Ordered and Sequential Binding of DnaA Protein to oriC, the Chromosomal Origin of Escherichia coli

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DnaA protein of Escherichia coli acts in initiation of chromosomal DNA replication by binding specific sequences, termed DnaA boxes in the chromosomal origin, oriC. On binding, it induces a localized unwinding to create a structure recognized by other replication proteins that act subsequently in the initiation process. In this report, we examined the binding of DnaA protein to each of the DnaA boxes in oriC. By gel mobility shift assays, DnaA protein formed at least six discrete complexes. ATP or ADP included in the reaction mixture prior to electrophoresis was required. Chemical cleavage of isolated complexes with 1,10-phenanthroline-copper revealed that DnaA protein binds in an ordered manner to the DnaA boxes in oriC. Preferential binding to one DnaA box (R4) was confirmed by demonstration that a DNA fragment containing it was bound with greater affinity than another DnaA box sequence (R1). In vitro replication activity correlated with a complex formed at a ratio of 30 DnaA monomers/oriC in which all DnaA boxes are occupied. The last site bound is DnaA box R3. This event may be critical in promoting initiation of DNA replication, as occupancy of R3 occurs at the time of initiation of chromosomal replication, whereas other DnaA boxes are bound by DnaA protein throughout the cell cycle (Cassler, M. R., Grimwade, J. E., and Leonard, A. C. (1995) EMBO J. 14, 5833–5841).

DnaA protein of Escherichia coli is a sequence-specific DNA-binding protein, proposed to recognize 9-mer sequences termed DnaA boxes, present in four copies within the chromosomal origin, oriC (Fig. 1) (1). At oriC, its binding promotes an ordered series of events to result in the initiation of chromosomal DNA replication (reviewed in Ref. 2). The binding of DnaA protein to oriC has been examined by a variety of methods. By electron microscopy (3, 4) and DNase I footprinting (1), a large nucleoprotein structure containing 20–30 monomers of DnaA protein is formed at oriC. Experiments to examine its interaction with individual DnaA boxes led to the conclusion that it bound to the two centrally located DnaA boxes in oriC (R2 and R3) with greater affinity than to the flanking DnaA boxes (R1 and R4) (5). This was based on an indirect assay that assessed the activity of DnaA protein as a transcriptional terminator in vivo. Expression from the lac promotor, located upstream to the mutant DnaA box being examined, was measured.

By contrast, gel mobility shift assays with oligonucleotides of 21 base pairs containing various DnaA boxes with natural flanking sequences indicated that DnaA protein binds to DnaA boxes R1 and R4 of oriC with higher affinity than R2 (6). DnaA box R3 was bound as poorly as nonspecific oligonucleotides. In addition to these in vitro findings, in vivo footprinting of oriC plasmids with dimethyl sulfate in exponentially growing cells revealed protection of DnaA boxes R1, R2, and R4 with little binding to R3 (7, 8). These observations, suggesting that the binding of DnaA protein to R3 is critical for the initiation process, is supported by the observation that occupancy of R3 occurs at the time of initiation of DNA replication in synchronized cultures (7). Another study reported that mutations in single DnaA boxes (R1 and R4) of oriC reduced binding of DnaA protein to respective sites (5) but only induced replication activity when both mutant sequences were present together (9).

The replication activity of oriC may tolerate an alteration of one of the binding sites (9), perhaps by the speculated cooperative binding of DnaA protein, or occupancy of all four DnaA boxes is not required for replication.

Other proteins have been characterized to bind to specific regions of oriC. IdA protein was isolated by its ability to bind specifically to 13-mer motifs near the left boundary of oriC (10) (Fig. 1). Its binding inhibits the initiation process. IHF$^*$ and Fis binding sites in oriC have been described (11–15). Rob protein binds to a region near the right boundary of oriC, but its significance is unknown (16).

The studies summarized above do not provide a clear understanding of whether DnaA protein binds to sites in oriC randomly or in an ordered and sequential manner in the process of initiation of chromosomal replication. If this event is ordered, the binding of other proteins to oriC may inhibit or augment the initiation process. In this report, we use gel mobility shift and DNA footprinting techniques to characterize complexes of DnaA protein bound to oriC. Results indicate that DnaA protein binds to oriC in an ordered manner. DnaA box R4 is bound first, then R1, and finally the two inner boxes. Formation of these discrete complexes was dependent on ATP or ADP. Replication activity correlates with binding to all four DnaA boxes.

Experimental Procedures

Gel Mobility Shift Assays (17, 18)—Unless noted, a SmaI-XhoI fragment containing oriC, gel-purified from pBSoriC (19) with a Qiaex DNA extraction kit (Qiagen) and quantified by absorbance at 260 nm, was 3'-end-labeled with the large fragment of DNA polymerase I (Boehringer Mannheim), and [$\text{a}^{32}\text{P}]\text{dATP}$, then combined with the same unlabeled fragment to adjust its specific radioactivity to $4 \times 10^3$ cpm/25 fmol of DNA. Reactions (10 μl) with the labeled oriC fragment (25 fmol) and indicated amounts of DnaA protein were incubated in buffer containing 20 mM HEPES-KOH, pH 8.0, 5 mM magnesium acetate, 1 mM EDTA, 4 mM dithiothreitol, 0.2% Triton X-100, 5 mg/ml bovine serum albumin, and 0.5 μM ATP (unless noted otherwise) at 20 °C for 5 min

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$^*$The abbreviations used are: IHF, integration host factor; ATP$\gamma$S, adenosine 5’-O-(thiotriphosphate).
Ordered Binding of DnaA Protein to oriC

The samples were electrophoresed in a 4% polyacrylamide gel (60 parts acrylamide:1 part bisacrylamide) (13.5 × 13.5 × 0.15 cm) in 45 mM Tris borate and 1 mM EDTA at 80 V for 3 to 4 h. Gels were dried and autoradiographed with Hyperfilm MP (Amersham Corp.) at −70 °C using a Cronex Quanta III intensifying screen, or image-analyzed with a Molecular Dynamics PhosphorImager.

In Situ Cleavage with 1,10-Phenanthroline-Copper (20)—Gel mobility shift assays were performed as above but scaled up 10-fold. After electrophoresis, the wet gel was immersed for 2.5 to 4.5 min in 200 ml of 42 mM Tris-HCl, pH 8.0, 0.2 mM phenanthroline, 38 μM CuSO4, and 5 mM 3-mercaptopropionic acid at room temperature. To quench the reaction, 2.9-dimethylphenanthroline was added to 2.3 mM followed by incubation for 2 min. The gel was quickly washed with water and autoradiographed for 1 h at room temperature. The developed film was used to guide excision of the complexes. DNA from the gel slices was eluted overnight at 37 °C in 500 μl of elution buffer (0.5 mM ammonium acetate, 0.2% SDS, 1 mM EDTA, 10 μg/ml Proteinase K, and 100 μg/ml tRNA). After recovering the elution buffer, an additional 200 μl of elution buffer was used to wash the gel slices, and both were pooled. The eluted DNA was ethanol-precipitated, washed with 70% ethanol, dried, and resuspended in 5 μl of 80% (v/v) formamide, 1 mM NaOH, 1 mM EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanid, heated to 95 °C for 2 min, and electrophoresed at 50 watts on a prerun 6% sequencing gel. After autoradiography, beta emission scanning was with a Molecular Dynamics PhosphorImager. Graphed with Excel (Microsoft), the radioactivity in each lane was normalized to the cleavage pattern of unbound DNA that was isolated from the gel.

DNA Replication Assays—Reactions (25 μl) were performed as described (21) with a crude protein fraction deficient in DnaA protein activity, M13oriC26 DNA (25 fmol) as a template, and the indicated amounts of DnaA protein. Incubation to measure DNA synthesis was at 30 °C for 20 min. Acid-insoluble incorporation of [3H]TTP was quantified by liquid scintillation counting.

RESULTS

Six Discrete Complexes Are Formed on Binding of DnaA Protein to oriC—By footprint analysis, and electron microscopy, the nucleoprotein complex of DnaA protein bound to oriC is estimated to contain 20–30 monomers organized to occupy the AT-rich 13-mers in the promoter region (18). The samples were electrophoresed in a 4% polyacrylamide gel (60 parts acrylamide:1 part bisacrylamide) (13.5 × 13.5 × 0.15 cm) in 45 mM Tris borate and 1 mM EDTA at 80 V for 3 to 4 h. Gels were dried and autoradiographed with Hyperfilm MP (Amersham Corp.) at −70 °C using a Cronex Quanta III intensifying screen, or image-analyzed with a Molecular Dynamics PhosphorImager.

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Six Discrete Complexes Are Formed on Binding of DnaA Protein to oriC—By footprint analysis, and electron microscopy, the nucleoprotein complex of DnaA protein bound to oriC is estimated to contain 20–30 monomers organized to occupy the four DnaA boxes (1, 3, 4)). Sequence comparison of these DnaA boxes reveal that R1 and R4 are identical, whereas R2 and R3 differ at the fifth and seventh positions, respectively (Table I). Inasmuch as other studies indicated that several nucleotide changes at each of the positions only marginally affected the ability of DnaA protein to bind to oriC and that mutant DnaA boxes of oriC did not affect in vivo replication activity when singly present (9), the binding of DnaA protein to oriC may not show a strong preference to one site relative to others. To investigate this, we examined the binding of DnaA

![Figure 1](image-url)

**The oriC region.** DnaA boxes R1-R4 are the 9-mers recognized by DnaA protein. DnaA box R5 (36) is also shown. Binding sites for IHF (11, 12) and Fis (13-15), 13-mer motifs recognized by ICa protein (10), and restriction enzyme sites (S. Smal; Hf; HinFii; A. AvaII; Hf, HinDIII; Ac, Acd; X, Xhol; and P, PstI) of pBSoriC are indicated.

### Table I

| DNA                  | Sequence^a | Position |
|---------------------|------------|----------|
| oriC R1             | GGATACCTTG | -10      |
| oriC R2             | GATGGAGGGG | -10      |
| oriC R3             | CAACCGTATG | -10      |
| oriC R4             | CCCTAGACG  | -10      |
| DnaA box in the dnaA promoter region | TACACTGGAG | 123456789 |
| DnaA box in M13mp18  | TACACTGGAG | 10       |

^a The polarity of respective DnaA boxes is from left to right.
that DnaA protein bound with about 3-fold higher affinity to a supercoiled oriC plasmid than to the linearized or relaxed form (26). To examine this issue with the gel mobility shift assay, unlabeled competitor DNA was added to reactions containing a fixed level of DnaA protein and radioactively labeled oriC fragment (Fig. 5). Addition of either supercoiled (estimated to be contaminated by ~10% nicked DNA by resolution by agarose gel electrophoresis and quantitative densitometry), linearized oriC plasmid, or the same unlabeled restriction fragment resulted in a comparable reduction of DNA binding to the labeled DNA, measured by densitometric analysis of the autoradiogram. By contrast, poly(dI-dC) was an ineffective competitor. These results indicate a comparable binding affinity of DnaA protein to supercoiled or linear DNAs containing oriC. The apparent discrepancy between these observations and the cited study may be due to the absence of ATP in the filter binding assays (26), whereas it was present in the gel mobility shift experiments (Fig. 5). We have not examined the effect of ATP on binding affinity to different topological forms of oriC-containing plasmids nor compared the effect of supercoiled and linear oriC-containing DNAs by each assay method.

Ordered Binding of DnaA Protein to oriC—To determine the sites bound by DnaA protein in the separated complexes, in situ footprint analysis was performed with 1,10-phenanthroline-copper (20). Complexes formed with DNA labeled in the top or bottom strand were examined (Fig. 6, A and B). Regions protected from chemical cleavage were identified by quantitative analysis of the resultant autoradiograms (Fig. 6, C and D). Complex I consisted of DnaA protein bound to R4. R1 was additionally protected in Complex II. Complex III differed from Complex II by protection of R2 as well as sequences to the left of R4 in vicinity of the AccI site. Binding to the region encompassing the AccI site is likely not responsible for the electrophoretic position of this and more slowly migrating complexes (described below). Gel mobility shift experiments with a DNA fragment lacking the AccI site resulted in a similar number and proportion of complexes (data not shown). The scarcity of Com-
Fig. 6. In situ footprinting of complexes with 1,10-phenanthroline-copper. In situ cleavage was performed as described under “Experimental Procedures” with a SmaI-XhoI fragment labeled at the XhoI site with the large fragment of DNA polymerase I and all 4 [α-32P] deoxyribonucleotides (top strand, panel A) or at the HinfI site of a HinfI-PstI fragment by incorporation of all 4 [α-32P] deoxyribonucleotides and subsequently cleaved with XhoI (bottom strand, panel B). The cleavage pattern of respective complexes was analyzed by beta emission scanning.
Differences in respective 9-mers in addition to the influence of R2 and R3 apparently is less and may be due to sequence (Fig. 6 sequence of R4 in Complex I (Fig. 6) sequences at and G of the region containing the DnaA box appear to contribute to binding affinity. The protection of R4 is identical to that of R1, sequences that flank the DnaA box from R2 to R4. The altered mobility of Complexes IV and V relative to III may be due to more stable binding of DnaA from R2 to R4. The altered mobility of Complexes IV and V relative to III may be due to more stable binding of DnaA protein to R2, and R3, possibly by interaction among proteins relative to III may be due to more stable binding of DnaA protein to R2, and R3, possibly by interaction among proteins. In summary, these findings indicate that DnaA protein binds to DnaA box R4 with greater affinity than to R1. An EcoRI0191-PstI restriction fragment (25 fmol) containing DnaA box R4 from pBSR4, radioactively labeled by end-filling at the EcoRI0191 site with [α-32P]dGTP, was incubated with the indicated amounts of the same unlabeled fragment or an unlabeled Smal-AvalI restriction fragment containing DnaA box R1. pBSR4 was constructed by insertion of the HindIII-PstI fragment (gel-purified) containing DnaA box R4 of oriC into corresponding sites of pBluescript II SK+ (Stratagene). DnaA protein (3 ng) was added and complexes resolved from free DNA as described under "Experimental Procedures." After autoradiography, the amount of free DNA remaining in each lane was quantified by emission scanning, then the amount of DNA bound was calculated. The amount of radiolabeled DNA bound in the absence of competitor was normalized to 1.

DnaA Protein Binds to DnaA Box R4 with Greater Affinity than to R1—Results from gel retardation and footprinting experiments indicated that DnaA box R4 is bound with greater affinity than R1 by DnaA protein. To determine the relative affinity of DnaA protein to restriction fragments containing only box R1 or R4, gel shift assays were performed with unlabeled DNAs as competitors. Addition of increasing amounts of unlabeled fragment containing R4 resulted in proportional inhibition of binding to the labeled R4 fragment present at a constant level (Fig. 7). By comparison, addition of unlabeled R1 fragment was less effective. These results, the average of three independent experiments, confirm that DnaA protein binds to R4 in oriC with greater affinity than R1. As the 9-mer sequence of R4 is identical to that of R1, sequences that flank the DnaA box appear to contribute to binding affinity. The protection of residues at –1 and –2 positions (Table I) outside the 9-mer sequence of R4 in Complex I (Fig. 6C) and of R1 in Complex II (Fig. 6D) supports this conclusion. Also, the binding affinity to R2 and R3 apparently is less and may be due to sequence differences in respective 9-mers in addition to the influence of flanking sequences (Table I).

A supercoiled template containing oriC is required for in vitro replication (25). Relating this requirement to structure 3, it was observed more frequently with supercoiled DNA than linear DNA (4). We have not determined whether complex VI forms more efficiently on supercoiled DNA than linear or relaxed DNA, despite attempts to resolve complexes formed on supercoiled DNA in low percentage agarose gels. In addition, we have been unsuccessful in demonstrating ordered binding of DnaA protein on a supercoiled oriC-containing plasmid. The method used was quantitative footprint analysis with DNase I or 1,10-phenanthroline of complexes formed in solution, followed by primer extension. Footprinting in solution provides an averaged picture of complexes formed. At lower ratios of DnaA protein to oriC where we expected to see preferential binding to DnaA box R4 then to R1, we presume that the amount of free DNA masks the protection pattern resulting from ordered binding. Also, we presume that this reason explains why ordered binding was not observed in previous reports (1, 24). Although solution footprinting on supercoiled DNA failed to detect ordered binding, competition experiments demonstrated that DnaA protein binds with a similar affinities to supercoiled or linear DNAs containing oriC (Fig. 5). This suggests that DnaA protein binds to either topological form by a similar mechanism.

Despite the identical 9-mer sequences of R1 and R4 (Table I), DnaA protein bound to R4 with about 3-fold higher affinity than to R1 (Fig. 7). Presumably, sequences that flank the 9-mer in R4 contribute to its higher binding affinity. Indeed, the protection of flanking sequences at −1 and −2 positions of R4, and R1, clearly seen in Complexes I and II (Fig. 6C and D), indicates that DnaA protein binds to residues outside of the core sequence. Whether the differences in sequences at the −2 position of R4 compared with R1 is responsible for the different binding affinities can be tested directly. Other evidence supports the notion that flanking sequences contribute to binding affinity. With a nitrocellulose filter binding assay, we found that DnaA protein bound 4-fold greater to the DnaA box in a

and compared with the cleavage pattern of the corresponding unbound fragment that was treated similarly (panels C and D). Maxam-Gilbert G and G + A reactions performed on the appropriate oriC fragment served as size markers. The sequence of R4 (panel C) and R1 (panel D) with sequences at −1 and −2 positions (Table I) is presented at the bottom.

**DISCUSSION**

By use of a gel retardation assay in conjunction with protection from cleavage by 1,10-phenanthroline-copper, the binding of DnaA protein to sequences in oriC was found to be ordered and sequential. The six discrete complexes (and material near the wells seen at higher levels of DnaA protein) may correlate with the seven unique structures detected by electron microscopy (4). At higher levels of DnaA protein, the inhibition of replication activity correlated with formation of material that entered the gel poorly (Fig. 3). Complex VI (Fig. 2) may correspond to structure 3 (4) as the formation of both correlated with optimal replication activity. Second, both structure 3 and complex VI formed more efficiently with ATP than with ADP in the reaction mixture. In the absence of nucleotide DnaA protein failed to form discrete complexes. This apparently is due to aggregation of DnaA protein that occurs on its incubation without ATP.2

By comparison, similar experiments with a dnaA promoter fragment containing a DnaA box identical to R4 of oriC (Table I) and a weak DnaA box have been performed.3 We observed two prominent complexes and two minor, more slowly migrating species.

3 C. Margulies and J. M. Kaguni, unpublished results.
ordered Binding of DnaA Protein to oriC

dNAa promoter-containing fragment than to a synthetic DnaA box (9-mer) inserted into the multiple cloning site of M13mp18 (Table 1). These observations are also supported by the 50-fold difference in binding affinity to a specific DnaA box when flanking sequences were varied (6).

The observations described here are in contradiction to the conclusion that DnaA protein bound with higher affinity to central DnaA boxes (R2 and R3) relative to the flanking sites (R1 and R4) (5). This deduction was based on an indirect method that measured expression of galK dependent on transcription from the lac promoter. The DnaA box sequence being assessed was positioned between the promoter and the galK gene. The relative ability of DnaA protein to function as a transcriptional terminator to affect galK expression was the basis of the assay. It is possible that differences in mRNA stability and/or translational efficiency may have influenced the results obtained.

Four lines of evidence correlate the initiation of DNA replication to the binding of DnaA protein to DnaA box R3. First, dimethyl sulfate treatment of an oriC plasmid carried in an exponentially growing strain revealed that DnaA box R3 was not bound whereas the remaining sites were (8). Such minichromosomes in which plasmid replication occurs from oriC are duplicated once per generation (27) and synchronously with the bacterial chromosome (28). Assuming that replication fork movement is the same as that of the bacterial chromosome, oriC plasmid replication should be completed within a few seconds. As dimethyl sulfate treatment was for 2 min, most plasmids should not be active in replication. These observations suggest two possibilities. One is that the level of DnaA protein throughout most of the cell cycle may be insufficient to bind to this site and that a critical level must be attained to promote initiation. Alternatively, this site may be occluded (see below). Second, in synchronous cultures, initiation of oriC plasmid replication correlated with the binding of DnaA protein to R3 (7). Third, elevated expression of DnaA protein stimulated initiation (29–31), possibly by increased occupancy of DnaA protein and rebinding of the nucleotide-free form occurs during electrophoresis. The significance of binding to this site is unclear as it is not part of the functional oriC sequences (37).

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