The structure of the initiation complex at the replication origin, *oriC*, of *Escherichia coli*

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ABSTRACT

Two distinct regions in the replication origin, *oriC*, of *Escherichia coli* are separately distorted upon initiation complex formation by the initiator protein DnaA. The AT-rich region in the left part of *oriC* and the start site region in the right part of *oriC*. Chemical modification of single-stranded DNA was observed at both regions whereas endonuclease recognition of DNA mini-bulges specifically occurred in the start site region. We show that the helical phasing of binding sites for DnaA protein in *oriC* is important for origin function. An insertion or deletion of one helical turn between the two rightmost binding sites does not alter the efficiency of replication initiation, whereas all modifications of distance by less or more than one helical turn result in inactivation of *oriC*. DnaA binding and helical distortions in the AT-rich region as well as in the start site region are not affected in the distance mutants irrespective of their functionality *in vivo*. We propose a specific compact nucleoprotein structure for the initiation complex.

INTRODUCTION

Binding of the initiator protein DnaA to its 4 (1) or 5 (2) binding sites (DnaA boxes) in the replication origin, *oriC*, is the initial step in the initiation of bacterial replication. A compact complex is formed in the presence of HU protein and ATP (3) which contains 10 – 40 monomers per *oriC* (4). This complex shows structural distortions and partial unwinding in the AT-rich region left in *oriC*, as shown *in vitro* and *in vivo* (5–7).

Accessory proteins with specific binding sites in *oriC* which bend the DNA upon binding, like IHF (integration host factor) and FIS (factor of inversion stimulation) might be involved in the initiation complex formation (8–13). DnaC assisted entry of the DnaB helicase results in the prepriming complex (3). Further addition of SSB, DNA gyrase and DnaG primase leads to priming at start sites for bidirectional DNA synthesis in the right half of *oriC* (14).

In this paper we analyze the changes in DNA structure upon DnaA binding to *oriC*. The ability of the DnaA protein to distort the DNA, to cause changes in DNA conformation which result

in non-B-DNA, to unwind the *oriC* DNA in an open complex, and to form higher order nucleoprotein structures was monitored using different sensitive chemical reagents and enzymes. Modification by these agents were localized using a PCR-linked primer extension reaction.

We also investigated the replication ability in a structurally altered *oriC* region. Defined insertions and deletions over a range of –12 to +24 bp were introduced in the spacer region between the two rightmost DnaA recognition sequences R3 and R4. Initiation complex formation of the mutants was analysed *in vitro* by Dnaase I footprinting and KMnO₄ footprinting. The functionality of these *oriC* mutants was tested *in vivo*.

MATERIALS AND METHODS

Bacterial strains and plasmids

WM1785 [= W3110] and its *polA* derivative WM1838 [*polA*, *fadA*::Tn10] were used as host strains in the *ori* assay. For cloning procedures we used the *recA* strains JM109 [D(*lac-proAB*), *endA1*, *gyrA96*, *hsdR17*, *recA1*, *relA1*, *supE44*, *thi-1*; F'*lac*: *lacI*^q, D(*lacZ*)M15, *traD36*, *proA*⁺, *proB*⁺] and XL1-Blue [*endA1*, *gyrA46*, *hsdR17*, *lac*, *recA1*, *relA1*, *supE44*, *thi*; F'*lac*: *lacI*^q, D(*lacZ*)M15, Tn10 (Tc), *proA*⁺, *proB*⁺].

For the construction of pOC161 an *oriC* containing fragment from SmaI to HincII (coordinates –44 to +1497; all *oriC* coordinates refer to ref. 15) was ligated via XbaI 8mer linkers with the HindIII-EcoRI fragment of pBR322. We used standard techniques for plasmid isolation and cloning (16).

Enzymes and proteins

Enzymes were purchased from Boehringer Mannheim except for Taq Polymerase (cloned, Perkin Elmer). Extracts of a DnaA overproducing strain were prepared from WM1492 harboring plasmid pLSK5 (17). DnaA protein was purified as described (10, 18).

Oligonucleotide-directed mutagenesis of *oriC*

The chromosomal 2052 bp PstI fragment spanning the *oriC* region was isolated from plasmid pOC51 (19) and inserted into the PstI site of M13 mp8 (20). This phage (pWO80R) was used to mutagenize the spacer region between DnaA boxes R3 and R4

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in *oriC* by oligonucleotide-directed mutagenesis (21). The mutant clones obtained were verified by sequence analysis (22). Oligonucleotides were synthesized by the solid phase phosphoramidite method on an Applied Biosystems model 380B DNA synthesizer.

Primer extension

KMnO₄ treatments, Dnase I footprints, and T4 endonuclease VII footprints, were performed on plasmid pOC161. Modified sites were detected in the different footprinting techniques by PCR-linked primer extension. The oligonucleotides for the extensions were complementary to the bottom strand at *oriC* coordinates -29 to -10, and to the top strand at 308 to 326, respectively.

The primers were end labeled with [γ -³²P] ATP and T4 polynucleotide kinase and purified by phenol/ chloroform extraction and 2×ethanol precipitation with 1 μ l of mussel glycogen. To about 500 ng of modified DNA, 1 ng of end labeled primer (300,000 cpm) was added. Primer extension was done in a PCR thermocycler using Taq Polymerase (Cetus, Perkin Elmer) in 70 mM Tris-HCl pH 8.8, 2 mM MgCl₂, 0.1 % Triton-X100 in a total volume of 50 μ l with 30 cycles of amplification (denaturation for 1.5 min at 95°C, primer annealing for 1 min at 56°C and primer extension for 2 min at 72°C). The extension products were separated on 8% sequencing gels.

KMnO₄ footprinting

Permanganate footprinting was carried out as described (6) with minor modifications. 0.5–1 μ g plasmid DNA were incubated in a total volume of 25 μ l under the buffer conditions of the *in vitro* replication system (23), i.e. in the presence of 4% PEG 6000, 2 mM ATP, 50 μ M NAD, creatinphosphate/creatinkinase, 8 mM Mg-acetate, 25 μ M cAMP and dNTP's in 25 mM Hepes-KOH pH 7.6, with 90–120 ng of purified DnaA protein or DnaA in an extract of a DnaA overproducing strain for 10 min at 37°C. If purified DnaA protein was added the reaction mixture also contained 100 μ g/ml BSA (nuclease free, Boehringer Mannheim). KMnO₄ was added from a 50 mM stock solution to a final concentration of 3 mM. After 3 min the reaction was quenched by addition of 1 μ l β -mercaptoethanol. The DNA was brought

to 50 μ l with water and phenol extracted. The aqueous phase was then loaded onto 1 ml of packed Sephadex LH60 (Pharmacia) in a 1.5 ml spin column (Mobitec) that had been preequilibrated with TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA pH 8.0) and centrifuged at 2000 rpm for 2.5 min perched on a 2 ml Eppendorf tube. The experimental sample was carefully applied to the center of the packed column, which was spun again for 5 min at 2500 rpm. The desalted eluate was brought to 70 μ l with water. 10 μ l of the eluate was loaded onto a 1% agarose gel. The remaining DNA was cut with XbaI. Samples were then phenol extracted, ethanol precipitated, and analysed by PCR-linked primer extension.

Dnase I footprinting

The footprinting procedure is largely that of Gralla (24). 120 ng of purified DnaA protein or 90 ng of DnaA in an extract of a DnaA overproducing strain (6) and 1 μ g of plasmid DNA were incubated in 25 ml using the buffer conditions of the *in vitro* replication system (23). Then 1 μ l containing 0.3 u of Dnase I (Boehringer Mannheim) was added to the reaction mixture for 1 min. The reaction was stopped with 4 μ l of 0.2 M EDTA and 35 μ l phenol and placed on ice. Purification of the DNA and primer extension was identical to the KMnO₄ footprinting protocol.

For conventional Dnase I footprinting of linear *oriC* DNA (25, 26) fragments were 5'-end-labeled using γ -[³²P]ATP and T4 polynucleotide kinase. Binding of purified DnaA protein was at room temperature for 5 min in DnaA binding buffer (40 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM magnesiumacetate, 1 mM DTT). The DNA was subjected to limited Dnase I cleavage. Resulting DNA fragments were analyzed on 8% acrylamide sequencing gels.

T4 endonuclease VII footprinting

Incubation was the same as described above for KMnO₄ and footprinting of supercoiled DNA except that after 10 min at 37°C, 0.1 μ l T4 endonuclease VII were added to the 25 μ l cocktail for 10 min. T4 endonuclease VII contained 20 units /ml in 10 mM Tris-HCl pH 8.0, 0.1 mM glutathion, 50% glycerol, 100 mg/ml BSA (nuclease free), and was a gift of Brries Kemper. The

Table 1. Transformation efficiency of spacer mutants.

insertion/ deletion	sequence	ratio <i>polA/polA</i> ⁺¹⁾	ori function
WT	240 ATCCAAGCTTC 250	100	+++
+04	240 ATCCAAGCTAGCTTC 250	0	—
+10	240 ATCCAAGCTCCCGGGAGCTTC 250	67	+++
+12	240 ATCCAAGCTGCCCGGGCAGCTTC 250	73	+++
	240 ATCCAAGCTGGAATTCAGCTTC 250		
+14	240 ATCCAAGCTCCCCGGGGAGCTTC 250	0	—
	240 ATCCAAGCTCGGAATTCAGCTTC 250		
+16	240 ATCCAAGCTCCCCGGGGAGCTTC 250	0	—
	240 ATCCAAGCTCCCGAATTCGGGAGCTTC 250		
+22	240 ATCCAAGCTCGGAATTCGGGAATTCAGCTTC 250	0	—
+24	240 ATCCAAGCTCGGAATTCGGGAATTCAGCTTC 250	0	—
-04	240 ATCCATC 250	0	—
-08	240 ATCCAGACAGAGT 260	0	—
-10	240 ATCCACAGAGT 260	17	++
-12	240 ATCCAGAGT 260	39	+++
-14	240 ATCCAGT 260	0	—

¹⁾ normalized: WT = 100.

reaction was stopped with 1 μ l of 500 mM EDTA (pH 8.0) and 35 μ l phenol. The DNA was purified and primer extended as described in the KMnO₄ footprinting protocol.

RESULTS

oriC function of insertion/deletion derivatives (*ori* assay)

Two categories of sequences are present in *oriC*, sequences which are highly conserved among the origins of enterobacteriaceae (27), and presumably are recognition sites for proteins involved in the replication initiation. Between the conserved regions are spacer regions with variable sequence in which base substitutions but not insertions or deletions are tolerated (28–32). Prominent among the conserved regions are the binding sites of DnaA protein (DnaA boxes).

Insertions and deletions that alter the distance between DnaA binding sites were done at the HindIII site (position 244) of pOC161 between the DnaA boxes R3 and R4. Insertions were introduced by manipulating the cohesive HindIII ends by either Klenow fill-in reaction or by Mung bean nuclease treatment followed by ligation with or without linkers. Linkers of different lengths resulted in plasmids carrying insertions from –4 to +24 bp (Table 1). SmaI and EcoRI linkers of the same size were analyzed in parallel constructs to ensure independence of the observed effects from the actual sequence inserted. *oriC* derivatives carrying deletions were obtained by oligonucleotide directed mutagenesis (21). The phage used (pWO80R) contained the chromosomal 2052 bp PstI fragment spanning the *oriC* region in M13mp8. All clones have been confirmed by sequence analysis (22). Mutated chromosomal origins, with deletions at the HindIII site, were subcloned into pOC161.

Figure 1. DnaI footprints of *oriC* BglII-XhoI fragments from insertion/deletion mutants. DnaI footprints were done as described for linear fragments in Material and Methods. Lanes 1–4: *oriC*–4 mutant; lanes 5–8: *oriC* wildtype; lanes 9–12: *oriC*+10 mutant. DnaA protein added: 0 (lanes 1, 5, 9), 30 ng (lanes 2, 6, 10), 60 ng (lanes 3, 7, 11), 90 ng (lanes 4, 8, 12). The marker lane gives *oriC* coordinates, bars indicate 9 bp DnaA boxes.

pOC161 derivatives with insertions or deletions were tested for *oriC* function by the ability to transform a *polA* host. The joint replicons are able to replicate in *polA*⁺ cells via the *E. coli* origin as well as via the ColE1 origin of pBR322, whereas in *polA* cells replication starting from the ColE1 origin is blocked (33). The ratio of the transformation frequencies into *polA* versus *polA*⁺ cells was used to quantitate the functional efficiency of *oriC* (Table 1). Insertions at the HindIII site of pOC161 of +4, +14, +16, +22 and +24 bp as well as deletions of –4, –8, and –14 bp gave a transformation ratio of <0.03 as compared to a joint replicon with wildtype *oriC*. The capability of pBR322 to transform a *polA* strain was <0.01. Therefore, these insertions and deletions inactivated the replication origin *oriC*.

However, inserting 10 bp and 12 bp, respectively, in pOC161 gave a transformation efficiency close to wildtype. Similar results were obtained with the deletion derivatives of *oriC*. Deletions of 10 bp and 12 bp were functional in the *ori* assay contrary to all other deletions. Thus, introduction or deletion of one helical turn into the spacer region between DnaA boxes R3 and R4 resulted in a functional *oriC* whereas inserting or deleting a part of a helical turn as well as inserting two turns destroyed the origin

Figure 2. DnaA dependent KMnO₄ modifications in the top strand of the AT-rich region in *oriC*. About 1 μ g pOC161 DNA was incubated with DnaA protein or with an extract of a DnaA overproducing strain under buffer conditions supporting *in vitro* replication. PCR-linked primer extension was done from a primer hybridizing to positions 308–326 to the right of *oriC*. Markers indicate *oriC* coordinates. **A:** Comparison of modifications with purified DnaA and with cell extract. Lane 1: pOC161 DNA with 120 ng DnaA in an extract of a DnaA overproducing strain (WM1492); lane 2: supercoiled plasmid without DnaA; lane 3: 120 ng purified DnaA. **B:** Comparison of wildtype and mutant *oriC*. Lane 1: pOC 161 (wildtype) without protein, lane 2: with 80 ng of purified DnaA protein, lane 3: *oriC*–4 mutant without protein, lane 4: with 80 ng of purified DnaA protein.

Figure 3. Mg^{++} dependence of distortions in the AT-rich region. pOC161 was incubated and modified products were primer extended as described in legend Fig. 1, except for lane 1 which shows the reaction in the presence of 2 mM Mg^{++} and without DnaA protein. Lane 2: plasmid DNA was incubated with 120 ng purified DnaA protein and in lane 3 without protein, both in the presence of 8 mM Mg-acetate. The marker indicates *oriC* coordinates.

function (Table 1; ref. 13). Similar results were obtained with EcoRI instead of SmaI linkers (not shown).

DnaA binding to wildtype and mutant *oriC* is similar

We analysed the DnaA-*oriC* interaction at the mutated origins *in vitro* using Dnaase I footprinting (25). The 454 bp *oriC* BglII-PstI fragment was used for Dnaase I footprints. DnaA protein bound to its five binding sites on the wildtype *oriC* fragment and on the mutant *oriC* fragments in a similar way (Fig. 1). There was no significant difference between wildtype *oriC* (Fig. 1, lanes 5–8), the functional *oriC* +10 insertion mutant (+10; lanes 9–12), and nonfunctional *oriC* mutants (–4; lanes 1–4).

DnaA mediated local unwinding in the AT-rich region of *oriC*

Local unwinding with (5) or without (34) DnaA protein was originally observed in the AT-rich region at the left side of *oriC* using single-strand specific nuclease. Helical distortion in this region of *oriC* had also been studied *in vitro* and *in vivo* using $KMnO_4$ as a chemical probe (6, 13). $KMnO_4$ oxidizes pyrimidines, preferentially thymine, in single-stranded regions or at kinks in DNA (35–37). An extract of a DnaA overproducing strain caused helical distortion, detected by $KMnO_4$ modification in the top strand as well as in the bottom strand (6). Here we determined whether purified DnaA protein alone, i.e. without accessory proteins, is able to form a complex with supercoiled *oriC* DNA which can be modified by $KMnO_4$ at similar positions. Using buffer conditions which allow replication *in vitro* (23) the complex of *oriC* with purified DnaA protein exposed nucleotides in the top strand at identical positions as a complex prepared with a cell extract from a DnaA overproducing strain (Fig. 2A). Oxidized bases were mainly in the right 13mer between positions 56 and 63 (all *oriC* coordinates refer to ref. 15). Local unwinding was strictly dependent on DnaA (Fig. 2A, lane 2). Modifications in the bottom strand were analysed using a primer complementary to the bottom strand at coordinates –29

Figure 4. DnaA mediated distortion in the right half of *oriC*, bottom strand, comparison between wildtype *oriC* and different *oriC* mutants. Primer extension reaction was done with a primer hybridizing to positions –29 to –10 left of *oriC*. Lanes 1–3: pOC161 (wildtype) incubated with 0, 30, 60 ng DnaA in an extract of a DnaA overproducing strain. Lanes 4–5: pOC161 (wildtype) incubated with 30, 60 ng purified DnaA protein. Lanes 6–9: *oriC* mutant +10 with 0, 30, 60, 90 ng DnaA in an extract of a DnaA overproducing strain; lanes 10 and 11: with 60 and 90 ng of purified DnaA protein. Lanes 12–14: insertion mutant +12 incubated with 0, 30, 60 ng DnaA in an extract of a DnaA overproducing strain; lanes 15 and 16: with 30 and 60 ng DnaA protein. The marker lane gives *oriC* coordinates.

to –10 for PCR-linked primer extension. As observed before (6) oxidized residues were found over a wider range in the middle and right 13mer (coordinates 36 to 68) and with a lower probability in the left 13mer (Fig. 7). Again there was no significant change in the pattern observed upon addition of purified DnaA protein or with cell extract (data not shown). Partially unwound regions detected by $KMnO_4$ footprinting covered approximately the same region as those detected by P1 nuclease sensitivity (5, 7). Both agents recognize a smaller single-stranded region in the top strand than in the bottom strand (Fig. 7, ref. no. 7).

We investigated whether the *in vivo* functions of the *oriC* derivatives carrying spacer mutations correlate with structural alterations in the initiation complex, as detected by $KMnO_4$. Fig. 2B shows that the distortion of the AT-rich region upon complex formation by DnaA was similar in the plasmids carrying the wildtype origin and a mutant origin. Mutant origins which are nonfunctional behaved the same way as mutant origins which are functional *in vivo*.

A region of helical instability, called a DNA unwinding element (DUE), was observed in the left part of the AT-rich region of *oriC*. Sensitivity in this region to nuclease or $KMnO_4$ was observed in the absence of Mg^{++} and DnaA protein (6, 34). Here we show that low concentrations of Mg^{++} (2 mM) still allow local unwinding in the absence of DnaA protein (Fig. 3). At 8 mM Mg^{++} , however, unwinding was strictly DnaA dependent. DnaA independent distortion was further to the left than DnaA mediated unwinding, at bases 33–43 in the top strand (Fig. 3) and at bases 12–24 in the bottom strand.

DnaA mediated helical distortions close to the start sites for DNA replication

Start sites for bidirectional DNA synthesis were observed in the right half of *oriC*, close to DnaA boxes R2, R3 and R4 (Fig 7; ref. 14). Addition of DnaA protein to *oriC* plasmid DNA (pOC161) resulted in $KMnO_4$ sensitive sites close to these start sites (Fig. 4). There was no difference between a complex prepared with purified DnaA (lane 5) or with a cell extract from a DnaA overproducing strain (lanes 2 and 3). Again the wildtype

Figure 5. Initiation complex formation as probed with T4 endonuclease VII. pOC161 DNA was incubated with purified DnaA protein for 10 min at 37°C as described in legend Fig. 1. 2 u of T4 endonuclease VII were added and incubation continued for 10 more min at 37°C. PCR-linked primer extension was done with a primer hybridizing to positions 308 to 326 to the right of *oriC*. Lane 1: without protein; lane 2: with 120 ng purified DnaA. The marker indicates *oriC* coordinates, arrows show major T4 endo VII restriction sites.

origin showed the same pattern as the mutant origins (Fig. 4). With the techniques used we could not see any differences in DnaA binding and in helical distortion in the mutant origins versus the wildtype origin

KMnO₄ modified bases were also observed in the IHF and FIS binding sites and close to DnaA box M (Fig. 7, ref. 6). All these modifications affected only single bases contrary to the KMnO₄ footprints in the AT-rich region. Therefore they must be due to kinks or bends in the DNA or to a much smaller region of local unwinding.

KMnO₄ sensitive sites close to the start sites of DNA replication, bends and kinks accessible for KMnO₄ modification inbetween this region and the unwound regions in the left part of *oriC* suggest that the DNA is folded around a higher order nucleoprotein complex of DnaA protein with *oriC* DNA. Bends are consistent with bent regions in the binding sites for the bending proteins IHF and FIS.

The DnaA-*oriC* complex shows mini-bulges in DNA close to the start sites of DNA synthesis

Information on structural changes upon formation of the DnaA-*oriC* complex was extended using T4 endonuclease VII

Figure 6. Dnase I footprints of the supercoiled initiation complex. pOC161 DNA was incubated with purified DnaA and restricted with Dnase I as described in Material and Methods. PCR-linked primer extension was done with a primer hybridizing to positions 308 to 326 to the right of *oriC*. Lane 1: Dnase I cleavage with no DnaA; lane 2: Dnase I footprint with 120 ng purified DnaA; lane 3: 120 ng DnaA in an extract of WM1492. The marker indicates *oriC* coordinates, bars show 9 bp DnaA boxes.

footprints. This enzyme specifically recognizes cruciform structures (38), bulges (39), replication forks (40), and to a minor extend strong bends and kinks (41). It cuts the DNA 3' of the non-B-DNA (42).

pOC161 DNA was incubated with purified DnaA protein, followed by cleavage with T4 endonuclease VII. Positions of nuclease cuts were then detected by PCR-linked primer extension as described above. T4 endonuclease VII footprints on supercoiled plasmid DNA showed a defined cleavage pattern in the initiation complex. Major restriction sites for T4 endonuclease VII with 2 to 4 adjacent cleavage sites in the *oriC* DNA were localized at position 215–218 between the DnaA boxes R2 and R3, and at positions 243–245 and 258–259, between DnaA boxes R3 and R4 (Fig. 5). These restriction sites are located close to the start sites for bidirectional DNA replication (Fig. 7). Additional targets for T4 endonuclease VII were at positions 95, 150, 180, and 238 (Fig. 5). These single cuts, which can also be seen at lower intensity in the control, may reflect bent DNA becoming more bent upon open complex formation with DnaA.

Figure 7. The *E. coli* replication origin, *oriC*. The positions of the DnaA boxes and their respective orientations within *oriC*, and the AT-rich regions are shown. The location of the IHF and FIS binding sites are also indicated. The right-angle arrows show the start sites of bidirectional DNA synthesis (14). Arrows of different shape show the different modifications.

All positions were defined by parallel sequence tracks using the same primers (not shown).

We postulate that the restriction sites at positions 215–218, 243–245 and 258–259 represent mini-bulges in the DNA. These sites were located 2–4 bp 3' to KMnO₄ sensitive single stranded regions, close to the start sites for DNA synthesis (Fig. 7). Since these KMnO₄ sensitive sites are single nucleotides the bulges must be very small. It is unlikely that the T4 endonuclease VII restriction sites observed are due to cruciforms because of the sequence context, and replication forks are not possible since no replication proteins were added.

No enhanced T4 endonuclease VII cleavage pattern could be observed in the left part of *oriC* in the AT-rich region, although the AT-rich region shows KMnO₄ hypersensitive sites on both strands.

Probing the structure of the initiation complex with DnaE I

DnaE I footprints are a frequently used tool for probing DNA structure. Besides protection from cleavage at protein binding sites DnaE I shows an enhanced cleavage pattern at transition points from a narrow minor groove to a wide minor groove (43). It also shows an enhanced cleavage at exposed base pairs. So far DnaE I footprints with *oriC* DNA and with DnaA were only done with linear fragments. This is due to the difficulty with supercoiled *oriC* plasmids to obtain sufficiently strong signals

under conditions in which the average frequency of DnaE I cleavage per plasmid must be less than 1, but only a small percentage of cuts is within the area of interest. The PCR-linked primer extension used in all experiments reported here overcomes this problem. The 5 DnaA boxes (1, 2) were protected from DnaE I cleavage upon DnaA binding on supercoiled plasmid in the same way as on linear fragments (compare Figs. 1 and 6 for DnaA boxes R2–R4). No other site showed a clear DnaA footprint, except, as observed before (10), there were some alterations in accessibility for DnaE I cleavage in the FIS binding site between DnaA boxes R2 and R3 (Fig. 6, lane 2). With extract from a DnaA overproducing strain which contains accessory protein and thus might reflect the initiation complex formation *in vivo* more accurately, the FIS binding site was more clearly protected from DnaE I cleavage (Fig. 6, lane 3), and the relative intensities of bands 255/256 and 267/268 were reversed. Otherwise the pattern was very similar to the one with purified DnaA protein. DnaA box R3 was clearly protected, contrary to results with DMS footprinting done *in vivo* (44).

Structural changes on supercoiled *oriC* DNA upon binding of purified DnaA protein or DnaA in a cell extract also resulted in DnaE I hypersensitive sites. The DnaE I footprint showed enhanced cleavage by DnaE I close to the DnaA boxes R2 at position 182 and around position 160, R3 at position 235, and R4 at position 255/256 (Fig. 6).

Figure 8. Model for the initiation complex at *oriC*. Details are given in the text. Large grey arrows represent DnaA boxes, small black arrows indicate KMnO_4 sensitive sites, white arrowheads DNA synthesis start sites.

DISCUSSION

We have analyzed the initiation complex between the replication origin, *oriC*, of *E. coli* and the DnaA protein using genetics, the functionality of mutant *oriC*, and various footprinting techniques.

DnaA-*oriC* interactions are helical-phase-dependent

In order to analyse the role of the precise distance between DnaA protein binding sites we introduced a defined range of insertions and deletions at the HindIII site in the spacer region between the two rightmost DnaA boxes R3 and R4. *oriC* function was completely destroyed by either insertion or deletion of sequences comprising a part of a helical turn. However, introducing or deleting a full helical turn resulted in functional origins, about as active as wildtype *oriC*. We conclude that DnaA boxes have to be on the correct side of the helix for *oriC* function, presumably allowing interaction between adjacent DnaA protein monomers. Insertions of 10 bp between the DnaA boxes R2 and R3 also resulted in a functional origin whereas an insertion between R1 and R2 inactivated *oriC* (12). Apparently, helical phasing of DnaA boxes is a necessary but not a sufficient condition, and there are regions in *oriC* where the spacing must be precisely maintained.

DnaA binding to *oriC* is not inhibited in *oriC* spacer mutants

We analyzed the DnaA-*oriC* complexes by Dnaase I footprinting. All five DnaA boxes in *oriC* were protected by DnaA from Dnaase I cleavage. The wildtype *oriC* fragment showed the same footprint as fragments with mutated *oriC*. There was also no difference between functional (insertions or deletions of 10 and 12 bp) and nonfunctional *oriC* mutants. Therefore, the initial binding of DnaA is possible irrespective of the helical phasing of DnaA boxes.

Three types of helical distortions are found in the DnaA-*oriC* initiation complex

We probed the complex between DnaA protein and *oriC* with three different footprinting techniques: i. KMnO_4 oxidizes pyrimidines in single-stranded or kinked regions (35, 36). ii. T4 endonuclease VII recognizes cruciforms (38), replication forks (40), DNA bulges (39), bends and kinks (41), and cleaves 2–4 nucleotides 3' of the distortion (42). iii. Dnaase I hypersensitivity is observed at transition points from a narrow minor groove to a wide minor groove (43). All modifications were visualized by PCR-linked primer extension, which is a linear amplification of fragments from a single labelled primer up to the modification site in a thermocycler using Taq DNA Polymerase. This technique is fast and very sensitive.

Local unwinding in the AT-rich region of the three 13mer repeats was detected by modification with KMnO_4 . The pattern of modifications in the AT-rich region was similar to those in previous reports (6, 13). The region in the top strand appeared smaller than the unwound region in the bottom strand (Fig. 7), as was observed before with P1 nuclease as a probe for single stranded regions (7). Surprisingly, this region was completely refractory to restriction by T4 endonuclease VII. Taken together, the results suggest that local unwinding over approximately 30 nucleotides occurs at the AT-rich 13mer repeats, mainly in the middle and right repeat, upon DnaA binding. The region seems to be partially shielded in the initiation complex (45), protecting part of the top strand from KMnO_4 action and the whole area from restriction by T4 endonuclease VII.

KMnO_4 footprints were used to monitor the non-B DNA conformation in the mutant origins in comparison to wildtype *oriC*. The distortions in the left part of *oriC* at the AT-rich region and in the right part of *oriC* close to the start sites of DNA synthesis were the same in the wildtype origin and the mutant

origins, irrespective of the functionality of *oriC* *in vivo*. We conclude that also local helical distortions as measured by KMnO_4 footprinting do not require a correct distance between DnaA boxes R3 and R4. Most likely the inability of the mutant origins to replicate is due to a later step in initiation of replication from *oriC*.

The second type of structure are mini-bulges observed in the right half of *oriC* close to the start sites for bidirectional DNA synthesis (14), close to DnaA boxes R3 and R4. Two to four adjacent phosphate bonds are cleaved by T4 endonuclease VII at positions 215–218, 243–245, and 258–259. Two to four base pairs to the left of each of these sites is a single oxidized T in the complementary strand (Fig. 7). The only explanation for such a pattern is that it represents mini-bulges. The mini-bulges must be very small since thymine residues close by were not oxidized.

A third type of distortions was found mainly between DnaA boxes R1 and R2 (Fig. 7). It is characterized by isolated oxidized T residues and isolated T4 endonuclease VII restriction sites with no obvious correlation with each other. Presumably both are due to bends or kinks.

Dnase I hypersensitive sites were not correlated with any of these three structures (Fig. 7). We assume that they are indicative of bent regions at the outside of the initiation complex. All structures were observed with DnaA protein alone. Addition of replication proteins and other proteins in cell extracts did not result in a significant change. This shows that subsequent initiation and replication structures must be very transient and thus represent only a small proportion of all molecules.

A model for the initiation complex at *oriC*

A model which explains and uses the results described in this paper, as well as additional information, assumes a tight complex of 10 DnaA monomers, arranged in a ring of 5 dimers with opposite orientation (Fig. 8). We suggest *oriC* DNA to be wrapped around and through this ring resulting in all DnaA boxes oriented in the same direction (large black arrows). In this structure IHF and FIS binding sites (black regions on the DNA) would be at positions which require bending. Most start sites of DNA synthesis (white arrowheads) are at the upper face of the ring opposing the AT-rich 13mers. A DnaB hexamer, attracted by the unwound region in the AT-rich 13mers, might be sandwiched inbetween the AT-rich region and the region where DNA synthesis starts, and then delivered to the mini-bulges. Although the hexameric DnaB is large in size (300 kd) DNA may not have to be single-stranded over a large distance to allow entry of DnaB. A model in which the helicase complex surrounds the DNA and moves along like a wheel while opening the strands was suggested for the T-Antigen helicase of SV40 (46) and might also apply to DnaB.

A number of observations are compatible with this model. Electron micrographs show a compact structure of many DnaA monomers in which approximately 220 bp DNA is hidden (4). We observe a transition reducing the length of the *oriC* region by 220 bp at a complex size of about 10 DnaA monomers (R. Lurz, R. Jueterbock, W. Messer, unpublished). The DnaA optimum in the *in vitro* replication system (23) is at about 10 DnaA monomers per *oriC*, and all experiments reported here were done at a molar ratio of 6–10, DnaA/*oriC*. Mutations which reduce or block DnaA binding to individual DnaA boxes were found to be without effect on *oriC* function (26). However changes in distance between DnaA boxes

inactivated the origin. Only insertions or deletions of one helical turn between DnaA boxes R3 and R4 or a one-turn insertion between R2 and R3 gave a functional origin. In the proposed model reduction of DnaA binding affinity at individual DnaA boxes might be tolerated by the coordinated structure. Disruption of the helical phasing, however, would destroy the complex.

DnaA boxes R3 and M are on the reverse side of the DNA helix compared to R1, R2, and R4. The minimal distance between R3 and R4 was found to be 20 bp in the –12bp deletion derivative between R3 and R4. Binding of IHF (8, 9) and FIS (10, 11) to their binding sites in *oriC* results in DNA bending. These features are incorporated into the model.

We assume that the initial wrapping and complex formation may not require supercoiled DNA because of the close similarity between Dnase I footprints with DnaA protein on linear *oriC* fragments and on supercoiled *oriC* DNA shown in this report. Any unwinding reactions, however, were strictly dependent on supercoiled DNA.

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