A hybrid bacterial replication origin

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We constructed a hybrid replication origin that consists of the main part of *oriC* from *Escherichia coli*, the DnaA box region and the AT-rich region from *Bacillus subtilis oriC*. The AT-rich region could be unwound by *E. coli* DnaA protein, and the DnaB helicase was loaded into the single-stranded bubble. The results show that species specificity, i.e. which DnaA protein can do the unwinding, resides within the DnaA box region of *oriC*.

INTRODUCTION

Bacterial initiator protein DnaA binds to iterated binding sites in replication origins, forming specialized nucleoprotein structures (Echols, 1990; Kornberg and Baker, 1992). The net result is the local unwinding of an AT-rich region, which in the case of *Escherichia coli* consists of an AT cluster and three 13mer repeats (Bramhill and Kornberg, 1988). This is the crucial reaction in the initiation of replication. The unwound region is the entry site for the replicative helicase, which in *E. coli* is loaded as a double-hexamers of the helicase DnaB and the loader protein DnaC (Fang *et al.*, 1999). DnaA and interaction between DnaA and DnaB are required for the loading reaction (Marszalek *et al.*, 1996; Seitz *et al.*, 2000). DnaB helicase extends the initial bubble allowing primase to enter the complex (Fang *et al.*, 1999). The resulting primer is extended by the DNA polymerase III holoenzyme (Kornberg and Baker, 1992).

We have shown recently that the mechanism of the unwinding reaction relies on a cooperative binding of the ATP-complexed form of DnaA protein to special binding sites in the AT-rich region of *E. coli oriC*, 6mer ATP–DnaA boxes, using the adjacent 9mer DnaA box as an anchor. ATP–DnaA then binds to the exposed single-stranded ATP–DnaA boxes, thereby stabilizing the single-stranded region (Messer *et al.*, 2001; Speck and Messer, 2001).

The region corresponding to the *E. coli* AT-rich 13mers in *Bacillus subtilis oriC* is a 27mer containing exclusively A and T residues, and AT-rich sequences close to it. The two origins

could only be unwound by the homologous DnaA protein (Krause *et al.*, 1997). The regions of *oriC* that are unwound by DnaA protein in *E. coli* and *B. subtilis* are thus AT rich, but otherwise quite different in sequence. Alignment of both regions using the adjacent DnaA box reveals, however, that the spatial pattern of unwinding is very similar. In both cases the first unpaired nucleotide, measured by its sensitivity to oxidation by KMnO_4 , is 14 bp from the border of the DnaA box, R1 in the case of *E. coli*. The following 28 bp are unwound without SSB, and 16–18 additional base pairs in the presence of SSB, both in *E. coli* and *B. subtilis oriC* (Figure 1) (Krause and Messer, 1999). In addition, the ATP–DnaA boxes, which mediate cooperative binding of ATP–DnaA to the AT-rich region, are present at corresponding positions in both origins (Figure 1) (Speck and Messer, 2001).

This similarity prompted us to construct a hybrid replication origin that contains the AT-rich region from *B. subtilis* and the main part of *oriC* from *E. coli*, to the right of DnaA box R1 (Figure 1), and to analyze which initiation reactions can be observed with such an origin.

RESULTS

Structure of pOCBS

For the construction of such an *E. coli*–*B. subtilis* hybrid origin we used plasmid pOC180. It contains the *oriC* region from *E. coli* and the replication origin of pBR322 (Skarstad *et al.*, 2000). The AT-rich region from this plasmid, encompassing the AT cluster and the three 13mers (*E. coli oriC* coordinates 6–79) (Buhk and Messer, 1983), was replaced by the PCR-amplified AT-rich region from *B. subtilis*, positions 2216–2064 in Moriya *et al.* (1985) (Figure 1). The fragment from *B. subtilis oriC* is larger than the *E. coli* fragment in order to include the complete region of helical instability (DNA unwinding element) (Krause *et al.*, 1997).

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Fig. 1. Alignment of AT-rich regions from *E. coli*, *B. subtilis* and pOCBS *oriC*. The regions are aligned using the adjacent DnaA box (shaded). AT cluster, 13mers and 27mers are shaded. Potential ATP-DnaA boxes are underlined. KMnO₄-reactive pyrimidines are indicated by arrows. *Escherichia coli* sequences from positions 10 to 93 (DDBJ/EMBL/GenBank accession No. K00826) and *B. subtilis* sequences positions 2706 to 2623 (DDBJ/EMBL/GenBank accession No. X02369) are shown. Colons are at every 10th position.

The hybrid origin is unwound by *E. coli* DnaA protein

Unwinding of the AT-rich region was monitored using the reactivity of unpaired T residues to KMnO₄ as described previously (Krause *et al.*, 1997; Krause and Messer, 1999). Supercoiled pOCBS was incubated with *E. coli* protein HU and different concentrations of purified *E. coli* DnaA protein, and subjected to oxidation by KMnO₄. The positions of modified bases were determined by primer extension using 5'-³²P-labelled primer A (see Methods), electrophoresis in a sequencing gel and detection in a PhosphorImager. A sequencing reaction with the same primer and pOCBS as template was run in parallel. The plasmid with the *E. coli oriC*, pOC180, served as a control.

With *E. coli* DnaA protein modified bases were observed with the hybrid origin from 14 to 41 bases upstream of the DnaA box, with signals of lower intensity up to 51 bases (Figure 2). This region of unwinding is identical to the unwound region observed with a plasmid containing a bona fide *B. subtilis* replication origin using *B. subtilis* DnaA protein (Krause *et al.*, 1997). The hybrid origin thus shows a normal unwinding pattern. With pOCBS we did not observe any modified bases using purified DnaA protein from *B. subtilis* (data not shown).

DnaA can load helicase to the unwound hybrid origin

The next step after the unwinding reaction is the loading of helicase. Therefore we tested whether helicase can be loaded to the hybrid origin. Loading of DnaB helicase, as a DnaB-DnaC double-hexamer, and the subsequent action of helicase can be monitored using the FI* test. DnaB helicase action on a negatively supercoiled plasmid in the presence of DNA gyrase changes the linking number of the plasmid. Such plasmids can be detected as a faster moving band (FI*) in agarose gels (Baker *et al.*, 1987; Konieczny and Helinski, 1997).

Plasmids pOCBS and pOC180 were incubated with purified proteins from *E. coli*: DnaA, DnaBC, SSB, HU and DNA gyrase. The reaction was stopped with EDTA-SDS and subjected to agarose electrophoresis. With the complete mixture, a FI* band was observed with both pOC180 and pOCBS (Figure 3, lanes 5 and 11). This shows that helicase can be loaded to the hybrid origin. Omission of DnaA, DnaBC, SSB or DNA gyrase abolished the reaction (Figure 3, lanes 2–4). Without protein HU, there was only a small amount of a FI* band with pOC180 (Figure 3, lane 6), which was further reduced by increasing the DnaA concentration or adding IHF protein (data not shown). With pOCBS, nearly as much FI* band was observed with and without HU (Figure 3, lane 12). The results show that the hybrid origin cannot only be unwound by DnaA protein, but DnaA-mediated loading of helicase is also possible.

The hybrid origin does not replicate *in vivo*

The ability of the hybrid replication origin to replicate *in vivo* was tested by transformation of a *polA1* strain. No transformants were found in multiple experiments, whereas the same plasmid preparation resulted in >1000 transformants per plate with a *polA*⁺ strain. pOC180 as a control gave comparable numbers of transformants (>1000 per plate) with both strains. The results show that DnaA-mediated unwinding and helicase loading are not sufficient to allow replication *in vivo*.

DISCUSSION

We show here that a hybrid replication origin, consisting of the DnaA box region from *E. coli oriC* and the AT-rich region from *B. subtilis oriC*, can be unwound *in vitro* by *E. coli* DnaA protein. The unwound bases, measured by KMnO₄ footprinting, cover an identical part in the AT-rich region of the hybrid as in *B. subtilis oriC*. In addition, helicase, *E. coli* DnaBC protein can be loaded onto such a bubble substrate, mediated by DnaA protein (Seitz *et al.*, 2000). DnaB helicase then extends the

Fig. 2. KMnO_4 footprinting of pOCBS and pOC180 *oriC*. The footprinting reaction was done with 1 μg ccc plasmid DNA and 0, 250, 500 and 750 ng DnaA as described in the Methods. Sequencing reactions of pOCBS were used as standards and obtained using the same labelled primer A as for the KMnO_4 footprints. The bar indicates the 28 bp region that is unwound in *E. coli* and *B. subtilis* (Krause and Messer, 1999) (also see Figure 1).

bubble (Fang *et al.*, 1999). Since this is a pre-requisite for the formation of the FI* structure, we can also conclude that this step occurs in the hybrid origin.

It has been shown previously that the unwinding reaction is species specific. Unwinding of *E. coli* *oriC* requires *E. coli* DnaA protein, likewise *B. subtilis* *oriC* is only unwound by *B. subtilis* DnaA (Krause *et al.*, 1997). The observation that the hybrid origin is unwound by *E. coli* DnaA protein, and only by *E. coli* DnaA, suggests that the species specificity resides in the DnaA box part of the origin, probably in the spatial arrangement of DnaA boxes.

Although it is possible to replace the AT-rich region of *E. coli* *oriC* by the corresponding region of another species, it cannot be completely random, and AT richness is not sufficient for its function (Speck and Messer, 2001). We have shown that several 6mer consensus sequences, ATP-DnaA boxes that are different from the 9mer consensus sequences of regular DnaA boxes, mediate the cooperative binding of the ATP-complexed form of DnaA protein. Such ATP-DnaA boxes are present in the AT-rich regions of both *E. coli* and *B. subtilis* origins in the same spatial arrangement (Figure 1). Binding of six or more ATP-DnaA protein molecules destabilizes the helix. ATP-DnaA protein then binds with high affinity to the exposed single-stranded ATP-DnaA boxes (Messer *et al.*, 2001; Speck and Messer, 2001), preparing them for the loading of helicase.

It is not clear why the hybrid origin is unable to replicate *in vivo*. One specific requirement for replication initiation

Fig. 3. FI* helicase loading assay. The FI* assay was done as described in the Methods.

in vivo is transcriptional activation (Lark, 1972; Messer, 1972). Crucial for transcriptional activation is the *gidA* promoter at the left boundary of *oriC*, and the spacing between *gidA* and *oriC* (Asai *et al.*, 1990). The different spacing between *gidA* and the AT-rich region in the hybrid origin is likely to be one reason for its inactivity *in vivo*.

Unwinding at the replication origin is the central reaction in the initiation of most origins. Most cellular origins, prokaryotic and eukaryotic, have AT-rich regions that are unwound with the help of an initiator protein, DnaA in the case of bacteria. The analysis of the hybrid *B. subtilis*/*E. coli* replication origin allows us to define that the DnaA box region of *oriC* contains the species-specific elements of this reaction.

METHODS

Bacterial strains and plasmids. *Escherichia coli* WM1963 [XL1-blue] (*endA1*, *gyrA46*, *hsdR17*, *lac*, *recA1*, *relA1*, *supE44*, *thi*; F'*lac*: *lacR*, *lacZ* Δ M15, Tn10, *proAB*⁺) (Sambrook *et al.*, 1989) and SCS1 [F⁻, *endA1*, *hsdR17*(r_K⁻, m_K⁺), *supE44*, *thi*-1, *recA1*, *gyrA96*, *relA1*; Stratagene, La Jolla, CA] were used for cloning and in the *polA* tests as control. WM1933 (wild-type except for *polA1*) was the strain used for the *polA* tests.

Plasmid pOC170 (Roth and Messer, 1995) contains the *oriC* region (coordinates -176 to +1497; coordinates refer to Buhk and Messer, 1983), the replication origin of pBR322 and the *bla* gene from pT7-7. Plasmid pOC180 is pOC170 with 24 bp of the multicloning region deleted. Plasmid DNA was purified using Qiagen purification kits (Qiagen, Hilden, Germany).

Proteins. Restriction enzymes, DNA polymerases and T4 DNA ligase were from New England Biolabs (Beverly, MA). Thermo-stable polymerase for PCR was from Promega (Madison, WI), DNA gyrase from Gibco BRL and *E. coli* SSB from Promega. DnaA protein was overproduced from expression vector pdnaA116 and purified as described previously (Schaper and Messer, 1995; Krause *et al.*, 1997). DnaB and DnaC were simultaneously overproduced from plasmid pPS562 and the

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DnaB–DnaC complex separated from DnaB and DnaC by chromatography on a Mono Q-column and gel filtration as described previously (San Martin *et al.*, 1995).

KMnO₄ footprinting. *In vitro* KMnO₄ footprinting was performed as described previously (Krause *et al.*, 1997) with minor modifications. All analysis contained 1 µg *oriC* plasmid, pOC180 or pOCBS, and as assisting protein 100 ng of *E. coli* HU protein in 75 µl. Purified DnaA protein was added on ice. Open complex formation and subsequent KMnO₄ modification was allowed for 2 min each as described previously (Krause *et al.*, 1997). Primer extension was performed using a 5′-³²P-labelled oligonucleotide complementary to the top strand of *oriC* (primer A, *oriC* coordinates 142–119: 5′-CATTACAGTTAATGATCCTTCC) as primer. The samples were run on sequencing gels and analyzed by a PhosphorImager (Molecular Dynamics).

FI* assay. Reactions (25 µl) contained 40 mM HEPES–KOH pH 7.6, 11 mM Mg⁺⁺-acetate, 2 mM ATP, 1 mM DTT, 0.1 pmol plasmid DNA, 23 pmol SSB protein (440 ng), 2.1 pmol HU protein (40 ng) and 10 pmol DnaA (540 ng). After incubation for 5 min at 4°C and 5 min at 32°C, 0.8 pmol DnaB**C* complex (410 ng) and 0.65 pmol DNA gyrase (125 ng) were added and incubation continued for an additional 30 min at 32°C. The reactions were stopped by adding 5 µl stop mix (10 mM EDTA, 2.5% SDS) followed by incubation at 65°C for 2 min. Twenty microlitres of the reaction was loaded onto a 0.8% agarose gel in 0.5× TBE buffer with 0.1 µg/ml ethidium bromide. The samples were electrophoresed for 20–24 h at a constant 1 V/cm, then stained with Vistra GreenTM and scanned.

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