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Mapping of the *in vivo* start site for leading strand DNA synthesis in plasmid R1

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We have previously constructed *Escherichia coli* strains in which an R1 plasmid is integrated into the origin of chromosome replication, *oriC*. In such *intR1* strains, *oriC* is inactive and initiation of chromosome replication instead takes place at the integrated R1 origin. Due to the large size of the chromosome, replication intermediates generated at the R1 origin in these strains are considerably more long-lived than those in un-integrated R1 plasmids. We have taken advantage of this and performed primer extensions on total DNA isolated from *intR1* strains, and mapped the free 5′ DNA ends that were generated as replication intermediates during R1 replication *in vivo*. The sensitivity of the mapping was considerably improved by the use of a repeated primer extension method (RPE). The free DNA ends were assumed to represent normal *in vivo* start sites for leading strand DNA synthesis in plasmid R1. The ends were mapped to a short region ~380 bp away from the R1 minimal origin, and the positions agreed well with previous *in vitro* mappings. The same start positions were also utilized in the absence of the DnaA protein, indicating that DnaA is not required for determination of the position at which DNA synthesis starts during initiation of replication at the R1 origin.

Key words: DnaA protein/initiation of DNA replication/plasmid R1/repeated primer extension/replication start site

Introduction

Initiation of DNA replication usually requires the binding of a replicon-specific initiator protein to its DNA recognition site in the origin region of the replicon (Bramhill and Kornberg, 1988b). Examples of such positively acting initiator proteins are the DnaA protein (reviewed by Georgopoulos, 1989) of *Escherichia coli*, which is necessary for initiation of chromosome replication, and RepA, the initiator protein of plasmid R1 (Masai *et al.*, 1983). The binding of the initiator protein facilitates unwinding of the origin region and is believed to direct the assembly of replisome components necessary for initiation of replication (reviewed in Bramhill and Kornberg, 1988b; McMacken *et al.*, 1987). Identification of the precise position at which incorporation of the first deoxyribonucleotide takes place *in vivo*, i.e. the start site for leading strand DNA synthesis, has been attempted only for a few of the replicons that form theta structures (these are described in Kornberg and Baker, 1992) during replication. These include the unidirectionally replicating plasmids ColE1 (Tomizawa *et al.*, 1977) and pAMβ1 (Bruand *et al.*, 1991) as well as the origin of bidirectional chromosome replication in *E.coli*, *oriC* (Kohara *et al.*, 1985). The *in vivo* mappings, together with *in vitro* results (Masai and Arai, 1989; Miyazaki *et al.*, 1988), suggest that the start site for DNA synthesis may be positioned outside the minimal origin of the replicon.

During initiation of bidirectional replication, leading strand DNA synthesis in the two opposite directions might be initiated simultaneously or, alternatively, with a time lag between the two initiation events. In the latter case, a free 5′ DNA end would be expected to be present as a replication intermediate until the second replication fork is initiated. This free DNA end would, however, be relatively short-lived and therefore difficult to isolate in sufficient quantities for analysis. In contrast, at origins from which unidirectional replication is initiated, such as that of plasmid R1 (Diaz and Staudenbauer, 1982), the free DNA end would be expected to persist for a whole round of replication of the plasmid, making isolation and analysis of replication intermediates easier.

We have constructed so-called *intR1* strains which contain a deletion at the left end of *oriC*, into which the R1 mini-plasmid pOU71 has been inserted (Bernander *et al.*, 1989; Figure 1). The deletion removes the leftmost of the three 13 bp repeats that are essential for unwinding *oriC* (Bramhill and Kornberg, 1988a), thereby preventing *oriC* from functioning in initiation of chromosome replication. In the *intR1* strains, chromosome replication is therefore controlled by the inserted R1 replicon, whereas *oriC* is inactive. Replication intermediates in *intR1* strains are considerably more long-lived than in non-integrated R1 plasmids, since the replication fork has to traverse the entire *E.coli* chromosome before the free 5′ DNA end at the start site for DNA synthesis can be sealed.

The properties described above make the *intR1* strains suitable for *in vivo* analysis of replication intermediates. In this communication, we report the mapping of free 5′ DNA ends generated *in vivo* during initiation of DNA replication from the integrated plasmid R1 origin, as well as from an unintegrated R1 plasmid. A repeated primer extension (RPE) method resulted in a many-fold increase in the sensitivity of the mapping. The same start sites for DNA synthesis were utilized in the absence of the DnaA protein, as well as during over-expression of the RepA initiator protein of plasmid R1.

Results

Repeated primer extension (RPE)

The strains were grown in minimal glucose medium and total DNA was extracted and purified. Short single-stranded DNA oligonucleotides complementary to various regions in and

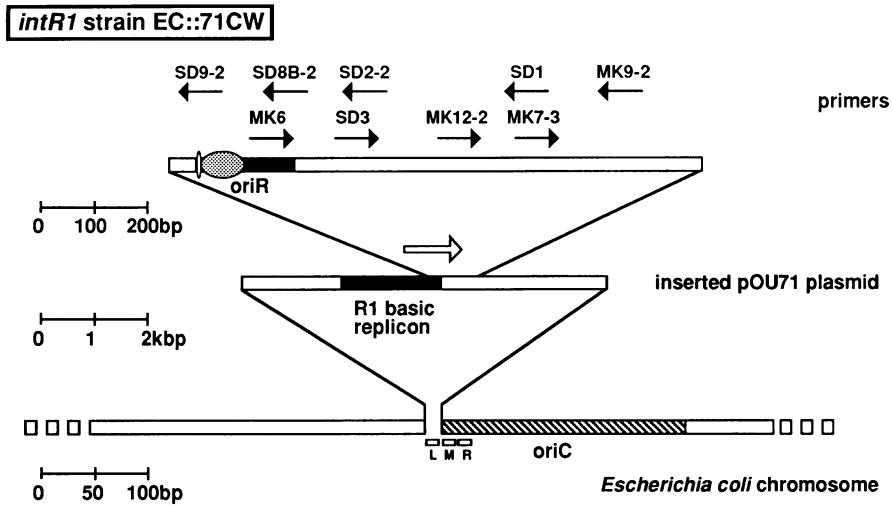

Fig. 1. Map of the origin region of the *intR1* strains and of the location of the various primers used. **Below:** chromosome DNA around *oriC*. Diagonal hatching shows the location of *oriC* and the open boxes the positions of the repeats essential for unwinding (Bramhill and Kornberg, 1988a). The lettering denotes the left (L), middle (M) and right (R) box, respectively. **Centre:** the inserted pOU71 plasmid that replaces the left unwinding element in strains EC::71CW and EC::71CC (Bernander *et al.*, 1989). The R1 basic replicon (filled box, open arrow above the box showing the direction of fork movement) is indicated. The orientation of the inserted plasmid, and therefore the direction of fork movement along the *E.coli* chromosome, is inverted in strain EC::71CC. **Above:** magnification of the region around the R1 minimal origin (a detailed description of the organization of the basic replicon of plasmid R1 is given in Figure 6). The filled box shows the minimal origin with the footprints of the DnaA and RepA proteins (Masai and Arai, 1987) shown as an open and a shaded ellipse, respectively. The various primers used to screen the origin region for free 5′ DNA ends are indicated by arrows, with the arrowheads showing the direction of extension. Note that different scales are used in the different parts of the figure and that the primers are not drawn to scale.

around the R1 minimal origin (Figure 1) were used in primer extensions with the isolated chromosome DNA as template (see Materials and methods for details). When chromosomal DNA is used as template for primer extensions, the amount of product that can be obtained is limited by the amount of template sequence in the reaction which, for practical reasons, usually is in the femtomole range (~3 μg) for *E.coli* chromosomal DNA. In addition, not all chromosomes will contain replication intermediates; some of the cells would have been harvested during the time period between termination of a round of chromosome replication and initiation of the next round. To increase the sensitivity, an RPE technique was therefore developed. In RPE, the standard polymerase chain reaction (PCR) protocol is used, i.e. repeated cycles of primer extension, denaturation, renaturation and new extension are performed, but only a single DNA primer is used. In contrast to PCR, RPE therefore results in a linear increase in the amount of product, which is single-stranded (see Materials and methods for details).

Free 5′ DNA ends in exponentially growing and stationary phase intR1 strains

Chromosomal DNA was isolated from the *intR1* strains EC::71CW and EC::71CC (Bernander *et al.*, 1989; Figure 1) and analysed with RPE as described above. Primer MK9-2 (see Figure 1) gave rise to discrete run-off bands in the primer extensions (Figure 2, lane 1), presumably corresponding to free 5′ DNA ends in the R1 origin region. The increase in sensitivity obtained with the RPE technique is apparent from a comparison with a single-round primer extension (lane 3). The bands obtained were considerably less intense when stationary phase cultures were used as the source of template DNA (lane 2). This shows that more free ends were generated during active growth and replication, as expected if they corresponded to replication intermediates.

The additional band that can be seen in the RPE lanes was not correlated with on-going replication, since the intensity of this band did not depend upon whether DNA from exponential or stationary cultures was used (cf. lanes 1 and 2). Instead, this band appeared to result from termination of the extensions at a putative hairpin structure (Figure 2), whose formation was apparently favoured in the RPE experiments, since the band was absent in single round extensions even after long exposure times (see also Figure 4, lane 3). In the RPE reactions, the extra band may therefore be used as an internal standard for the amount of sample that was loaded on the gels; note that in occasional samples the RPE reaction efficiency was decreased in Figures 3 and 5. To determine the positions of the free 5′ DNA ends, a DNA sequence ladder (lanes G, A, T and C) was prepared using the same primer. The major extension products were mapped to the C residues at positions 1984–1987 according to the numbering of Masai and Arai (1989). These positions were in good agreement with previous *in vitro* results (Masai and Arai, 1989; Miyazaki *et al.*, 1988), indicating that the run-off products represented normal replication intermediates generated during initiation at the R1 origin *in vivo* as well as *in vitro*. A more extensive analysis (Figure 3) revealed that the same start positions were utilized regardless of the orientation of the integrated pOU71 plasmid in the chromosome (cf. lanes 1 and 3). Furthermore, the free 5′ DNA ends were absent when a control strain without the R1 plasmid was used as the source of template DNA (lanes 5 and 6).

Free 5′ DNA ends in an unintegrated R1 plasmid

We wanted to investigate whether the same *in vivo* start sites for DNA synthesis were also utilized in an R1 plasmid not integrated into the chromosome. In our hands, primer extensions to map free 5′ DNA ends generated *in vivo* in unintegrated plasmids usually resulted in a large number of

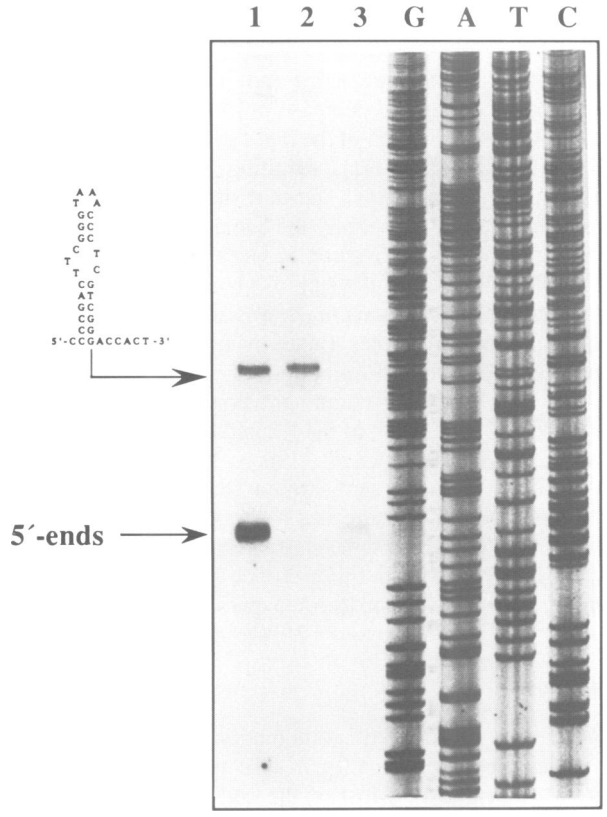

Fig. 2. Detection of free 5′ DNA ends generated as replication intermediates in *intR1* strain EC::71CW. Approximately 0.2 μg (~0.07 fmol) of *E.coli* chromosomal DNA containing an integrated R1 plasmid was used as template in extensions with primer MK9-2 (Figure 1). Repeated primer extensions were run for 30 cycles, and one-third of the resulting samples were loaded on the gel. Lane 1, template DNA from an exponentially growing culture of strain EC::71CW; lane 2, EC::71CW stationary culture; lane 3, same as lane 1 but single round primer extension and all of the resulting sample loaded; G, A, T and C, sequence ladder obtained with the same primer using purified, unintegrated, plasmid DNA as template. The band corresponding to free 5′ DNA ends generated as replication intermediates during initiation of replication from the integrated plasmid R1 origin is indicated. An additional band, resulting from termination of the repeated primer extensions at a putative hairpin structure (see second paragraph in the Results section for details) in the DNA, shown to the left, is also indicated.

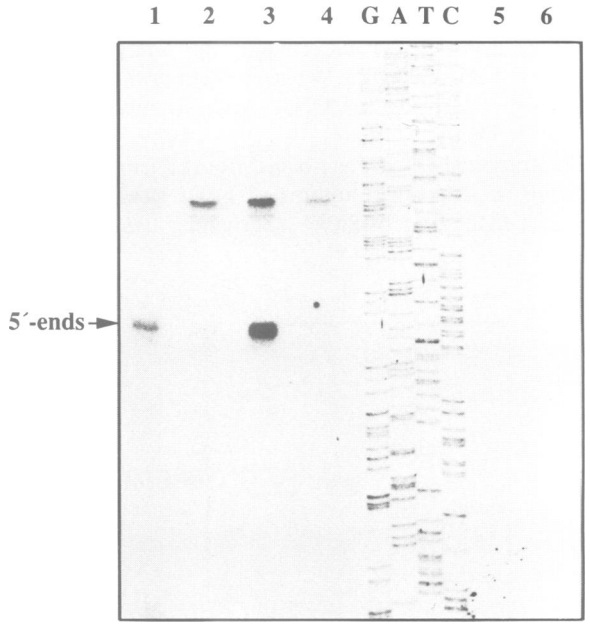

Fig. 3. Repeated primer extensions on chromosomal DNA from exponentially growing and from stationary cultures. The extensions were performed as described in Figure 2, using primer MK9-2 (Figure 1). Lane 1, exponential culture of *intR1* strain EC::71CW; lane 2, EC::71CW stationary culture; lane 3, *intR1* strain EC::71CC, exponential culture; lane 4, EC::71CC stationary culture; G, A, T and C, sequence ladder (see Figure 2); lane 5, wild-type control strain EC1005, exponential culture; lane 6, EC1005 stationary culture. The free 5′ DNA ends representing start sites for DNA synthesis are indicated. (The upper band is discussed in the legend to Figure 2 and in the second paragraph of the Results section.)

extension products of different sizes (cf. Figure 4, lanes 1 and 2). The reason for this is not clear, but it could be due to structural differences, e.g. in the level of supercoiling, between isolated chromosomal and plasmid DNA. In support of this, restriction enzyme digestion of the plasmid DNA resulted in a reduction in the number of extension products (not shown). We reasoned that it should be possible to identify the bands that represented replication intermediates in an unintegrated plasmid by comparing them with the results obtained with the chromosomal DNA from the *intR1* strains. Plasmid pRB107 (Bernander *et al.*, 1989) was chosen for two reasons. First, it is fairly large (~20 kb) and the replication intermediates are therefore more long-lived than those of smaller R1 derivatives, such as the pOU71 plasmid (~7 kb) which is integrated into the chromosome of strains EC::71CW and EC::71CC. Secondly, the plasmid contains two functional origins from different plasmids, and initiation of replication from the R1 origin is conditional (see legend to Table I). Therefore, the relative amount of replication intermediates before and after induction of initiation of replication from the R1 origin can be compared.

An exponentially growing strain containing pRB107 was treated such that on-going replication should be instantaneously halted (see Materials and methods), to avoid runout of plasmid replication during the isolation procedure. Run-off products from single round primer extensions on plasmid DNA isolated from uninduced (growth temperature 30°C) and from induced (42°C) cultures were electrophoresed together with a sample from the repeated extensions performed on *intR1* chromosomal DNA. Of the various bands obtained (Figure 4), some increased in relative amount when plasmid DNA from induced cultures was used, as compared with DNA from uninduced cultures (cf. lanes 2 and 1). The positions of these bands were compared with the *intR1* sample (lane 3), and the result shows that the same free 5′ DNA ends were formed as replication intermediates whether or not the plasmid was integrated into the chromosome.

Free 5′ DNA ends in the absence of the DnaA protein

Initiation of DNA replication at the R1 origin *in vivo* can take place in the absence of the DnaA protein [Bernander *et al.*, 1991; Tang *et al.*, 1989; see references cited in Bernander *et al.* (1991) for dependence on DnaA *in vitro*]. The role of DnaA during initiation of R1 replication may, therefore, be limited to increasing the efficiency of initiation, e.g. by facilitating origin unwinding and subsequent entry and assembly of the various replisome components. In other replicons, it has been suggested that the DnaA protein is directly involved in priming of leading strand DNA

synthesis (Masai *et al.*, 1990b; Seufert and Messer, 1987). To investigate further the role of the DnaA protein in initiation of R1 replication, we performed primer extensions to map the start site for DNA synthesis in *intR1* strains devoid of DnaA.

Chromosomal DNA was isolated from either exponentially growing or stationary cultures of *intR1* strains, carrying the *dnaA850*::Tn*10* allele (Bernander *et al.*, 1991).

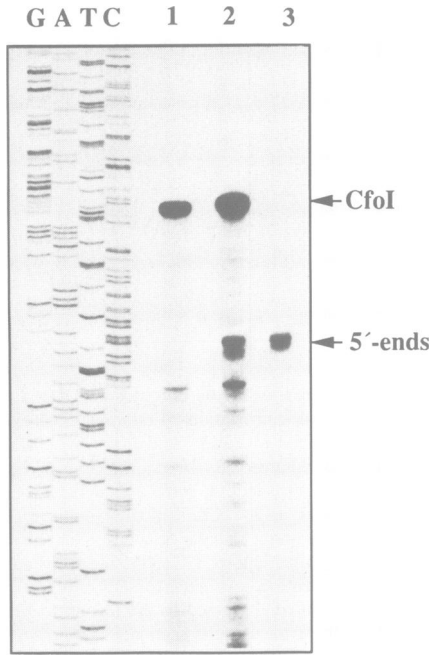

Fig. 4. Single round primer extensions on unintegrated plasmids. Repeated primer extension was found not to be necessary, since the amount of template DNA was considerably higher than when chromosomal DNA was used (see Materials and methods). The plasmid DNA was cleaved with the restriction enzyme *Cfo*I before primer extension (see text), and the extensions were performed with primer MK9-2 (Figure 1). Lane 1, plasmid pRB107 (in strain EC1005), not induced for initiation of replication from the R1 origin; lane 2, pRB107 induced; lane 3, chromosomal DNA from an exponential phase culture of *intR1* strain EC::71CC for size comparison of extension products; G, A, T and C, sequence ladder (see Figure 2). Extension products representing start sites for DNA synthesis are indicated. The high molecular weight band resulting from primer extension up to the *Cfo*I site is also indicated

Repeated primer extension was found to be essential in these experiments, since no detectable signals were obtained with single round extensions (not illustrated). This was presumably due to the decrease in initiation efficiency that results from inactivation of the *dnaA* gene in the *intR1* strains (Bernander *et al.*, 1991), resulting in a lower yield of replication intermediates. The start sites for DNA synthesis were found to be essentially the same in the wild-type and *dnaA* mutant strains (Figure 5, lanes 1, 2 and 4). Again, the relative amount of the run-off products decreased when DNA from stationary cultures was used, as expected for replication intermediates (lanes 3 and 5). At 38°C, the lowered efficiency of initiation of replication from the R1 origin in the *dnaA* mutant strains is compensated for by an increased production of the R1 initiator protein RepA (for details see Bernander *et al.*, 1991). The primer extension results showed that the start sites for DNA synthesis remained the same also during increased *repA* expression (lanes 6 and 8). In conclusion, the same start sites for initiation of DNA synthesis were used in the presence and in the absence of DnaA, and at different RepA expression levels.

Discussion

The *intR1* system in combination with RPE offers considerable advantages for *in vivo* mapping of start sites for DNA synthesis. Most of the cells in an exponentially growing *intR1* culture contain replication intermediates, which are more long-lived than those of unintegrated plasmids. The background level of extension products not related to replication is low in DNA isolated from *intR1* strains, and the same approach, integration of a functional origin region into the chromosome followed by RPE, should also be applicable to other replicons. RPE should be useful for mapping free 5′ DNA ends in organisms other than *E.coli*, and the high sensitivity allows for the detection of small amounts of free DNA ends; free ends in 0.1 fmol of *intR1* chromosomal DNA were easily detected.

This report shows that free 5′ DNA ends are generated as replication intermediates during initiation of replication at the plasmid R1 origin *in vivo*. We believe that the mapped 5′ DNA ends represent *in vivo* start sites for DNA synthesis. More free ends were present in exponentially growing cells than in stationary phase cells, in agreement with

Table I. *E.coli* strains and plasmids used in this study

	Parent	Relevant markers[a]	Reference
Strain			
EC1005		*metB1, nalA, relA1, spoT1*, λ^r, F^-	Grinsted *et al.* (1972)
EC::71CW[b]	EC1005	Δ*oriC*::pOU71, Ap^r, Rep-Td, clockwise fork direction	Bernander *et al.* (1989)
EC::71CC[b]	EC1005	Δ*oriC*::pOU71, Ap^r, Rep-Td, counterclockwise fork direction	Bernander *et al.* (1989)
EC::71CW*dnaA*::Tn*10*	EC::71CW	*dnaA850*::Tn*10*	Bernander *et al.* (1991)
EC::71CC*dnaA*::Tn*10*	EC::71CC	*dnaA850*::Tn*10*	Bernander *et al.* (1991)
Plasmid			
pRB107[c]		Ap^r,Su^r,Rep-Td	Bernander *et al.* (1989)
p71CW		Ap^r,Rep-Td	Bernander *et al.* (1989)

[a]Abbreviations: Ap, ampicillin; Su, sulphonamide; Rep-Td, replication temperature-dependent.
[b]See Materials and methods and Figure 1 for a description of strains EC::71CW and EC::71CC; these strains carry pOU71 inserted so that the direction of fork movement from the R1 origin in the chromosome is clockwise and counterclockwise on the genetic map, respectively.
[c]Plasmid pRB107 contains two functional replicons, from plasmids R1 and RSF1010, respectively. The R1 origin is not active at temperatures below 34°C, whereas at higher temperatures initiation of replication from the R1 origin is induced (Bernander *et al.*, 1989).

the assumption that the proportion of cells with on-going rounds of replication should be larger in actively growing populations. The positions of the ends correlated well with those previously identified *in vitro* in plasmid R1 (Masai and Arai, 1989) and in the closely related R100 plasmid

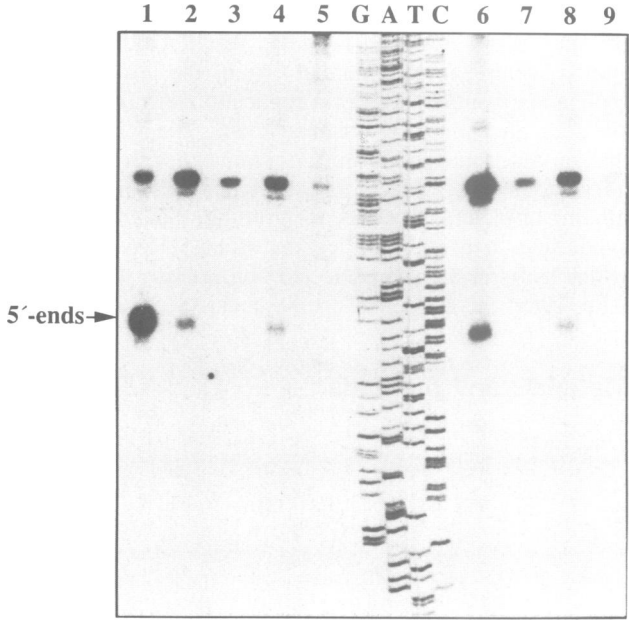

Fig. 5. Repeated primer extension on chromosomal DNA from *intR1* strains containing a *dnaA*::Tn*10* allele. The extensions were performed as described in Figure 2, using primer MK9-2 (Figure 1). Lane 1, exponential phase culture of strain EC::71CW grown at 34°C, for size comparison of extension products; lane 2, EC::71CW *dnaA*::Tn*10*, exponential phase culture grown at 30°C; lane 3, EC::71CW *dnaA*::Tn*10*, stationary phase culture at 30°C; lane 4, EC::71CC *dnaA*::Tn*10*, exponential phase culture at 30°C; lane 5, EC::71CC *dnaA*::Tn*10*, stationary phase culture at 30°C. G, A, T and C, sequence ladder (see Figure 2); lane 6, EC::71CW*dnaA*::Tn*10*, exponential phase culture grown at 38°C; lane 7, EC::71CW*dnaA*::Tn*10*, stationary phase culture grown at 38°C; lane 8, EC::71CC *dnaA*::Tn*10*, exponential phase culture grown at 38°C; lane 9, EC::71CC *dnaA*::Tn*10*, stationary phase culture grown at 38°C. Extension products representing start sites for DNA synthesis are indicated. (The upper band is discussed in the legend to Figure 2 and in the second paragraph in the Results section.)

(Miyazaki *et al.*, 1988), indicating that the *in vitro* system accurately reflects the *in vivo* situation (see Figure 6). The same start sites were obtained whether or not the plasmids were integrated into the chromosome, and no free DNA ends could be demonstrated with primers directed against other regions in and around the R1 origin. Several extension products of slightly different length were identified, and the main products were mapped to positions 1984–1987 (Figure 6). The heterogeneity could be due to trimming or limited degradation of the free DNA ends, or to remaining ribonucleotides at the 5′ end of some template DNA molecules. In an analysis of priming of leading strand DNA synthesis in the closely related R100 plasmid *in vitro* (Masai *et al.*, 1990a), variation in the length of the RNA primers was observed. The heterogeneity may, therefore, reflect natural variation in the position at which leading strand DNA synthesis is initiated *in vivo*.

In strain EC::71CW, chromosome replication is mainly bidirectional. The replication fork initiated at the integrated R1 origin in this strain travels towards the remaining part of *oriC*, and we have speculated that when the *oriC* region is unwound, bidirectional replication may be induced (Bernander *et al.*, 1989; Nordström *et al.*, 1991). In support of this, strain EC::71CC, in which the replisome travels away from *oriC*, is more biased towards unidirectional replication than EC::71CW. If the R1 replication fork in EC::71CW is aborted after induction of bidirectional replication from *oriC*, the free DNA ends at the R1 origin might persist for some time in the cells before being degraded. This might explain why similar amounts of free 5′ ends were detected in the two *intR1* strains, despite their different degrees of bidirectionality of chromosome replication.

Priming of leading strand DNA synthesis in plasmid R1 is carried out by DNA primase (Ortega *et al.*, 1986; Masai and Arai, 1989), the product of the *dnaG* gene. The positions of the primers synthesized *in vitro* at the origin of plasmid R100 (Masai *et al.*, 1990a) roughly agreed with start of DNA synthesis at the sites mapped in this study. The leading strand start sites in plasmids R1 and R100 reside within a single strand initiation (*ssi*) sequence (Bahk *et al.*, 1988; Masai *et al.*, 1990a; Nomura *et al.*, 1991), which also includes the region in which the RNA primer synthesis is initiated, as well as sequences homologous to binding sites

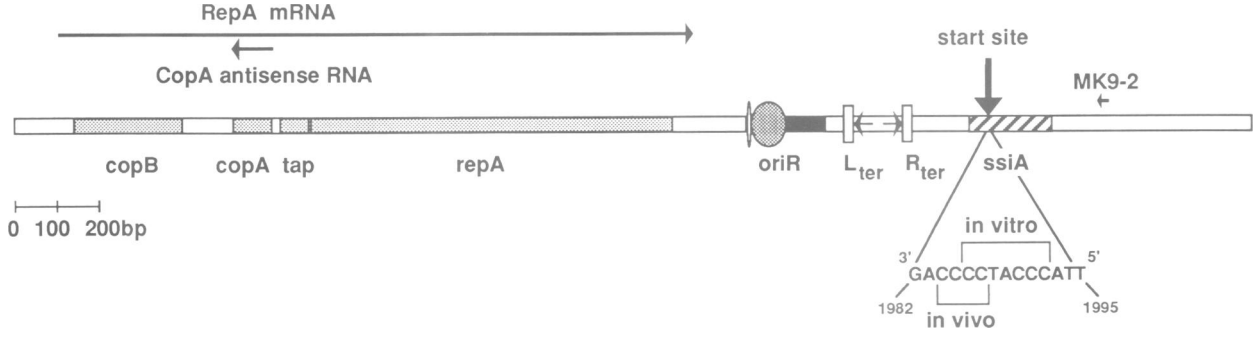

Fig. 6. Organization of the basic replicon of plasmid R1 and summary of *in vitro* and *in vivo* mappings of the start sites for DNA synthesis. Relevant features: *copB*, *copA*, *tap* and *repA* genes (shaded boxes), RepA mRNA and the antisense RNA CopA (filled arrows above the genes), R1 minimal origin (filled box) as defined by Masai *et al.* (1983), DnaA and RepA protein footprints (Masai and Arai, 1987) within the minimal origin (open and shaded ellipse, respectively), *ter* sites (Hill *et al.*, 1988) located downstream of the minimal origin (open rectangles, arrows indicating the polarity of replication fork blockage); *ssi* (Bahk *et al.*, 1988; Masai *et al.*, 1991; Nomura *et al.*, 1991) region (striped box), start site for DNA synthesis (vertical filled arrow), primer MK9-2 (horizontal filled arrow). A summary of *in vivo* (this work) and *in vitro* (Masai and Arai, 1989) mappings of the start sites for leading strand DNA synthesis in plasmid R1 is given in the lower right part of the figure. The brackets indicate the region in the template strand where DNA synthesis starts, with coordinates according to Masai and Arai (1989). All features are drawn to scale.

for the PriA (n′) protein, which is known to be important for initiation of DNA synthesis in other replicons. Although priming at the R1 *ssi* site *in vitro* only requires the Ssb and DnaG proteins (Masai and Arai, 1989), it was found that R1 plasmids could not be maintained in *priA* mutant cells *in vivo* (Lee and Kornberg, 1991). It is therefore not clear which elements normally take part in defining the start site, and whether additional positional or other information resides within the *ssi* region. Interestingly, the start sites in plasmids R1 and R100 are located outside the minimal origin, i.e. if these sites are deleted, DNA synthesis is initiated from a different position in the DNA. Also, when the region containing the start sites for plasmid R100 was inverted, *in vitro* DNA synthesis was initiated from several new sites, located closer to the minimal origin than the original start sites (Miyazaki *et al.*, 1988). Although this may be taken to indicate that defined start regions are dispensable *in vivo*, another possibility is that other, fortuitous, *ssi*-like sequences may instead have been recruited in these constructs. Furthermore, the *ssi* sequence might be used also in conjugal initiation of replication in plasmid R1. In conjugation, the DNA entering the recipient cell is single-stranded, and it is conceivable that in this single-stranded DNA, *ssi* sites are exposed and used to direct initiation of DNA synthesis of the complementary strand.

The DnaA protein fulfils an important specificity function during initiation of replication from *oriC* by unwinding the origin and facilitating replisome assembly (reviewed in McMacken *et al.*, 1987; Bramhill and Kornberg, 1988b; Georgopoulos, 1989). The specificity function at the R1 origin appears to be performed by the plasmid-encoded RepA initiator protein (Masai and Arai, 1987), and DnaA may modify initiation quantitatively; the efficiency of initiation has been shown to increase when the DnaA protein is present (Bernander *et al.*, 1991). In accordance, the free DNA ends were mapped to the same positions in the absence of the DnaA protein, indicating that DnaA is not required for determining the position of the start site for leading strand DNA synthesis in plasmid R1. This confirms that priming of R1 DNA synthesis is not carried out by a mechanism involving DnaA (see Masai and Arai, 1989), such as the 'ABC-primosome' (Masai *et al.*, 1990b). In addition, when expression of the *repA* gene was increased, the start sites remained the same. This indicates that the increased frequency of initiation of replication from the integrated R1 origin that results from the higher level of RepA production (Bernander *et al.*, 1991) occurs through the normal priming mechanism.

As a result of this and other reports, an overall understanding of the mechanism of initiation of replication in plasmid R1 is beginning to emerge (Figure 6). The RepA mRNA and the antisense RNA CopA are constitutively synthesized (Nordström *et al.*, 1984; Womble *et al.*, 1985), and binding between these two RNAs prevents expression of the *repA* gene (Persson *et al.*, 1988). If, however, translation of a leader peptide, *tap*, is initiated before CopA has bound to the mRNA, translation of the *repA* reading frame can take place. This occurs by translational coupling between the *tap* and *repA* reading frames in the mRNA (Blomberg *et al.*, 1992). Translation results in formation of the RepA protein which binds to the R1 minimal origin, preferentially *in cis* (Masai and Arai, 1988), thereby facilitating binding of the DnaA protein to an adjacent site (Masai and Arai, 1987). Binding of the RepA and DnaA proteins presumably results in unwinding of the origin region, in analogy with the scheme presented for initiation of replication at *oriC* (Bramhill and Kornberg, 1988b), and in assembly of other factors needed for initiation. The main unresolved question is how this makes the *ssi* region, located almost 400 bp away from the minimal origin, accessible for priming. Also, it should be noted that if DNA synthesis started within the R1 minimal origin, the leading strand replication would encounter a replication termination (*ter*) site soon after initiation, whereas the *ter* site is bypassed if the start sites mapped in this communication are used. As a result of the activation of the *ssi* region, which may include binding of factors necessary for priming, the DnaG protein synthesizes a primer for leading strand DNA synthesis, which starts at the sites detected both *in vitro* (Masai and Arai, 1989; Miyazaki *et al.*, 1988) and *in vivo* (this report).

Materials and methods

Bacterial strains and plasmids

The *E.coli* K-12 strains used in this study are described in Table I and Figure 1. The construction and characterization of the *intR1* strains EC::71CW and EC::71CC and their *dnaA*::Tn*10* derivatives, as well as plasmid pRB107, have been described previously (see references in Table I). In the *intR1* strains, chromosome replication is controlled by an integrated R1 plasmid, pOU71, and the initiation frequency from the inserted plasmid can be controlled by temperature. At growth temperatures below 35°C, the copy number is close to that of a wild-type R1 replicon, or about one per chromosome equivalent. At temperatures above 34°C, the copy number gradually increases with temperature, and temperatures above 38°C are lethal due to over-replication of the chromosome. The replication initiated within the integrated plasmid proceeds clockwise and counterclockwise with respect to the genetic map of the *E.coli* chromosome in strains EC::71CW and EC::71CC, respectively.

Media and growth conditions

The bacteria were grown at 34°C (except strains containing *dnaA*::Tn*10*, see below) in M9 medium (Maniatis *et al.*, 1982) supplemented with 0.2% glucose (w/v). Methionine and ampicillin were added to final concentrations of 50 μg/ml and 20 μg/ml, respectively, when appropriate. Optical density was monitored with a Klett-Summerson colorimeter at 640–700 nm. The cultures were harvested in exponential growth phase (~40 Klett units) to maximize the relative amount of replication intermediates. Stationary phase cultures were obtained by continuing the incubations overnight, such that samples were collected ~20 h after the exponential phase samples. The strains containing the *dnaA*::Tn*10* allele were grown either at low (30°C) or high (38°C) temperature. At low temperature, solid medium was used to avoid the spreading of more rapidly growing derivatives through the cultures (for details see Bernander *et al.*, 1991). At 38°C, however, liquid medium was used also for these strains.

Isolation of chromosomal and plasmid DNA

For isolation of chromosome and plasmid replication intermediates, the bacteria were grown as described above. At harvest, a solution of KCN (final concentration 15 mM), sodium azide (15 mM) and EDTA (15 mM) was rapidly added to inhibit on-going replication, and the cultures were immediately chilled. In this way, the yield of replication intermediates was improved when unintegrated plasmids were analysed, whereas the yield from the *intR1* strains was essentially the same whether or not on-going replication was inhibited. Total DNA was isolated essentially as described by Marmur (1961), regardless of whether chromosomal or plasmid DNA was analysed, and the plasmid DNA samples therefore also contained chromosomal DNA.

Single round primer extension and DNA sequencing

Single-stranded DNA primers, 17–25 nucleotides in length, were synthesized on a Gene Assembler Plus (Pharmacia) or on a 394 DNA/RNA synthesizer (Applied Biosystems). The sequence of primer MK9-2, used in all experiments shown, was 5′-TCTGGCTCCCGAACTAATG-CCCCAT-3′. Single round primer extensions were performed using a modified *Taq*-track kit protocol (Promega). Template DNA (~0.1 fmol of chromosomal DNA from the *intR1* strains, 10- to 100-fold more when

plasmid DNA was used) was heat-denatured at 100°C together with excess ^{32}P end-labelled primer, in a reaction mixture containing $MgCl_2$ (10 mM), Tris–HCl (50 mM, pH 9.0) and deoxyribonucleotide triphosphates (35 μM). After chilling on ice for 10 min, *Taq* polymerase (1.5 units) was added and the primer extensions were carried out at 70°C for 10 min. The reactions were terminated by addition of stop solution supplied with the kit. The samples were heat-denatured and electrophoresed on 6% polyacrylamide gels together with sequence ladders obtained with the same primer, using an unintegrated plasmid as template. Plasmid p71CW (Bernander *et al.*, 1989), which was used for the construction of strain EC::71CW, was chosen for this purpose, and the sequencing was performed as recommended for the Sequenase 2.0 kit (USB).

Repeated primer extension

The RPE reactions were carried out in an ordinary DNA thermal cycler essentially as recommended for PCR by the manufacturer (Perkin Elmer Cetus). The reaction mixtures contained $MgCl_2$ (1.5 mM), KCl (25 mM), Tris–HCl (20 mM; pH 8.3), deoxyribonucleotide triphosphates (50 μM), template DNA (see single round extensions above), excess of primer end-labelled with ^{32}P, and *Taq* polymerase (3 units). Up to 30 cycles of denaturation at 94°C, annealing at 65°C and extension at 72°C (1 min at each temperature) were tested. More cycles can easily be run, if necessary. As for PCR reactions, it is crucial that the reaction conditions, mainly the renaturation temperature and the choice of reaction buffer, are carefully optimized for each primer, since we observed either no signal at all or a large array of additional extension products when the reaction conditions were suboptimal (not illustrated).

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