13). Chromatography of the ammonium sulfate fraction (fraction II) on Bio-Rex 70 and elution with a salt gradient separated activity into two broad peaks; the second contained more than half of the starting activity and was 50–80% pure as judged by $NaDodSO_4$/polyacrylamide gels stained with Coomassie blue (unpublished data). When activity that had been eluted in the high-salt peak was rechromatographed on Bio-Rex 70, 80% was eluted again with high salt.

When dnaA protein eluted by high salt (0.8–0.9 M KCl) from Bio-Rex 70 was chromatographed by HPLC, two peaks of replication activity were observed (Fig. 2), each corresponding to the abundance of the 52,000-dalton band in $NaDodSO_4$/polyacrylamide gel electrophoresis. The excluded (or just slightly included) protein in the first peak is presumably a multimer or aggregate; the second peak (eluted just after the position of bovine serum albumin) corresponds to monomeric dnaA protein. Both forms of dnaA protein were active in the replication (complementation) assay, although interconversion of these forms was not observed by HPLC after exposure to pH values of 6.0–7.5 and salt levels of 0.2–1.0 M KCl. Monomeric dnaA protein, about 95% pure as judged by the gel pattern, had a 2.5-fold higher specific activity in the replication assay than did the high molecular weight form. One circle is replicated per 15 monomeric dnaA protein molecules added to the replication assay.

Early Action of dnaA Protein in Initiation. In an *oriC* replication system partially reconstituted from purified components (unpublished data), dnaA protein was observed to act early (Fig. 3). As in the crude *dnaA* complementation assay (14), there was a lag of 3–5 min before DNA synthesis began. Prior incubation ("preincubation") of all components in the absence of dNTPs eliminated this lag; DNA synthesis began immediately upon addition of dNTPs. When either DNA gyrase (*E. coli* topoisomerase II) or RNA polymerase was added only after the preincubation (along with dNTPs), there was no reduction in lag time (unpublished data), implying that both are required for the earliest events. When dnaA protein was omitted from the preincubation, DNA synthesis began with an abbreviated but significant lag (about 1.5 min) after its addition (Fig. 3). Thus, dnaA protein participates in an early stage of the reaction but perhaps after the action of gyrase and RNA polymerase.

Specific Binding of dnaA Protein to the *oriC* Sequence. Partially purified dnaA protein specifically retained restriction fragments containing *oriC* in a nitrocellulose filter binding assay (25). However, dependence on DNA gyrase (13) and relative inactivity of linearized *oriC* template (unpublished data) imply that the optimal template for *in vitro* replication is supercoiled *oriC* plasmid DNA. Binding of dnaA protein to supercoiled M13*oriC*26, an M13–*oriC* chimera that can utilize *oriC* both *in vivo* and *in vitro* (13, 14, 16), was measured by Millipore filter binding (26). M13*oriC*26Δ221, having a small deletion that removes the *oriC* sequence, (16) served as a control. dnaA protein retained both ^3H-labeled RF Is, and the titration curve of dnaA protein was sigmoidal in each case (Fig. 4A). A 3-fold preference for M13*oriC*26 RF I (Fig. 4A) was accentuated to greater than 30-fold by addition of heterologous,

Table 1. Purification of dnaA protein

Fraction No.	Description		Protein, mg	Activity, units × 10^{-5}	Specific activity, units/mg × 10^{-3}	Yield, %	
I	Cleared lysate		9,120				
II	Ammonium sulfate		3,300	210	6.2	(100)*	
III	Bio-Rex 70†						
		A	55	63	116	30	98
		B	25	105	415	50	
		C	8.0	37	458	18	
IV	HPLC‡	A§	2.3	13	590	6	75¶
		B§	1.1	15	1,400	7	

Strain N4830 (pBF110) was grown in 200 liters of L broth (18) containing thymine, 25 μg/ml; glucose, 0.2%; and ampicillin, 25 μg/ml, at 30°C to an A_{595} of 0.8, shifted to 39°C, and harvested by centrifugation after 1.5 hr at an OD_{595} of 2.4. The cell paste was resuspended in buffer C containing 250 mM KCl to an OD_{595} of 400, frozen in liquid nitrogen, and stored at −80°C. Thawed cell suspension (408 g) was diluted 2-fold with buffer C containing 250 mM KCl; brought to 20 mM in spermidine·HCl, 200 μg/ml in egg white lysozyme, and 0.1 μg/ml in T4 lysozyme; left at 0°C for 30 min; and lysed in 250-ml batches in centrifuge bottles placed at 37°C for 4 min and inverted each min. Lysates were chilled to 0°C and centrifuged 70 min at 14,000 rpm in a Beckman JA14 rotor, and the supernatant was collected (570 ml, fraction I). $(NH_4)_2SO_4$ (160 g) was added to fraction I, and the suspension was stirred at 0°C for 30 min and centrifuged as above. The pellets were resuspended in 34 ml of buffer C′ to a final volume of 50 ml (fraction II), dialyzed for 2 hr against 1 liter of buffer C′, and diluted with 215 ml of buffer C′ to a conductivity equivalent to that of buffer C′ containing 50 mM KCl. Dialyzed fraction II was loaded at 4°C onto a 250-ml Bio-Rex 70 (100–200 mesh) column equilibrated with buffer C′ containing 50 mM KCl by first stirring with 80 ml of the packed resin and then pouring this slurry on the column containing the remaining resin. The column was washed with 7 column volumes of buffer C′ containing 50 mM KCl, and activity was eluted with a gradient (2 liters) of 50 mM to 1 M KCl in buffer C in two broad peaks between 0.3 and 0.5 M KCl and 0.6 and 0.9 M KCl. Fractions with comparable specific activity were pooled (A, 0.4–0.5 M KCl; B, 0.5–0.8 M KCl; C, 0.8–0.9 M KCl) and precipitated with the addition of 0.35 g of $(NH_4)_2SO_4$ per ml. Of the dissolved precipitate of pool C, 1.4 ml (18%) was concentrated 2-fold by vacuum dialysis (25,000-dalton-cutoff collodion bag, Schleicher & Schuell) against buffer D and chromatographed (HPLC) at 0°C at a pressure of 500 psi (1 psi = 6.89 kPa) and a flow rate of 0.6 ml/min by three successive injections on a TSK 3000 SW column (Altex, M_r exclusion of 200,000–400,000) equilibrated with the buffer D; 0.3-ml fractions were collected.

* Yield and purification are based on fraction II because activity could not be reliably measured in fraction I.

† A, B, and C are the pools of Bio-Rex fractions after concentration with ammonium sulfate.

‡ Only 18% of fraction IIIC was carried through to fraction IV; the values are corrected for this by a factor of 5.6.

§ A includes HPLC fractions 5 and 6; B includes HPLC fractions 15–18.

¶ Yield in fractions IVA and B combined, relative to fraction IIIC, was 75%.

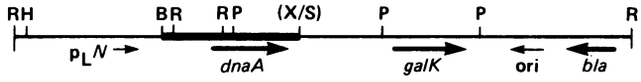

FIG. 1. Organization of the *dnaA* plasmid pBF110. B, *Bam*HI; R, *Eco*RI; H, *Hin*dIII; P, *Pvu* II; (X/S), junction of *Bam*HI–*Xho* I fragment containing *dnaA* [the *Hin*dIII–*Xho* I *dnaA* fragment from pBF101 (13) (▬) converted to a *Bam*HI–*Xho* I fragment with addition of *Bam*HI linkers] to *Sal* I–*Bam*HI vector fragment. The vector pAD329 is identical to pMA22 (20) except that it lacks the *Bgl* II fragment containing the phage λ cII gene. Arrows refer to the directions of genes and transcripts.

unlabeled competitor DNA (Fig. 4*B*). These results imply that dnaA protein recognizes a site present in M13*oriC*26 but absent from M13*oriC*26Δ221, probably within the minimal *oriC* sequence. Binding activity specific for *oriC* coincided with both the abundance and replication activity of dnaA protein in the HPLC column fractions (Fig. 2). The ratios of *oriC* binding to replication activities were similar in the excluded and monomer peaks.

Characteristics of the dnaA Protein–*oriC* Complex. Formation of a dnaA protein–*oriC* complex was complete in less than 30 sec. When complex formed with 660 fmol of monomeric dnaA protein (27 nM) and 63 fmol of ^{3}H-labeled *oriC* plasmid (2.5 nM; DNA was at saturation) was challenged with excess unlabeled *oriC* plasmid (250 fmol), the amount of [^{3}H]-DNA retained (16 fmol) was reduced by 45% in 60 min, indicating considerable stability of the complex. Complex formation was relatively salt resistant; in 300 mM KCl, binding was reduced by about 50% compared to standard conditions (150 mM KCl). When ^{3}H-labeled M13*oriC*26 was titrated to saturation in the presence of constant levels of dnaA protein, half-saturation was achieved with a concentration of free plasmid molecules of 0.3 nM at 27 nM dnaA protein (monomer) and with 0.6 nM free plasmid at 53 nM dnaA protein. At saturation, the ratio of dnaA monomers to *oriC* molecules was 50:1 and 20:1 for the two dnaA protein concentrations.

Influence of Superhelicity on Binding. When either ^{3}H-labeled M13*oriC*26 RF I or ^{3}H-labeled M13*oriC*26Δ221 RF I was linearized by *Eco*RI cleavage at a site distant from *oriC* (16), retention of DNA was reduced significantly compared to the corresponding supercoiled DNA, yet the preference for *oriC*26 versus *oriC*26Δ221 was maintained (Fig. 4*A*). This suggests that dnaA protein binding may be accompanied by the bending or partial unwinding of the DNA favored in negatively supercoiled DNA.

Protection of the *Hin*dIII Site Within *oriC* by dnaA Protein. M13*oriC*26 contains single sites for cleavage by *Eco*RI and *Hin*dIII. The *Eco*RI site is within sequences derived from phage G4, whereas the *Hin*dIII site is within the highly conserved, minimal *oriC* sequence (27, 28). Comparison of binding to M13*oriC*26 and M13*oriC*26Δ221 DNAs (Fig. 4) indicated that a dnaA protein binding site or sites lay within or overlapped the region lost in the deletion Δ221. The protection afforded by dnaA protein binding against *Hin*dIII cleavage directly demonstrates *oriC* specificity. Cleavage by *Hin*dIII but not by *Eco*RI

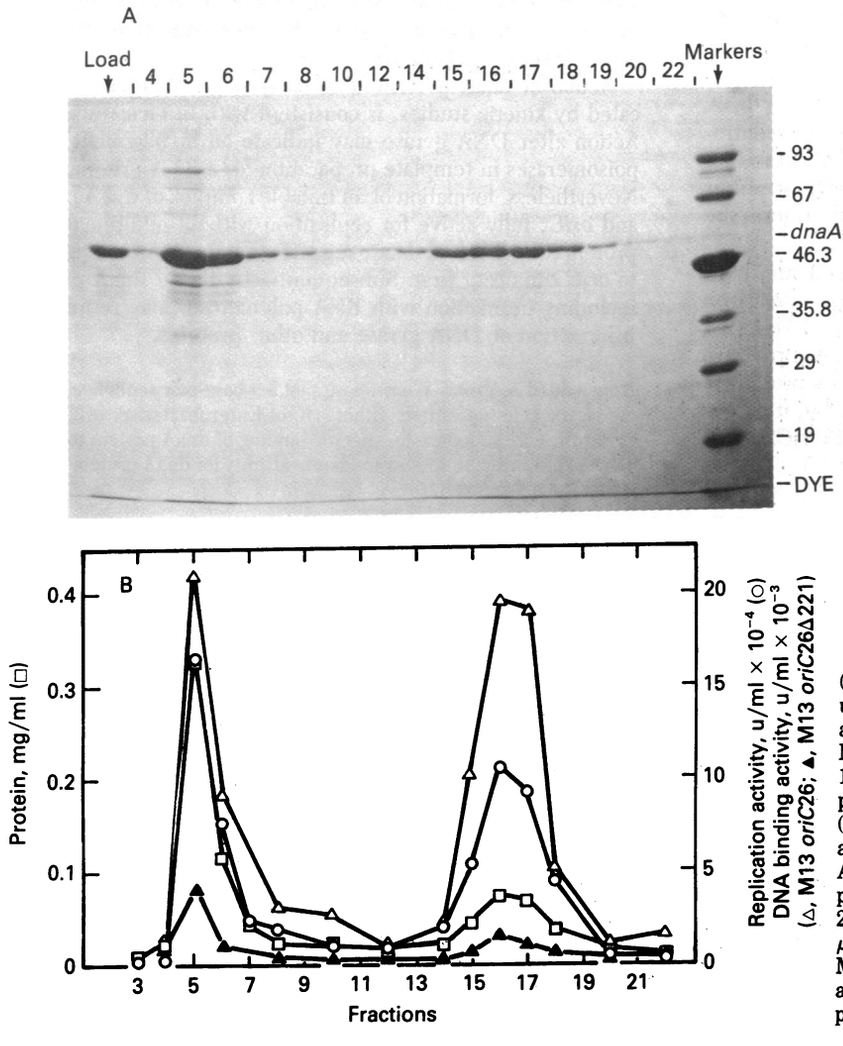

FIG. 2. Purification of dnaA protein by HPLC. (*A*) Bio-Rex pool C (LOAD lane, 10 μl) or HPLC column fractions (lanes 4–22, 100 μl) were precipitated at 0°C with 8% trichloroacetic acid and loaded on a NaDodSO$_4$/polyacrylamide gel (4% stacking and 12.5% body gel) (22). M_r markers (shown × 10^{-3}) were phosphorylase b (93,000); bovine serum albumin (67,000); φX174 capsid proteins F (46,300), H (35,800), and G (19,000); and carbonic anhydrase (29,000). (*B*) Assays for replication and binding activities of dnaA protein. Binding assays were incubated at 30°C for 2 min in 25 μl containing 1 μg of ColE1 DNA and 0.2 μg of either ^{3}H-labeled M13*oriC*26 or ^{3}H-labeled M13*oriC*26Δ221 RF I. One unit (u) of DNA binding activity corresponds to retention of 1 fmol of *oriC* plasmid under the conditions of the assay.

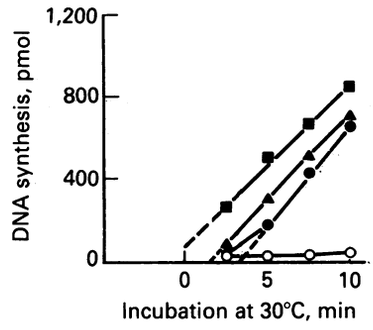

FIG. 3. Stages in initiation with a reconstituted system. Reactions (75 μl) contained buffer, ribonucleoside triphosphates, $Mg(OAc)_2$ polyvinyl alcohol, creatine kinase, and M13*oriC*26 RF I at the same concentrations as in the *dnaA* complementation assay except that creatine phosphate was 6 mM. Two protein fractions, FrIII red A agarose (Amicon) bound fraction (14 μg) and FrIII red A agarose flow-through fraction (8 μg), were added to furnish required factors (unpublished data) in addition to the following purified proteins [units measured in the φX174 single strand-to-RF assay (23)]: single-stranded DNA binding protein, 120 units; dnaB protein, 120 units; dnaC protein, 75 units; DNA polymerase III holoenzyme, 150 units; primase, 165 units; protein i, 90 units; and protein n′, 90 units; also added were RNA polymerase holoenzyme, 0.5 μg; gyrA protein, 0.1 μg; gyrB protein, 0.5 μg; *E. coli* topoisomerase I, 75 ng; and protein HU, 0.2 μg (24). Reaction mixtures, assembled at 0°C, were "preincubated" ($t = -5$ min to $t = 0$ min) at 0°C (●) or 30°C (■, ▲, ○) prior to initiation of DNA synthesis with addition of four dNTPs to 100 μM at $t = 0$ min. dnaA protein (200 units of fraction IV monomer) was added at $t = -5$ min (●, ■), $t = 0$ min (▲), or not at all (○); 10 μl was removed at the indicated times, and incorporation of [^{3}H]dNMP was determined. At 30 min, incorporations of 1,880 (●), 1,820 (■, ▲), and 90 (○) pmol were observed.

was inhibited by prior incubation with increasing levels of dnaA protein (Fig. 5). Dependence on the dnaA protein concentration in protection of the *Hin*dIII site is nearly identical to that in binding ^{3}H-labeled M13*oriC*26 RF I in the Millipore filter binding assay (compare Fig. 4*A* with Fig. 5*B*); furthermore, the curves are sigmoidal for both assays.

To judge whether the *Hin*dIII site is required for dnaA protein binding, ^{3}H-labeled M13*oriC*26 DNA linearized with *Hin*dIII and ^{3}H-labeled M13*oriC*26 and ^{3}H-labeled M13*oriC*26Δ221 DNAs linearized by *Eco*RI were compared in the binding assay. Not only was binding by dnaA protein of *Hin*dIII-cleaved M13*oriC*26 significantly reduced, but it proved to be little better than that of *Eco*RI-cleaved M13*oriC*26Δ221 (Fig. 4*A*).

DISCUSSION

Long-standing questions about the nature of the *dnaA* gene product, the site of its action, and whether its role in the initiation of a cycle of *E. coli* chromosomal replication is positive or negative have now been answered. The dnaA protein was purified to near homogeneity as a soluble polypeptide of 52,000 daltons with a strong tendency to aggregate. Its action is absolutely essential in an enzyme system that initiates bidirectional replication at the *E. coli* chromosomal origin contained in small plasmids and that also replicates plasmids containing homologous *oriC* sequences of numerous other species of Enterobacteriaceae (28). Thus dnaA protein is a required factor that acts positively. dnaA protein binds specifically to the *oriC* sequence within plasmid molecules with a preference for the supercoiled form and protects the unique *Hin*dIII site within *oriC*. The action of dnaA protein precedes DNA synthesis but may follow earlier actions of RNA polymerase and DNA gyrase. dnaA protein action is presumably directed to *oriC* by the specificity of its binding.

Although monomeric dnaA protein is active for *oriC* binding and replication, it cannot be concluded that it functions simply as a monomer. The sigmoidal titration of dnaA protein in binding to *oriC* suggests cooperativity, either in binding to DNA or in association of monomers into an active oligomeric form. The high ratio of added dnaA protein monomers to bound *oriC* and the dependence of that ratio on the concentration of dnaA protein also suggest that many molecules of dnaA protein are bound to *oriC*; studies with labeled dnaA protein are needed for a more accurate determination. Sites of moderate affinity for dnaA protein binding appear to exist in M13*oriC*26Δ221 as well as in other plasmids (unpublished data).

Action of dnaA protein at an early stage of initiation, indicated by kinetic studies, is consistent with *in vivo* studies (3). Action after DNA gyrase may indicate an involvement of topoisomerases in template preparation for a RNA priming step. Nevertheless, formation of an isolable complex of dnaA protein and *oriC*, fully active for replication without additional dnaA protein (unpublished data), suggests that dnaA protein binding to *oriC* can occur first. Subsequent activities of dnaA protein, including interaction with RNA polymerase, may require the prior action of DNA gyrase and other proteins.

Note Added in Proof. We propose that a 9-base-pair sequence, highly conserved at four positions within *oriC* of Enterobacteriaceae (28) comprises the core sequence for specific binding of dnaA protein to duplex DNA. Plasmids with sites of moderate affinity for dnaA protein contain

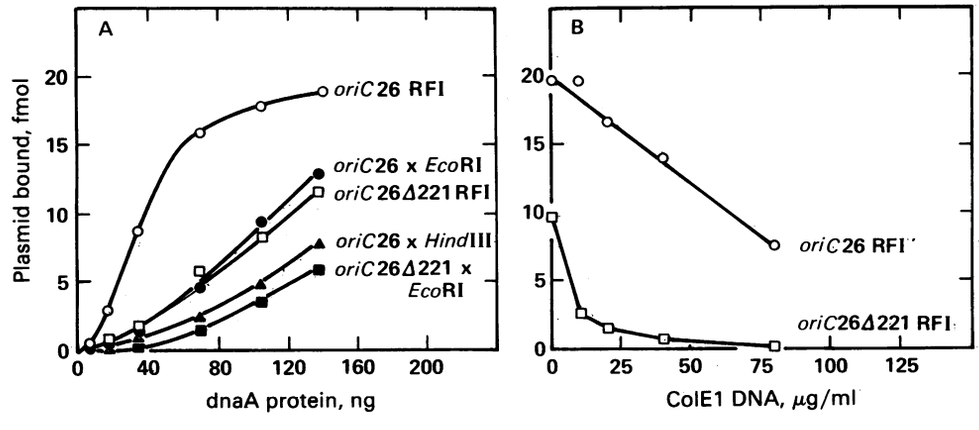

FIG. 4. DNA binding of dnaA protein. (*A*) Titration of dnaA protein., fraction IV monomer, in binding to various DNAs. "x *Eco*RI" and "x *Hin*dIII" indicate plasmid DNA linearized by *Eco*RI and *Hin*dIII, respectively. Incubations were for 10 min at 30°C. Each point is the average of four determinations. (*B*) Influence of competitor ColE1 DNA concentration on binding was determined as above with 70 ng of dnaA protein, fraction IV monomer. Each point is the average of two determinations.

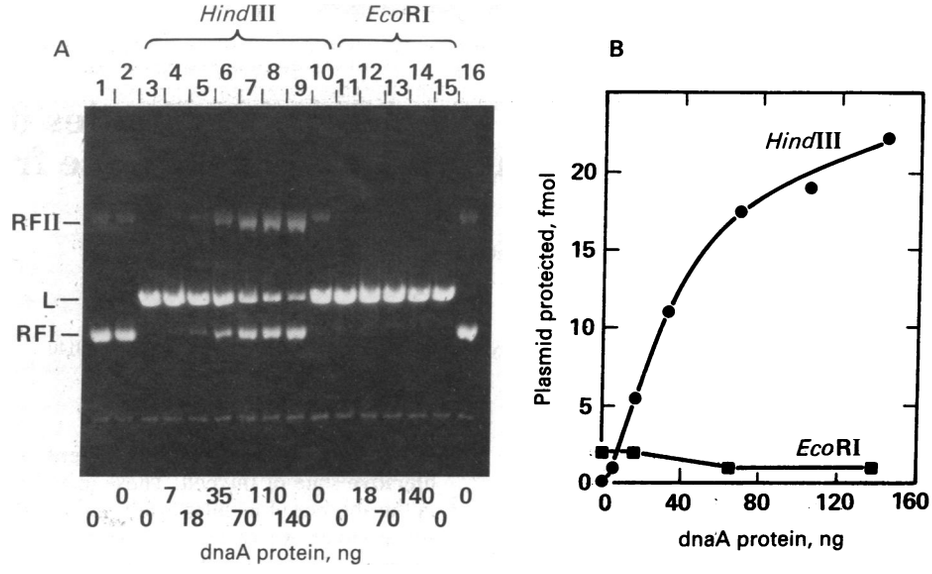

FIG. 5. Protection of *Hin*dIII site within *oriC* by dnaA protein. Binding reactions were as described except that 200 ng of unlabeled M13*oriC*26 RF I was added and concentrations of Hepes·KOH and KCl were lowered to 20 mM and 50 mM, respectively. The mixtures were preincubated in the presence or absence of dnaA protein (fraction IV, monomer) as indicated for 15 min at 30°C. Either no restriction enzyme (lanes 1, 2, and 16) or 2 units of *Hin*dIII (lanes 3–10) or of *Eco*RI (lanes 11–15) was added, and incubation was continued for 30 min. Reactions were stopped by addition of EDTA to 20 mM and $NaDodSO_4$ to 0.5%; the reaction mixtures were incubated at room temperature for 30 min and on ice for 10 min and were centrifuged for 2 min in a Brinkmann microfuge before being loaded onto a 0.5% agarose gel in 100 mM Tris·borate, pH 8.3/1 mM EDTA/0.5 μg of ethidium bromide per ml; electrophoresis was for 12 hr at 15 mA. (*A*) Reaction mixture in lane 1 was not incubated, and those in lanes 10 and 15 contained 2 μl of HPLC buffer (buffer D) during preincubation. The gel was photographed under a 254-nm UV light source with Polaroid-type 665 negative-print film. (*B*) Individual lanes on the negative were scanned with a Quick Scan (Helena Laboratories, Beaumont, TX) densitometer, and the sizes of RF I peaks were determined relative to the control lanes 1, 2, and 16. The *Hin*dIII and *Eco*RI curves were calculated by using the data from lanes 3–9 and lanes 11–14, respectively.

a copy of at least an 8/9 match of the consensus sequence T-T-A-T-$\frac{C}{A}$-C-A-C-A. From ^{32}P-end-labeled *Taq* I digests of a variety of plasmid DNAs, dnaA protein retains on Millipore filters only those that contain the 9-base-pair sequence. Fragments containing this sequence within or near the following sequences are specifically bound: *oriC*, the promoter of a 15.5-kilodalton protein coding sequence adjacent to *oriC* (found in both M13*oriC*26 and M13*oriC*26Δ221), the region between the two promoters for the *dnaA* gene (12), the internal repeat IR_L sequence of Tn5 (29), the region between the origin of DNA synthesis and the n′ (Y) ATPase site on the L-strand of pBR322 (30), and the origin of replication of pSC101. Consistent with the results of this paper, fragments containing *oriC* with its four 9-base-pair sequences are bound more tightly than other fragments containing only one. We suggest that dnaA protein binding may have a positive role at certain sites (e.g., *oriC*, *ori*-pSC101) and a negative role at others (*ori*-pBR322, *ori*-ColE1 and the control region for the *dnaA* gene.

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