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Initiation of *Escherichia coli* minichromosome replication at *oriC* and at protein n′ recognition sites. Two modes for initiating DNA synthesis *in vitro*

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The start sites for leading and lagging DNA strands were determined *in vitro* with minichromosomes as templates. Fragments from replication intermediates were analyzed by hybridization to single-stranded probes. Leading strand synthesis in the counterclockwise direction was found to originate in or close to (position 248 to −44) the minimal origin. Complementary lagging strand synthesis started several positions to the left outside of *oriC*. The results suggest in addition a concerted synthesis of leading and lagging strands following the *dnaA* directed assembly of initiation proteins at double-stranded *oricC* DNA (pre-replisome). In addition, DNA synthesis could initiate at protein n′ recognition sequences located within and clockwise to the *asnA* gene. Initiation at n′ sites was dependent on protein i activity, whereas leading and lagging strand initiation in the *oriC* region was not affected by protein i. Our results argue against an involvement of the ΦX174-type primosome in the initiation of discontinuous DNA synthesis at *oriC*. An alternative function is suggested.

Key words: dnaA/n′ site/pre-replisome/primosome/protein i

Introduction

A complex series of reactions involved in the process of initiation controls bacterial DNA replication and subsequent cell division. Bidirectional replication of the *Escherichia coli* chromosome requires assembly of two identical replication complexes during initiation. Moreover, primer molecules for leading and lagging strand synthesis at both replication forks, moving in opposite direction, must be generated. So far, neither the primers nor the start sites of all DNA strands are known.

For *E. coli* lagging strand initiation the ΦX174-type primosome, involving the proteins n, n′, n″, i, DnaB, DnaC and DnaG (primase), was an attractive model (Kornberg, 1978). However, neither the replication origin, *oriC*, nor its close vicinity (position −677 to 2006; for nucleotide positions see Buhk and Messer, 1983) support primosome assembly on single-stranded phage DNA *in vivo* (van der Ende *et al.*, 1983). This is in contrast to segments located 2000 to 3300 bp clockwise from *oriC* on both strands (Stuitje *et al.*, 1984). Replication intermediates of minichromosomes isolated *in vivo* did not contain extensive single-stranded regions (Meijer and Messer, 1980). This shows that lagging strand synthesis must be efficiently initiated close to *oriC* by a different mechanism.

A soluble enzyme system has been developed (Fuller *et al.*, 1981) which is able to replicate specifically minichromosomes − plasmids containing and replicating from *oriC*. Recent reconstitution of enzymatic replication exclusively with purified proteins (Kaguni and Kornberg, 1984; Ogawa *et al.*, 1985; van der Ende *et al.*, 1985) allowed the distinction of functional classes of proteins required for minichromosome replication *in vitro*. However, information on the replication products of the *in vitro* system is still limited, and only recent electron-microscopic analysis suggested that continuous leading as well as discontinuous lagging strand DNA synthesis occurred in this system (Funnell *et al.*, 1986).

In order to gain insight into the initial events leading to bidirectional replication we analyzed replicative intermediates of *E. coli* minichromosomes, generated *in vitro*. The extent of DNA synthesis in different regions of the template plasmid, separate for each strand, was analyzed by hybridization to cloned single-stranded fragments. We define start sites for leading and lagging strand DNA synthesis within and close to *oriC*, demonstrating that concerted synthesis of both strands does occur in the *in vitro* system. In addition, we demonstrate lagging strand initiation at *oriC* to be independent of the ΦX174-type primosome under conditions where this primosome does prime initiations at protein n′ recognition sites (n′ sites) distant from *oriC*. An alternative function for the ΦX174-type primosome system in chromosomal replication is discussed.

Results

Minichromosome replication *in vitro* was done using the crude system (fraction II), complemented with DnaA protein (Fuller *et al.*, 1981). This system reflects *E. coli* chromosomal replication as judged from the requirements for proteins DnaA, DnaB, DnaG and RNA polymerase, from its specificity for *oriC*, and from the bidirectionality of replication (Fuller *et al.*, 1981, Kaguni and Kornberg, 1984). We verified these criteria for the system used here (data not shown). In addition, we found the products of the complemented crude system to be predominantly monomeric circular covalently-closed plasmids, observed so far only in the *in vitro* system reconstituted from purified proteins (Funnell *et al.*, 1986).

In most experiments the minichromosome pOC51 was used as a template. pOC51 (Buhk and Messer, 1983) represents a circularized form of the *oriC* region, consisting exclusively of *E. coli* chromosomal DNA, the *oriC* containing and the *asnA* containing *Pst*I fragments (position −1564 to 2566; see maps in Figures 1−3 and 5). Therefore, any possible influence of vector sequences on the initiation process are avoided. In the absence of chain terminators, identical amounts of both single-strands of pOC51 were synthesized, as judged by identical signals of complementary strands in denaturing polyacrylamide gel electrophoresis of replication products after restriction enzyme digests (Figure 1A, tracks 1, 2 and 3). In this analysis we took advantage of the different cleavage patterns of restriction enzymes (protruding versus blunt ends). Complementary single-strands of several fragments could thus be separated due to their different lengths (Figure 1C).

Procedure for the quantitative analysis of replication intermediates

For an analysis of the start sites of DNA strands we quantified the extent to which individual regions of the template plasmid

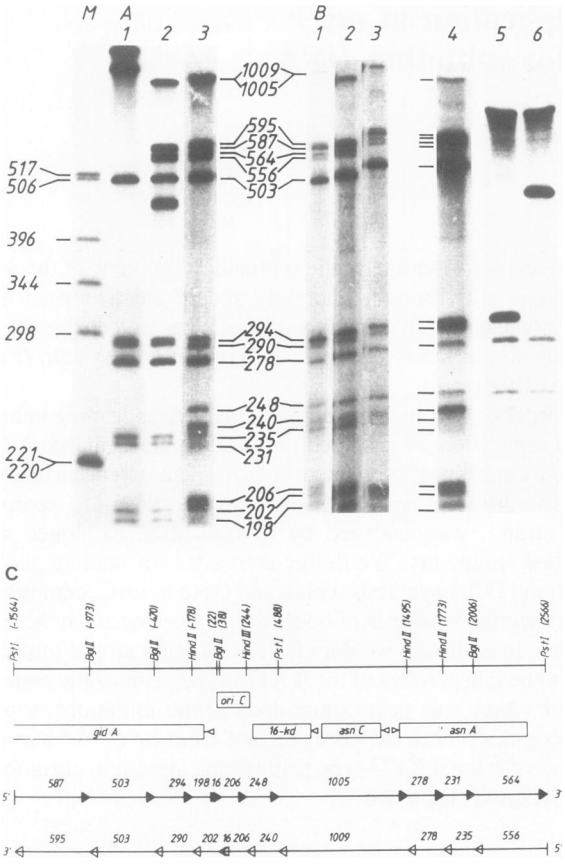

Fig. 1. Denaturing PAGE of replication products (**A**) and replication intermediates (**B**) and expected lengths of fragments (**C**). M: pBR322/*Hin*fI marker; A1: pOC51/*Bgl*II+*Hin*dII; A2: pOC51/*Bgl*II+*Hin*dII+*Pst*I; A3: pOC51/*Bgl*II+*Hin*dII+*Hin*dIII+*Pst*I; B1,2,3: pOC51/*Bgl*II+*Hin*dII+*Hin*dIII+*Pst*I synthesized in the presence of 60 μM, 100 μM, 150 μM ddTTP, respectively; B4: pOC52/*Bgl*II+*Hin*dII+*Hin*dIII+*Pst*I, 150 μm ddTTP; B5: pOC77/*Eco*RI+*Hin*dIII+*Pst*I, 200 μM ddTTP; B6: pOC150/*Eco*RI+*Hin*dIII+*Pst*I, 200 μM ddTTP.

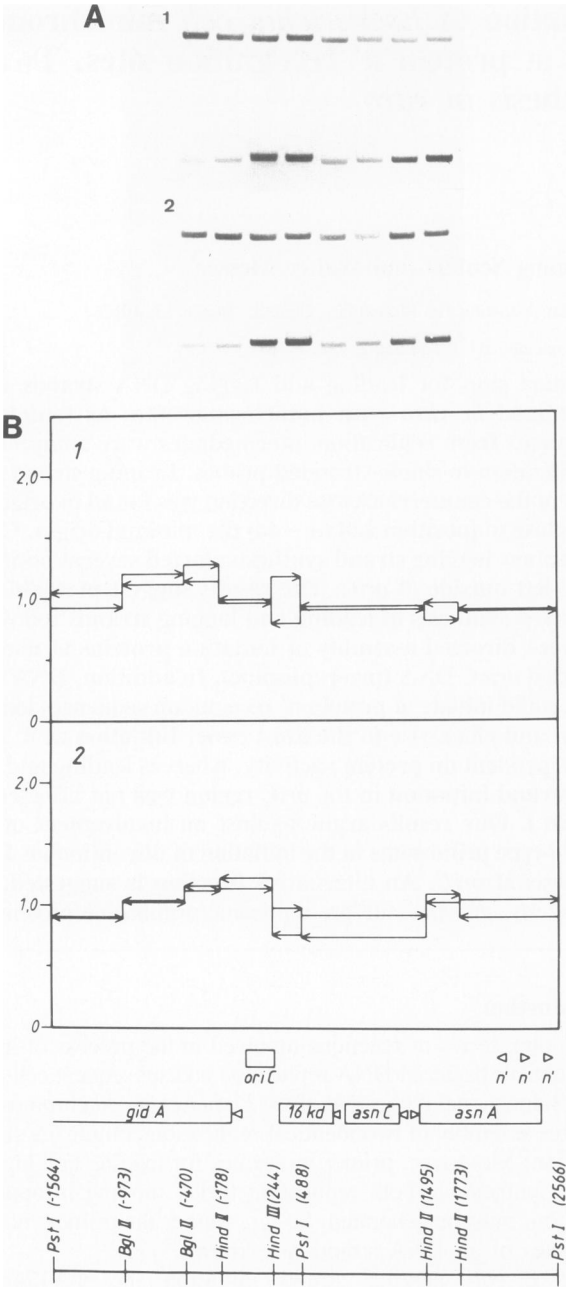

Fig. 2. Hybridization signals of uniformly labeled plasmid pOC51. (**A**) Autoradiogram of plasmid pOC51 labeled by nick-translation (**1**) or by replication *in vitro* (**2**). Labeled DNA was digested with the restriction enzymes shown in the map and hybridized to single-stranded probes constructed from the same fragments. The order of probes on the gels corresponds to the sequence of restriction fragments from left to right. The first one of a pair of probes gives counterclockwise DNA synthesis, the second one clockwise synthesis. The first two tracks of each lane (no signal) are single-stranded vector DNA (M13mp8 and mp9). (**B**) Extent of DNA synthesis in individual regions of the template. Individual signals from (A) were quantified using a laser scanner, the resulting values divided by the number of labeled nucleotides, and expressed in relation to the mean of all signals (=1). Direction of arrows: 5′−3′.

had been replicated during limited elongation. Replicative intermediates were isolated from the *in vitro* system, cut with the restriction enzymes defining the investigated segments, and hybridized to cloned single-stranded fragments of the template plasmid. For the construction of hybridization probes different fragments of the minichromosome pOC51 were inserted into the cloning vector M13mp8 and mp9 (Messing and Vieira, 1982). Resulting hybrid plasmids were identified by restriction enzyme analysis. Single-stranded viral DNAs of M13 hybrid clones were used for hybridization after transfer from an agarose gel to the hybridization membrane (Chomczynski and Qasba, 1984). This procedure gave more reproducible results than simple dot hybridization.

In order to demonstrate that the chosen procedure is suited for quantitative analysis we used plasmid pOC51, labeled either by nick translation (Figure 2.1) or by *in vitro* replication (Figure 2.2) for hybridization. Both procedures are intended to produce uniformly labeled plasmid molecules. The single-stranded vectors M13mp8 and mp9 did not produce a signal indicating specific hybridization. As expected, the signals produced by individual probes were proportional to the length of their plasmid pOC51 inserts. For further evaluation, individual signals were densitometrically scanned and resulting signal intensities were corrected by the number of nucleotides available for labeling and then normalized, thereby facilitating comparison of different experiments. Figure 2A shows the hybridization autoradiograms and Figure 2B the normalized specific hybridization intensities that reflect DNA synthesis in different regions of the minichromosome, resolved at the level of single strands. Uniformly labeled plasmids are expected to yield a straight line at the relative value 1.0, which is in good agreement with the experimental results.

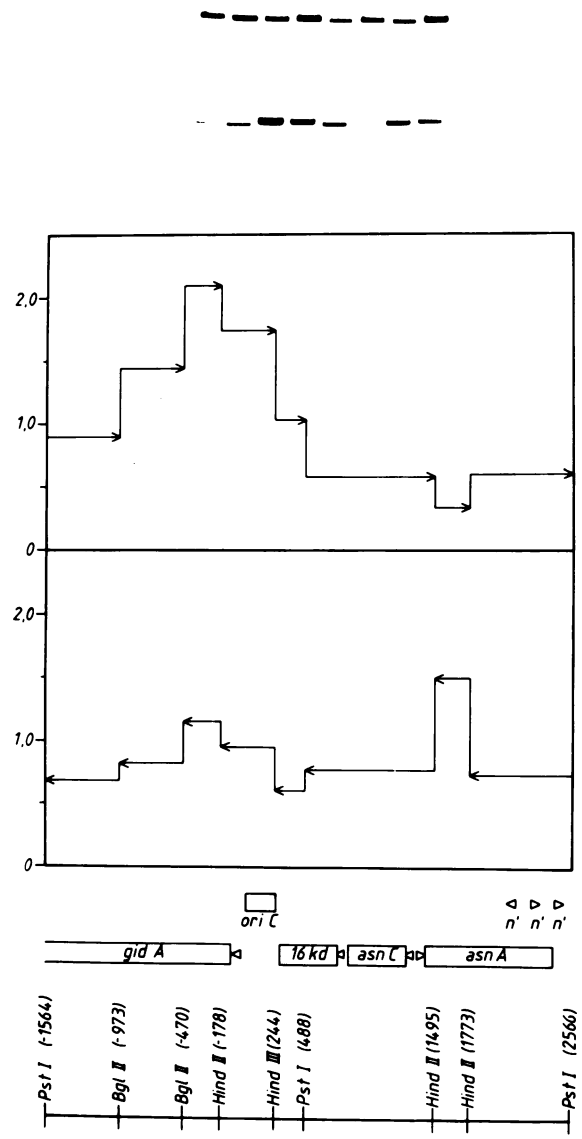

Fig. 3. Hybridization signals of replicative intermediates of pOC51. Replicative intermediates were generated in the presence of 150 μM ddTTP. See Figure 2 for details.

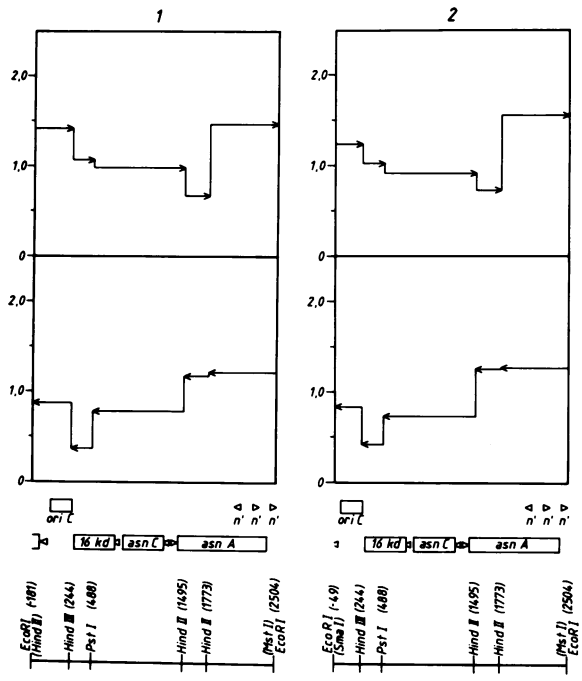

Fig. 4. Hybridization signals of replicative intermediates of pOC150 (1) and pOC77 (2). Replicative intermediates were generated in the presence of 200 μM ddTTP. See Figure 2 for details.

Localization of start sites for DNA synthesis

Start sites for leading and lagging strand synthesis at both replication forks were analyzed using replicative intermediates of the minichromosome pOC51 as template. Figure 3 shows the hybridization autoradiogram and its analysis, which illustrates different amounts of DNA synthesis in individual regions of the template plasmid. There was maximum synthesis in the clockwise direction in a fragment to the left of *oriC* (position −470 to −178). Its decline in the direction of synthesis (clockwise to position −178) reflects the expected termination function of the dideoxynucleotides used in the *in vitro* system for creating intermediates. To the left of this maximum a stepwise increase in the amount of DNA synthesis in 5′−3′ direction was observed. Such a stepwise increase can only occur if there is initiation of DNA synthesis throughout this region, becoming less frequent with increasing distance from *oriC*. The strand synthesized clockwise in this region thus reflects discontinuous DNA synthesis. Moreover, this synthesis in the *gidA* region distinctly predominated complementary counterclockwise synthesis in contrast to balanced synthesis of both strands during non-limited replication, as judged both from hybridization and denaturing polyacrylamide gel electrophoresis of replication products (Figures 2.2 and 1A) and intermediates (Figures 3 and 1B).

For DNA synthesis in the counterclockwise direction, we found the fragment immediately to the right of *oriC* (position 488−244) to be synthesized least frequently. We assign the start for counterclockwise DNA synthesis to the *Hin*dIII(244)−*Hin*dII(−178) fragment, containing most of the minimal origin (Oka *et al.*, 1980), although maximum synthesis occurred in the next fragment. Note that average synthesis in the initiation fragment will be less compared to the following fragment, if DNA synthesis does not start exactly at the beginning of the fragment. Again, replication in the direction of synthesis decreased due to chain termination.

We ascertained DNA synthesis in the counterclockwise direction to originate in or very close to the minimal origin using deletion derivatives of pOC51, pOC77 and pOC150, missing the region to the left of the *Sma*I(−44) or the *Hin*dII(−178) site, respectively (Figure 4). Initiation of counterclockwise synthesis occurred to the right of the *Sma*I(−44) restriction site, as judged by the two step increase of DNA synthesis in the *oriC* region of plasmid pOC77, followed by the expected decrease in the direction of synthesis (Figure 4). Comparing intermediates of plasmid pOC77 to pOC150, we found indentical amounts of DNA being synthesized in corresponding regions. It is obvious that sequences to the left of the *Sma*I site, although being conserved among the origins of Gram-negative bacteria (Zyskind *et al.*, 1983), do not contain information relevant for the initiation of DNA synthesis.

DNA synthesis start at protein n′ recognition sites

Surprisingly, intermediates of plasmid pOC51 exhibited another maximum for counterclockwise DNA synthesis (Figure 3), indicating initiation in a region roughly opposite to *oriC* on the

circular plasmid. By analysis of replication intermediates using denaturing polyacrylamide gel electrophoresis after restriction digests, the start for DNA synthesis in the *asnA* gene was localized to the right of the *Bgl*II(2006) restriction site (Figure 1B, tracks 1, 2 and 3). The single strand (235 bases) of the *Bgl*II(2006)–*Hin*dII(1773) restriction fragment with 5′–3′ orientation pointing leftward (Figure 1C) was synthesized substantially during limited elongation, in contrast to the complementary single strand (231 bases; Figure 1B). The region where the observed DNA synthesis initiated coincides with an n′ site, localized in the *Bgl*II(2006)–*Bam*HI(2191) fragment on the template strand for counterclockwise DNA synthesis (Stuitje *et al.*, 1984). The ΦX174-type primosome is able to synthesize primer molecules immediately near the n′ site where it is assembled (Arai and Kornberg, 1981), in addition to its ability to move in anti-elongation direction on single-stranded DNA and synthesize primers in variable regions.

E. coli chromosomal n′ sites, inserted into the vector M13mp8 (Messing and Vieira, 1982) did not promote any DNA synthesis (pWO8-10, pWO8-10R), in contrast to the minimal origin (Oka *et al.*, 1980) in the same vector (pWO8-13, pWO8-13R), demonstrating a requirement for *oriC* in cis for the observed initiation signal (Table I).

If the start for counterclockwise DNA synthesis in the *asnA* gene is caused by primosome assembly, this initiation event should be prevented by inactivation of any of the primosomal components. Since there is no *oriC* plasmid replication *in vitro* in the absence of the primosomal proteins DnaB, DnaC or DnaG (Kaguni and Kornberg, 1984), we analyzed replicative intermediates of plasmid pOC51 generated in the presence of anti-protein i antibodies.

The efficiency of non-limited replication was not affected by the absence of protein i (114 pmol DNA synthesized in the presence of anti-protein i antibodies versus 103 pmol with neutral antibodies). However, the initiation pattern of replicative intermediates was different after inactivation of protein i. Initiation of counterclockwise DNA synthesis in the *asnA* gene did not occur, whereas initiation of continuous counterclockwise synthesis in or near *oriC* was not affected nor was clockwise synthesis (Figure 5). Virtually identical to the results obtained with intermediates synthesized in the presence of protein i we found a stepwise increase in the amount of clockwise DNA synthesis in the *gid*A region, indicating several positions of initiation typical for discontinuous strand synthesis. Thus, neither leading nor lagging strand initiation in the *oriC* region was dependent on protein i activity, in contrast to initiation at an n′ site.

Influence of different locations on n′ site recognition

DNA synthesis initiated in the presence of protein i near an n′ site localized approximately opposite to *oriC* on the template plasmid pOC51. Plasmids pOC77 and pOC150 contain the same n′ site which does, however, not result in an obvious initiation signal (Figure 4). Apparently, an n′ site located close to the left of *oriC* on the template strand for continuous DNA synthesis does not promote initiation efficiently.

To analyze this position effect, we isolated replicative intermediates of plamsid pOC52 (Figure 6), carrying *asnA, asnC* and part of the 16 kd gene in inverted orientation with respect to plasmid pOC51. We again found inactivity of the n′ site located on the template strand for continuous (clockwise) synthesis, in this case close to the right of *oriC*. But two n′ sites on the complementary strand in the *Hin*dII(1281)–*Pst*I(488) fragment obviously did promote initiation of counterclockwise synthesis

Table I. Template activity of different plasmids

Plasmid	Inserted fragment	DNA synthesis
M13mp8 RF	none	5
pWO 8-10	*Pst*I(488)–*Pst*I(2566)	4
pWO 8-10 R	as pWO 8-10, reverse orientation	5
pWO 8-13	*Sma*I(−44)–*Acc*I(285)	222
pWO 8-13 R	as pWO 8-13, reverse orientation	227

(Figure 6). Using denaturing polyacrylamide gel electrophoresis to analyze replicative intermediates, we found significantly more counterclockwise synthesis in the *Pst*I(488)–*Hin*dIII(244) fragment (244 bases) than clockwise synthesis (248 bases) (Figure 1B, track 4). The opposite was true for intermediates of plasmid pOC51 (Figure 1B, tracks 1, 2 and 3).

We conclude that n′ sites promote initiation of DNA synthesis only when they are localized in regions of the template plasmid which become intermittently single-stranded during replication. This may occur either because they are localized on the template for lagging strand synthesis as in the case of pOC52, or because of a location downstream of a replication fork which was blocked by a chain terminator, followed by local unwinding, as in pOC51.

Discussion

Initiation of replication in the oriC region

We resolved early events during the initiation of bidirectional replication at *oriC*. Counterclockwise leading strand DNA synthesis was found to start within or very close to the minimal origin (position 248 to −44). Initiation of clockwise DNA synthesis occurred throughout the region to the left outside *oriC*, indicating discontinuous replication of this strand. These start sites correspond to RNA-DNA transition sites mapped *in vivo* (Hirose *et al.*, 1983; Kohara *et al.*, 1985), demonstrating identical initiation patterns *in vitro* and *in vivo*. Our data do not allow the definition of a precise start for the leading strand of the clockwise replication complex starting at *oriC*. Instead, since there was a progressive reduction of clockwise synthesis starting to the left of *oriC* and continuing across *oriC* (Figures 3–5), the data are compatible with the suggestion that the lagging strand from the counterclockwise moving replication complex becomes the leading strand of the clockwise moving fork (Hirose *et al.*, 1983).

Our results, as well as the *in vivo* results (Kohara *et al.*, 1985), are in conflict with experiments reported by Tabata *et al.* (1983), who defined a bidirectional replication start *in vitro* in the vector sequence of a joint replicon 67–128 bp to the left of the minimal origin. The analysis was based on the amount of radioactivity incorporated into double-stranded restriction fragments. Due to the complex pattern of start sites for leading and lagging strand synthesis found here, the analysis of double-stranded fragments gives misleading results; e.g. maximum incorporation into double-stranded fragments in our experiments is between positions −470 and −178, clearly misplacing the leading strand start site.

The observation that leading and lagging strand DNA synthesis in the counterclockwise replication direction are reduced with increasing distance from *oriC*, with the same kinetics, demonstrates that a block of leading (counterclockwise) DNA synthesis by a chain terminator eventually stops lagging strand (clockwise) synthesis as well. This result supports the model of a dimeric replication complex for synchronous parallel synthesis

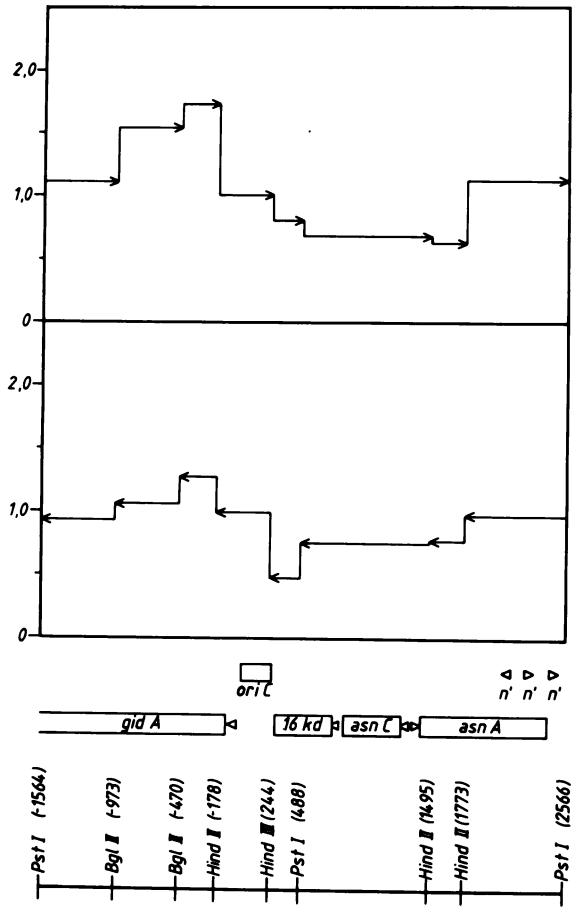

Fig. 5. Hybridization signals of replicative intermediates of pOC51 in the absence of protein i activity. Replicative intermediates were generated in the presence of 150 μM ddTTP and anti-protein i antibodies. See Figure 2 for details.

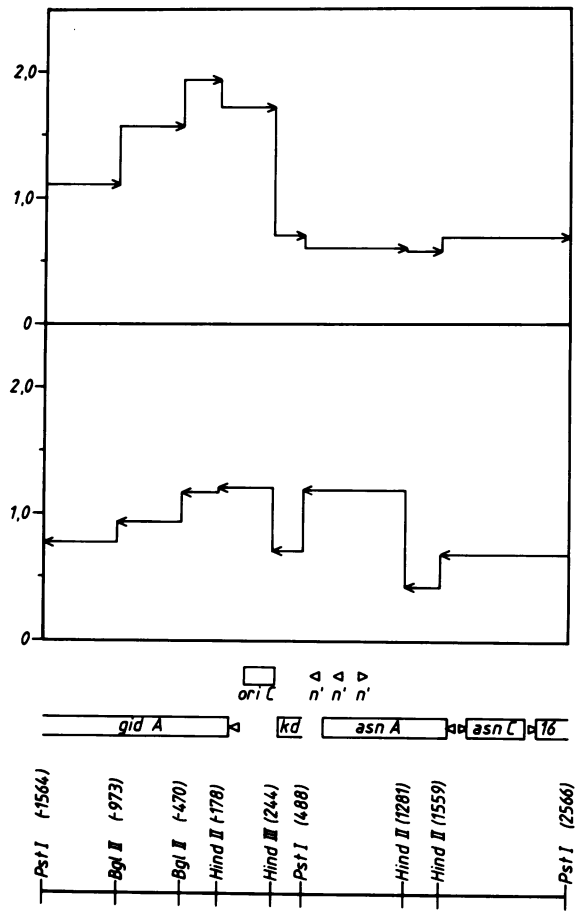

Fig. 6. Hybridization signals of replicative intermediates of pOC52. Replicative intermediates were generated in the presence of 150 μM ddTTP. See Figure 2 for details.

of leading and lagging strands, developed for T4 replication (Sinha *et al.*, 1980) and proposed for *E. coli* replication (Kornberg, 1982). It is also compatible with the kinetic properties of DNA polymerase III holoenzyme (McHenry, 1986). In addition, there is a plausible explanation for the excess of clockwise (lagging strand) over counterclockwise (leading strand) synthesis in this region. A block of leading strand synthesis may allow replication of lagging strand to continue for a certain distance, possibly because the Okazaki fragment ahead of the replication fork may be already primed and can finish synthesis.

We found leading and lagging strand initiation at *oriC* to be independent of the primosomal protein i. The ΦX174-type primosome is, therefore, not responsible for *E. coli* lagging strand initiation. Baker *et al.* (1986) reached a similar conclusion from the protein n′ independent unwinding of minichromosomes during initiation *in vitro*. Thus we have to conclude that an initiation complex consisting of DnaB, DnaC and DnaG proteins is assembled at double-stranded *oriC* DNA with the help of DnaA protein. We call this complex a pre-replisome. In conjunction with DNA polymerase III holoenzyme, this complex is able to direct the concerted synthesis of leading and lagging DNA strands.

Initiation at n′ sites

In addition to initiation of leading and lagging strand synthesis in the *oriC* region, we observed initiation of DNA synthesis at chromosomal n′ sites, located in regions potentially becoming single-stranded during replication. Protein i dependence of this process allowed unequivocal distinction between initiation at *oriC* and at n′ sites. Initiation at n′ sites required the presence of *oriC* on the template plasmid, and – on the template strand for leading strand synthesis – a certain distance between *oriC* and the n′ site. Presumably, the helicase activity of DnaB protein, together with DNA gyrase (LeBowitz and McMacken, 1985; Baker *et al.*, 1986) continues to unwind the DNA ahead of a stalled fork, exposing n′ sites in single-stranded form.

This observation suggests a possible function for n′ sites and primosomal proteins. Replication terminated prematurely for any reason might be reinitiated by assembly of a ΦX174-type primosome at an n′ site in the single-stranded region, as observed in this study with minichromosomes *in vitro*.

In addition to this auxiliary function for chromosomal replication, proteins i, n, n′, n″ and the n′ sites are possibly involved in replication in the absence of *de novo* protein synthesis (stable replication), that can be induced in *E. coli* wild-type cells by various treatments (Kogoma and Lark, 1975). It has been shown recently that protein i is encoded by the *dnaT* gene (Masai *et al.*, 1986), characterized by mutations which prevent induction of stable replication (Lark and Lark, 1978). The temperature-sensitive phenotype of *dnaT* mutants (Lark and Lark, 1978; Masai *et al.*, 1986) might be due to an absolute requirement for the postulated auxiliary function of the ΦX174-type primosome at temperatures approaching the upper limit for growth.

Materials and methods

General techniques

Cloning procedures, electrophoretic techniques, nick translation, and preparation of plasmid and phage DNA were performed as described (Maniatis *et al.*, 1982).

Denaturing polyacrylamide gel electrophoresis

Denaturing gels were 5% polyacrylamide, 7 M urea. Electrophoresis was in TBE buffer (Maniatis *et al.*, 1982). Gels were fixed with 10% acetic acid, washed with water, dried, and autoradiographed on Kodak XAR-5 film.

Replication in vitro

oriC dependent replication was performed as described by Fuller *et al.* (1981) with minor modifications (Lother *et al.*, 1985); KCl was 20 mM. Strain WM433 (*E. coli* B/r, *arg*-28, *deoB*23, *dnaA*204, *gal*-11, *his*-47, *hsdS*-K12, *lac*-11, *leu*-19, *mal*, *met*-55, *pro*-19, *rbs*, *rpsL*56, *sul*-1, *thyA*59, *trp*-25) was used for the preparation of the DnaA deficient receptor extract (fraction II). The DnaA complementing extract (fraction III) was prepared from strain WM1492 (*E. coli* K12, *lacI*^{Q}L8, *recA*56, *srlC*300::Tn*10*, pLSK5) harboring the plasmid pLSK5 (H.Kunze, C.Kücherer, H.Lother and W.Messer, submitted) in which transcription of the *dnaA* structural gene is controlled by the *tac* promoter. Induction was for 2.5 h at 37°C by adding 1 mM IPTG to the culture at a cell density of 1×10^8 ml^{-1}.

For generation of replicative intermediates the chain terminator ddTTP at a final concentration of 150 or 200 μM (see figure legends) was added to 100 μl of standard mixture. DNA used for hybridizations was labeled for [α-^{32}P]dATP (5 μM, 50 μCi/100 μl). DNA was isolated from the assay after digestion with RNase A (20 μg/ml, 30 min at 30°C) by phenolization. Prior to ethanol precipitation non-incorporated nucleotides were removed by passage through Sephadex LH-60.

Analysis of replication intermediates by hybridization

DNA recovered from the *in vitro* replication assay was digested with the restriction enzymes defining the borders of the hybridization probes (*Bgl*II, *Pst*I, *Hin*dII, *Hin*dIII in the case of pOC51 and pOC52) in the presence of RNase A (50 μg/ml), phenolized and precipitated with ethanol (Maniatis *et al.*, 1982).

Single-stranded hybridization probes (300 ng) were subjected to electrophoresis in a 0.8% agarose gel and transferred to the hybridization membrane (Gene Screen, New England Nuclear) by an alkaline procedure (Chomczynski and Quasba, 1984). Hybridization buffer contained 50% deionized formamide, 300 mM NaCl, 30 mM Na-citrate, 50 mM Na_2HPO_4/NaH_2PO_4 (pH 6.5), 0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin, 0.2 mg/ml denatured salmon sperm DNA. Pre-hybridization with hybridization buffer was for at least 3 h. More than 200 000 c.p.m. of *in vitro* replicated, restriction enzyme digested, DNA in hybridization buffer were hybridized at 42°C in a sealable plastic box with slow shaking for 30–40 h.

The membrane was washed twice in 60 mM Tris–HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA at room temperature for 5 min, once in 60 mM Tris–HCl, pH 8.0, 300 mM NaCl, 2 mM EDTA, 1% SDS at 47°C for 30 min, and once in 6 mM Tris–HCl, pH 8.0, 30 mM NaCl, 0.2 mM EDTA at room temperature for 30 min. The dried membrane was exposed (Kodak XAR-5 film) and the autoradiograph densitometrically scanned (Ultroscan Laser Denistometer, LKB).

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