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Localized DNA melting and structural pertubations in the origin of replication, *oriC*, of *Escherichia coli in vitro* and *in vivo*

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The leftmost region of the *Escherichia coli* origin of DNA replication (*oriC*) contains three tandemly repeated AT-rich 13mers which have been shown to become single-stranded during the early stages of initiation *in vitro*. Melting is induced by the ATP form of DnaA, the initiator protein of DNA replication. $KMnO_4$ was used to probe for single-stranded regions and altered DNA conformation during the initiation of DNA replication at *oriC in vitro* and *in vivo*. Unpairing in the AT-rich 13mer region is thermodynamically stable even in the absence of DnaA protein, but only when divalent cations are omitted from the reaction. In the presence of Mg^{2+}, *oriC* melting is strictly DnaA dependent. The sensitive region is distinct from that detected in the absence of DnaA as it is located further to the left within the minimal origin. In addition, the DNA is severely distorted between the three 13mers and the IHF binding site in *oriC*. A change of conformation can also be observed during the initiation of DNA replication *in vivo*. This is the first *in vivo* evidence for a structural change at the 13mers during initiation complex formation.

Key words: DnaA/DNA conformation/DNA helix distortion/replication initiation

Introduction

The initiation of chromosomal DNA replication in *Escherichia coli* is a precisely timed event that occurs only once during the cell cycle (Helmstetter, 1968; Skarstad *et al.*, 1986). Replication is initiated at a unique site, *oriC*, on the chromosome when the cells have reached a set ratio between mass and origins (Donachie, 1968). The intracellular concentration of DnaA was shown to determine the initiation mass (Loebner-Olesen *et al.*, 1989).

The main features of the *oriC* sequence are illustrated in Figure 5. The origin contains four binding sites for the initiator protein DnaA (DnaA boxes) which are present in an inverted orientation with respect to each other and three AT-rich 13mers at the left border of the origin which have been implicated in DNA melting in the presence of DnaA protein (Bramhill and Kornberg, 1988a).

Furthermore the origin contains a binding site for IHF (Filutowicz and Roll, 1990; Polaczek, 1990). We have recently identified a binding site for the FIS protein between DnaA box R2 and R3 (Messer *et al.*, 1990). FIS was originally identified on the basis of its capability to stimulate the inversion of the G segment of bacteriophage Mu (Kahmann *et al.*, 1985; Koch and Kahmann, 1986). Both proteins have been shown to bend the DNA strongly (Prentki *et al.*, 1987; Thompson and Landy 1988; Hübner *et al.*, 1989). Their role for the initation process is still under investigation.

After DnaA binding to the origin the protein has been shown to cause local unwinding *in vitro* if ATP is bound to it (Sekimizu *et al.*, 1987; Baker and Kornberg, 1988; Bramhill and Kornberg, 1988b; Hwang and Kornberg, 1990). The reaction is strictly dependent on negative supercoiling and requires a minimum temperature. The process is stimulated by low levels of HU and IHF (Dixon and Kornberg, 1984; Skarstad *et al.*, 1990). A transcriptional event is also required *in vivo* (Lark, 1972; Messer, 1972). However, whereas the crude *in vitro* system is sensitive to rifampicin (Fuller *et al.*, 1981), in the reconstituted assay replication proceeds efficiently in the absence of RNA polymerase (van der Ende *et al.*, 1985). *In vitro*, the unwinding is stimulated by transcription under adverse conditions such as high levels of HU, even transcription at a distance (Skarstad *et al.*, 1990). Transcription from the *mioC* promotor, which is located immediately to the right of *oriC*, was shown to affect minichromosome maintenance (Stuitje *et al.*, 1986; Loebner-Olesen *et al.*, 1987). However, to date there has been no evidence for an involvement of *mioC* in chromosomal replication.

Next, DnaB binding to the single-stranded region, facilitated by DnaC, leads to further unwinding (Baker *et al.*, 1986; Funnell *et al.*, 1987). After addition of DnaG the priming complex is able to synthesize RNA primers in the right half of *oriC* where DNA synthesis is then initiated (Seufert and Messer, 1987).

We have investigated the requirements for initiation complex formation at *oriC in vitro* and *in vivo*. In the presence of divalent cations DNA melting in the 13mer region was strictly DnaA dependent. The occurrence of unpaired regions in *oriC* during initiation *in vivo* was analyzed after alignment of logarithmically growing cultures at the initiation stage using a *dnaC* mutant.

Results

In the absence of divalent cations the 13mer region adopts an unusual conformation

Previous studies indicated that unpairing of the AT-rich 13mer region in the left part of *oriC* was thermodynamically stable even in the absence of DnaA protein (Kowalski and Eddy, 1989). In these studies supercoiled plasmids containing *oriC* were probed with mung bean nuclease *in vitro*.

We decided on the use of potassium permanganate ($KMnO_4$) as a reagent to probe the structure of the *oriC* region under various conditions. $KMnO_4$ has been shown to react preferentially with pyrimidines in single-stranded DNA (Hayatsu and Ukita, 1967) and can be used *in vivo* as well as *in vitro*. The 5,6 double bond in thymine rings

is preferentially oxidized. As other chemical probes of DNA structure it yields high resolution information and is relatively independent of buffer constraints. The reagent has been employed to study the mechanism of DNA melting at the *lac* promotor *in vivo* (Sasse-Dwight and Gralla, 1989) and unpairing in the SV40 origin of replication *in vitro* (Borowiecz and Hurwitz, 1988). Permanganate reacts to a lesser extent with regions that are strongly bent (Borowiecz *et al.*, 1987). After the reaction modified residues are detected by termination of primer extension with Klenow polymerase from a ^{32}P-labeled primer. The extension products are then separated on standard sequencing gels.

The joint replicon pOC161 contains the *oriC* region in a pBR322 derivative (B.Woelker and W.Messer, unpublished). Plasmid DNA was subjected to modification by $KMnO_4$ under various conditions (Figure 1). The linearized plasmid showed no sensitivity (lane 1). However if the supercoiled plasmid was incubated in TE buffer or water a strong hypersensitivity in the 13mer region appeared. The reactivity was completely abolished when 5 mM $MgCl_2$ or 5 mM Mg-acetate were included in the reaction (lanes 3 and 4). When 10 mM EDTA were added the sensitivity was reconstituted (lane 5). Furthermore 50 mM $BaCl_2$ and 10 mM $ZnCl_2$ prevented modification by permanganate although not quite as efficiently as Mg^{2+} (not shown). The $KMnO_4$ sensitive structure could not be stabilized by addition of single-strand binding protein (SSB) when Mg^{2+} was present (not shown).

DnaA dependent 13mer melting in vitro

Addition of DnaA to supercoiled *oriC* plasmids results in localized unwinding at the three AT-rich 13mers as has previously been demonstrated by P1 nuclease sensitivity *in vitro* (Bramhill and Kornberg, 1988b; Hwang and Kornberg, 1990). In order to investigate the structural changes in the origin more precisely we made use of the potassium permanganate technique.

Constant amounts of plasmid DNA were incubated with increasing amounts of crude preparations of DnaA protein in the presence of ATP, under conditions where *oriC* dependent replication proceeds efficiently *in vitro* (Fuller *et al.*, 1981; Lother *et al.*, 1985). Upon the addition of DnaA protein a region of permanganate sensitivity was observed that was different from that detected in the absence of Mg^{2+}. It was larger and mapped slightly to the left (Figure 2). No sensitivity was observed when identical preparations from a *dnaA204* strain that does not harbor the DnaA overproducing plasmid pLSK5 (Schauzu *et al.*, 1987) were added (lane 6).

In order to determine the exact position of modified residues a second primer which hybridized to the left of the 13mers was employed (Figure 3). Upon addition of DnaA a region spanning 26 bp was melted. The M and R 13mers showed bands of strong intensity with weaker bands in between. Furthermore isolated single and double bands appeared close to DnaA box R1 and near the IHF binding site. These are strongly indicative of DNA bending or loop

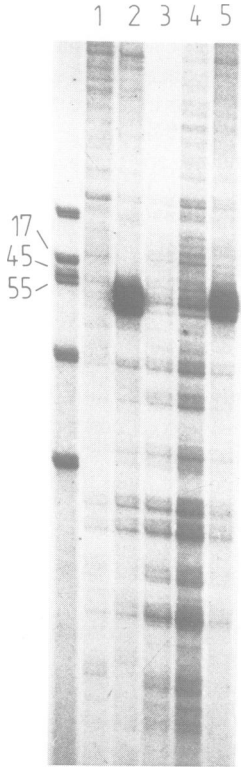

Fig. 1. Unusual structure in *oriC*. Plasmid DNA (pOC161; 20 μl) was incubated for 5 min at 30°C and 3 mM $KMnO_4$ were added. After 3 min the reaction was stopped by addition of 1 μl β-mercaptoethanol. Primer extension was carried out from a primer hybridizing to positions 308–326 to the right of *oriC*. Lane 1: linearized plasmid; lane 2: supercoiled plasmid in TE buffer; lane 3: in 5 mM $MgCl_2$; lane 4: 5 mM Mg-acetate; lane 5: 5 mM $MgCl_2$ and 10 mM EDTA. Numbers on the left marker lane indicate *oriC* coordinates.

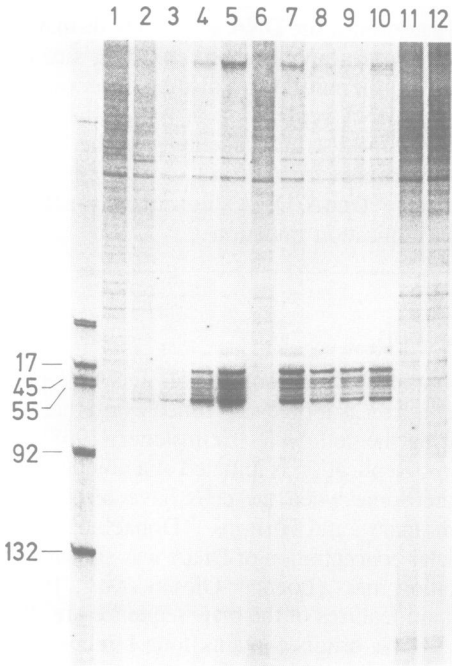

Fig. 2. DnaA dependent unpairing at *oriC*. Plasmid pOC161 was incubated with increasing amounts of DnaA protein under conditions supporting *in vitro* replication as described in Materials and methods. Samples were extracted with phenol–chloroform and analyzed by primer extension as above. Lanes 1–5, contained 0, 1, 2, 4 and 6 μl DnaA; lane 6 contained 4 μl of a control extract prepared from a *dnaA204* strain. Lane 7–10: Plasmid DNA was preincubated with 4 μl DnaA for 10 min, and 1, 2, 4 and 6 μl extract from a *dnaA*204 mutant strain were added for further 5 min prior to modification. Lanes 11 and 12 contained no protein and 10 μl DnaA buffer, respectively.

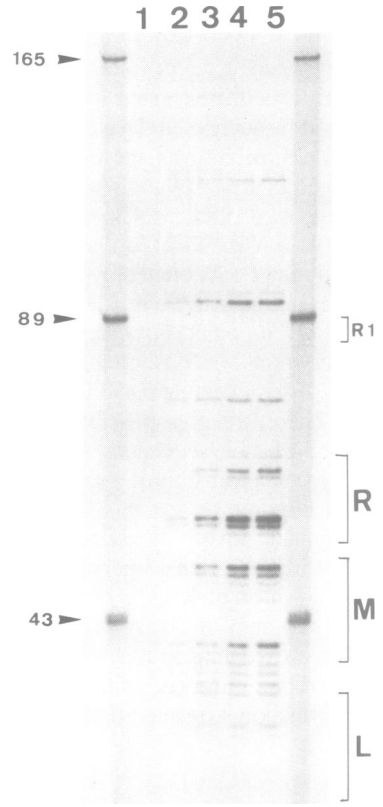

Fig. 3. DnaA dependent unpairing and helix distortion in *oriC*. pOC161 was incubated with DnaA protein and probed with $KMnO_4$ as in Figure 2A, except that a primer which hybridizes to *oriC* position −29 to −10 was used. The letters on the right indicate the positions of the left, middle and right 13mer and the leftmost DnaA box R1; the numbers on the left are *oriC* coordinates. Lanes 1−5 contained 0, 1, 2, 4 and 6 μl DnaA.

formation (Borowiecz *et al.*, 1987). The DNA in this region appears to be under torsional stress, probably facilitating processive melting towards the right part of *oriC*.

In order to allow detection of intermediates after the open complex [e.g. prepriming or priming complexes (Bramhill and Kornberg, 1988a)] *in vitro* replication was initiated by addition of the rest of the replication proteins in the form of an extract prepared from the *dnaA*204 mutant strain (Fuller *et al.*, 1981; Lother *et al.*, 1985). No significant reduction in 13mer sensitivity could be detected after 5 min incubation with the complementing replication proteins (Figure 2, lanes 7−10). Also we were unable to detect more extensively melted intermediates.

The 13mer region is the primary site of permanganate reactivity in vivo

Permanganate can be used to obtain high resolution structural information of DNA unwinding at a promoter during the initiation of transcription *in vivo* since open complexes can be collected by addition of rifampicin (Sasse-Dwight and Gralla, 1989; Sasse-Dwight and Gralla, 1990).

Since DnaA boxes R1, R2 and R4 are continuously bound by DnaA protein throughout the cell-cycle (Samitt *et al.*, 1989) we tried to detect single-stranded regions at *oriC* in exponentially growing cultures bearing plasmid pOC161. However no sensitivity could be detected at any point (Figure 4B, lane 1). Clearly, DnaA binding at R1, R2 and R4 alone does not lead to melting at the 13mers. Therefore we made use of the *dnaC2* mutant strain PC2 (Carl, 1970) as a host for pOC161.

Upon a shift to the non-permissive temperature, rounds of replication continue but no new initiation events occur. Replication origins are blocked at a stage after the action of DnaA protein but before DnaC and DnaB proteins enter the initiation complex. After one generation at 40°C all

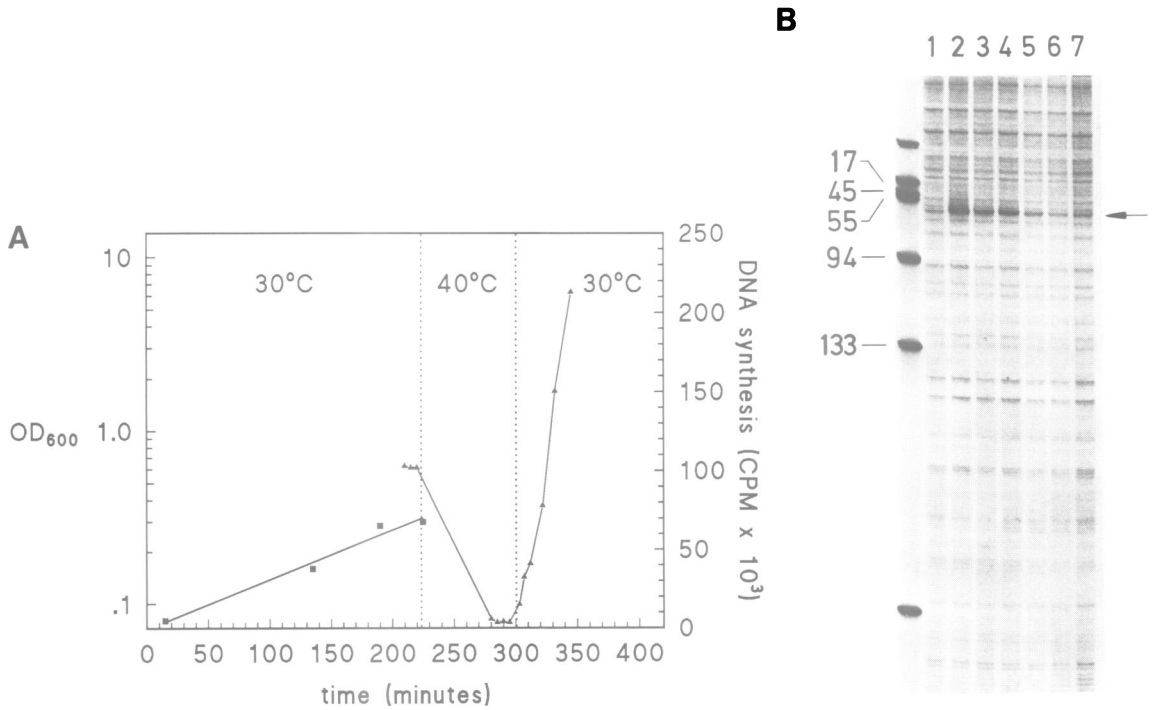

Fig. 4. Unpairing *in vivo*. **A**. Strain PC2 (*dnaC2*) was grown and shifted to 40°C for 60 min at OD_{600}=0.3 (squares) as described in Materials and methods. The rate of DNA synthesis was monitored by pulse-incorporation of [^{3}H]thymidine into TCA-precipitable material (triangles). **B**. A primer which hybridizes to positions 308−326 was used to detect single-stranded regions at *oriC in vivo*. Aliquots were taken during exponential growth at 30°C (lane 1), after 60 min at 40°C (lane 2) and 2, 5, 11, 16 and 30 min after return to the permissive temperature (lanes 3−7).

origins are at this stage, and replication has stopped. A shift to the permissive temperature 30°C results in a synchronous initiation of nearly all origins. The alignment at 40°C as well as the reinitiation was monitored by pulse-labeling with [^{3}H]thymidine (Figure 4A). Aliquots were treated with $KMnO_4$ as indicated (Figure 4B). Plasmid DNA was isolated and analyzed by primer extension. The primer corresponding to positions 308−326 in *oriC* was used in order to detect potential hypersensitive sites throughout *oriC*. After 60 min at the nonpermissive temperature a permanganate sensitive complex had accumulated (Figure 4B, lane 2). The hypersensitivity gradually disappeared after return to the permissive temperature (lanes 3−6).

The *in vivo* unpairing mapped exclusively to the right 13mer. It was not possible to detect any extensively melted regions in *oriC* during the reaction time (3 min). Apparently further DnaB dependent unwinding is too transient to be visualized by this method. In studies determining the mechanism of melting at the *lac* promotor *in vivo* open complexes could still be readily detected when only a fraction of 16% of the total plasmids were melted at their promotor regions (Sasse-Dwight and Gralla, 1989).

Open complex formation may be the rate-limiting step during the initiation of replication *in vivo*. Once the open complex is formed the ensuing prepriming complex formation and RNA/DNA synthesis occur rapidly since intermediates were not detectable.

Discussion

We provide the first evidence for a direct involvement and a structural change at the three AT-rich 13mers in the left part of *oriC* during the initiation of replication *in vivo*.

DnaA independent melting at oriC

In vitro a strong permanganate sensitivity can be observed on the supercoiled plasmids in the absence of DnaA protein if divalent cations are excluded from the reaction (Figure 1). Addition of 5 mM Mg^{2+} completely prevented modification by permanganate.

Extrusions of cruciforms from AT-tracts have been observed at various sequences and have been studied by two-dimensional gel electrophoresis and probing with different single-strand specific nucleases and reagents (Greaves *et al.*, 1985; Panyutin *et al.*, 1985; McClellan and Lilley, 1987; Furlong *et al.*, 1989). Cruciform formation requires negative supercoiling and may be facilitated by the addition of salts, notably Mg^{2+} (Haniford and Pulleyblank, 1985; McClellan and Lilley, 1987). In other instances the appearance of cruciforms at AT-tracts was observed at low salt conditions (Panyutin *et al.*, 1985; Diekmann and Lilley, 1987; Furlong *et al.*, 1989). Therefore it is possible that the observed unpairing at the AT-rich sequences in *oriC* at low ionic strength is due to the extrusion of a cruciform but clearly, further work is required to test this hypothesis.

This permanganate sensitivity reflects helical instability which gives the three AT-rich 13mers a propensity to melt, *in vitro* in the absence of Mg^{2+}, or under physiological conditions with the help of DnaA protein.

These results also demonstrate that the presence of a single-stranded region in *oriC* can not be taken as an indication for a functional open complex when Mg^{2+} levels are low.

DnaA dependent unpairing at oriC

In the presence of Mg^{2+} unpairing of the 13mers was completely dependent on the addition of DnaA protein. The region that was melted upon addition of DnaA is centered in the middle 13mer but extends into the R and L 13mers (Figure 5) thereby encompassing about 26 bp.

DnaB helicase protects ~80 nucleotides of single-stranded DNA *in vitro* (Arai and Kornberg, 1981). Therefore it appears unlikely that the amount of unpairing generated by DnaA alone can accomplish the loading of the helicase onto

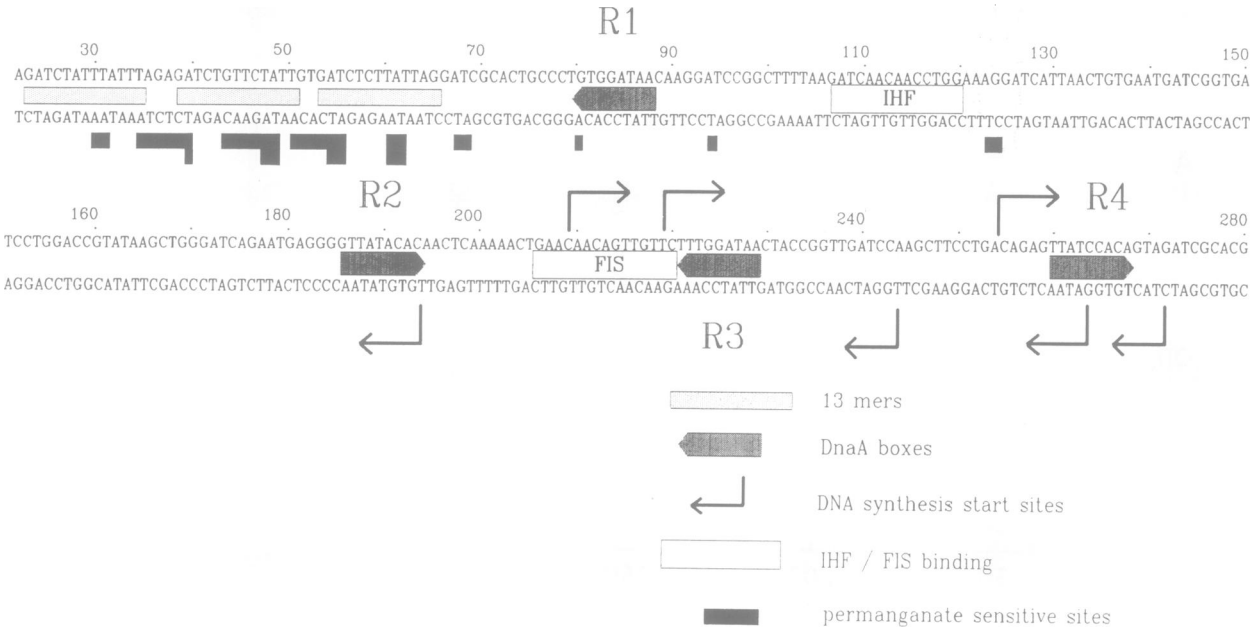

Fig. 5. The *E.coli* replication origin, *oriC*. The position of the 4 DnaA boxes and their respective orientations within *oriC*, and the 3 AT-rich 13mers are shown. The location of the IHF and FIS binding sites are also indicated. The right-angle arrows show the start sites of bidirectional DNA synthesis (Seufert and Messer, 1987). Solid boxes below the sequence indicate the positions of permanganate sensitivity *in vitro*.

the DNA. This is especially unlikely because the negative superhelicity facilitating DNA melting may be lower *in vivo* as the DNA is bound by various proteins (reviewed in Pettijohn, 1988).

In vitro, the region to the right of the 13mers is strongly bent upon the addition of DnaA protein as indicated by isolated permanganate sensitivities up to the IHF binding site (Polaczek, 1990). These isolated sensitivities indicate severe helix distortions that are likely to facilitate processive unwinding towards the right of *oriC*. Bidirectional synthesis is initiated between DnaA boxes R2 and R4 in the right part of *oriC* (Figure 5) (Seufert and Messer, 1987).

It has been shown that bending can drastically reduce the energy required to melt DNA (Ramstein and Lavery, 1988). Therefore it is likely that further distortion of the helix by IHF and FIS induced bending lowers the energy required for more extensive duplex opening even further. However, if the action of these proteins can be compensated by higher amounts of DnaA, their function may escape detection in the *in vitro* system.

Melting at oriC in vivo

Initially we tried to detect single-stranded regions in *oriC* in exponential cultures. However, although the DnaA boxes R1, R2 and R4 are continuously bound by DnaA throughout the cell cycle (Sammit *et al.*, 1989) no $KMnO_4$ sensitivity could be observed. This is in contrast to studies on the α and β replication origins of plasmid R6K, which show $KMnO_4$-sensitive regions in exponentially growing cells (Flashner and Shafferman, 1990). These distortions correlate with origin function as they are affected by *cis*-acting mutations. Probably, the higher initiation frequency at the R6K origins results in a larger fraction of origins undergoing initiation during the time of modification by permanganate. Therefore initiation complexes were collected in a *dnaC*(Ts) strain at the non-permissive temperature. The region that became single-stranded upon arresting a *dnaC2* mutant strain at the initiation stage was smaller than the unwound region observed *in vitro* with DnaA protein, and was located exclusively in the right 13mer (compare Figures 1 and 4B).

Interestingly, the L 13mer or the L and M 13mers can be replaced by a heterologous AT-rich sequence (Kowalski and Eddy, 1989; Asai *et al.*, 1990). Only the R 13mer is specifically required for origin function *in vivo* and can not be replaced or modified. It was proposed from these and earlier studies that DNA unwinding is initiated at the right 13mer (Bramhill and Kornberg, 1988; Asai *et al.*, 1990). The *in vivo* $KMnO_4$ reactivity pattern clearly supports this model.

In vivo the 13mer sensitivity rapidly disappeared after return to the permissive temperature (Figure 4). *In vitro* however, the open complex seemed to persist although the other replication proteins were added (Figure 2) under conditions where *oriC* dependent DNA replication proceeds efficiently *in vitro* (Fuller *et al.*, 1981; Lother *et al.*, 1985). A slow conversion of open complexes to prepriming complexes may be the reason for continued DNA synthesis for more than 60 min in the *in vitro* system (Sekimizu *et al.*, 1987). Therefore, whereas *in vivo* the 13mers are rapidly cleared, the initiation seems to proceed more slowly *in vitro*. In addition, the region that became reactive during the non-permissive temperature *in vivo* was considerably smaller than the sensitivite region that was observed *in vitro*. Apparently DNA melting is not easily achieved *in vivo*, possibly due to the lower amount of supercoiling. Alternatively, the action of a recently identified 33 kd protein, known to prevent duplex opening at the 13mers *in vitro*, could be responsible for the limited region of $KMnO_4$ sensitivity *in vivo* (Hwang and Kornberg, 1990). This may also be the reason why transcriptional activation is mandatory *in vivo*.

Neither *in vitro* nor *in vivo* was it possible to detect more extensively unwound intermediates after the open complex. Apparently these complexes are very transient, except when specifically arrested. Also, the regions that showed bending *in vitro* were not detected *in vivo*. This is not surprising since in studies on the bent *lac* repression loop $KMnO_4$ also failed to detect sites *in vivo* that were sensitive *in vitro* (Sasse-Dwight and Gralla, 1989). It was proposed that the loop is bent more smoothly *in vivo* due to the presence of other proteins such as HU.

DnaA mediated unpairing at the 13mer region is the first detectable event following the binding of the initiator protein DnaA to the replication origin *in vitro* and, as shown here, *in vivo*. AT-rich regions adjacent to reiterated binding sites for specific initiator proteins are a general feature of many replication origins, and may be subject to a similar change in conformation.

Materials and methods

Reagents

All reagents where obtained from sources previously described (Lother *et al.*, 1985) except for $KMnO_4$ which was purchased from FLUKA (microselect).

Enzymes

All enzymes were purchased from Boehringer Mannheim except for Klenow polymerase which was obtained from Amersham. SSB was from USB. Fractions enriched for DnaA were prepared from overproducing strain WM1492 harboring plasmid pLSK5 as described (Lother *et al.*, 1985).

Plasmids and oligonucleotides

$KMnO_4$ treatment and primer extension reactions were performed on plasmid pOC161 which is a joint replicon containing the origin region from −44 to +1497, including the *mioC* and *asnC* genes, in a pBR322 derivative (B.Woelker and W.Messer, unpublished).

The oligonucleotides from which the extension reactions were carried out correspond to *oriC* coordinates 308 to 326 and −29 to −10, respectively.

Permanganate footprinting and primer extension

Permanganate footprinting was carried out as described (Sasse-Dwight and Gralla, 1989) with minor modifications. In the *in vitro* reaction with DnaA protein 0.5−1.0 μg plasmid DNA were incubated in a total volume of 25 μl with protein under *in vitro* replication conditions, i.e. in the presence of 4% PEG 6000, 2 mM ATP, 0.05 mM NAD, creatin-phosphate/creatinkinase, 0.025 mM cAMP and dNTPs in 25 mM HEPES−KOH pH 7.6 as described (Lother *et al.*, 1985). When an extract from a *dnaA204* strain was added rNTP's were also present at 0.4 mM. Samples were incubated at 37°C for 5 min and $KMnO_4$ was added from a 50 mM stock to a final concentration of 3 mM. After 3 min the reaction was quenched by addition of 1 μl β-mercaptoethanol.

For the *in vivo* studies a fresh culture of strain PC2 (*dnaC2*; Carl, 1970) harboring plasmid pOC161 was diluted 1:100 into M9 medium supplemented with 0.25% casamino acids and grown at 30°C. When the cells had reached an OD_{600} of 0.3 the culture was shifted to 40°C for 60 min. It was returned to 30°C and an equal amount of fresh medium at 20°C was added to achieve a rapid temperature transition. Samples of 20 ml were withdrawn as indicated and treated with 3 mM $KMnO_4$. Plasmid DNA was prepared as described (Holmes and Quigley, 1981) and analyzed by primer extension (Sasse-Dwight and Gralla, 1989). The extension products were separated on 8% wedge-shaped sequencing gels.

Rate of DNA synthesis

Methyl-[^{3}H]thymidine (2 μCi, 83 Ci/mmol) was preincubated for 3 min at the indicated temperature (Figure 4A) and 48 μl cell suspension were added. After 4 min 800 μl cold TCA (10%) were added and the samples were precipitated on ice for 10 min. The precipitated material was collected on cellulose nitrate filters (Sartorius, 0.45 μm). The filters were washed and counted in Ready Organic (Beckmann).

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