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The 245 base-pair *oriC* sequence of the *E. coli* chromosome directs bidirectional replication at an adjacent region

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ABSTRACT

The replication origin of the E.coli K-12 chromosome has been isolated as autonomously replicating molecules(oriC plasmid), and the DNA region essential for replicating function(oriC) has been localized to a sequence of 232-245 base-pairs(bp) by deletion analysis. In this report, the functional role of oriC was analysed by using an in vitro replication system and various OriC$^+$ and OriC$^-$ plasmids previously constructed. The results obtained were summarized as follows:(1) The oriC sequence contained information enough to direct bidirectional replication. (2) The actual DNA replication began at a region near, but outside, oriC and progressed bidirectionally. (3) Initiation of DNA synthesis at the specific region required the dnaA-complementing fraction from cells harboring a dnaA-carrying plasmid.

INTRODUCTION

A DNA region carrying autonomously replicating function(oriC) has been isolated from the replication origin of the E.coli K-12 chromosome (1-5). Since DNA segments carrying oriC can be maintained in E.coli cells as plasmids, the minimal size of oriC was determined by deletion analysis. As a consequence, oriC was successfully localized to a sequence of minimum 232 base-pairs(bp) and maximum 245 bp in length(6). We have further analysed the sequence organization in oriC by introducing various mutations and provided evidence that multiple recognition sequences have been arranged precisely in oriC(6-9). Kornberg's group has recently developed an in vitro replication system(10), and shown by electron microscopy that oriC plasmids replicate bididirectionally around oriC in the in vitro system(11). In this report, we have investigated the functional role of the 232-245 bp oriC sequence more precisely by using a similar in vitro system and various OriC$^+$ and OriC$^-$ plasmids previously constructed.

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MATERIALS AND METHODS

oriC plasmids

oriC plasmids used were pTSO182(6), pTSO290(6), pTSO236(6), pKA22(8) and pMY129(constructed by M.Yamada). The chromosomal moieties carried by these plasmids are shown in Fig1. These oriC plasmids are recombinants of pBR322, and can replicate in both PolA$^-$ and PolA$^+$ cells, whereas pBR322 replicates only in polA$^+$ cells(5-9). By assaying the replication ability in PolA$^-$ cells, a number of OriC$^-$ mutants have been identified. OriC$^-$ mutants used in this study were pTSO279(7 bp deletion at the BglII site)(7), pTSO202(4 bp insertion at the BamHI site)(6), pTSO207(15 bp deletion at the BamHI site)(6), pTSO190(4 bp insertion at the HindIII site)(6), pTSO209(5 bp deletion at the HindIII site)(6), pKA32(3 bp insertion at the AvaII site)(8) and pKA62(3 bp deletion at the AvaII site)(8)(see Fig.1 for restriction sites).

Enzyme fractions

The enzyme fractions used were essentially identical to those described by Fuller et al.(10), except for their sources. Fraction II was prepared either from E.coli HMS83 (F$^-$ polA polB rha lys thyA lacZ str)(12) or JE107251

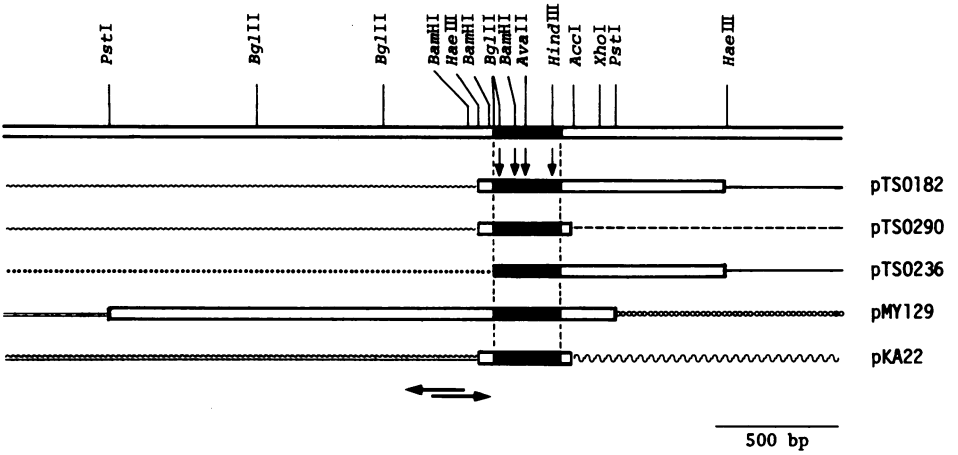


Fig.1 Restriction maps of oriC plasmids. Filled and open boxes represent the oriC sequence and remaining chromosomal moieties, respectively, and other lines indicate pBR322 moieties. Differences in pBR322 sequences adjacent to chromosomal moieties were shown by changing the shapes of lines. Relevant restriction sites in the vicinity of oriC were indicated, and sites at which OriC$^-$ mutations had been introduced were shown by vertical arrows. Horizontal arrows below the map indicate the approximate region where bidirectional replication starts.

(HfrP4X8 dnaA725 thy str)(13), and fraction III(a dnaA complementing fraction) was prepared from E.coli JE6087(F⁻Δ proB lac) which harbors a dnaA-carrying plasmid pSY405(13). The chromosomal gene carried by pSY405 is assumed to be only dnaA, as additional chromosomal sequences in pSY405 were about 400 bp at the 5'-side and a part of the dnaN gene at the 3'-side of the dnaA gene.

Reaction conditions

The reaction mixture(25 µl/tube) contains 40 mM Hepes buffer(pH 7.6), 11 mM Mg acetate, 2 mM ATP, 500 µM each of CTP, GTP and UTP, 21 mM creatine phosphate, 2.5 µg creatine kinase, 50 µM each of dATP, dGTP, and dCTP, 20 µM dTTP containing 5-methyl(^{3}H)dTTP(220 cpm per pmol of total dTTP), 1.25 µg bovine serum albumin, and 5% polyvinyl alcohol(W/V). 0.2 µg of oriC plasmids, 0.12 mg of fraction II from either HMS83(polA polB) or JE107251(dnaA725) and 0.05 mg of fraction III were added. When

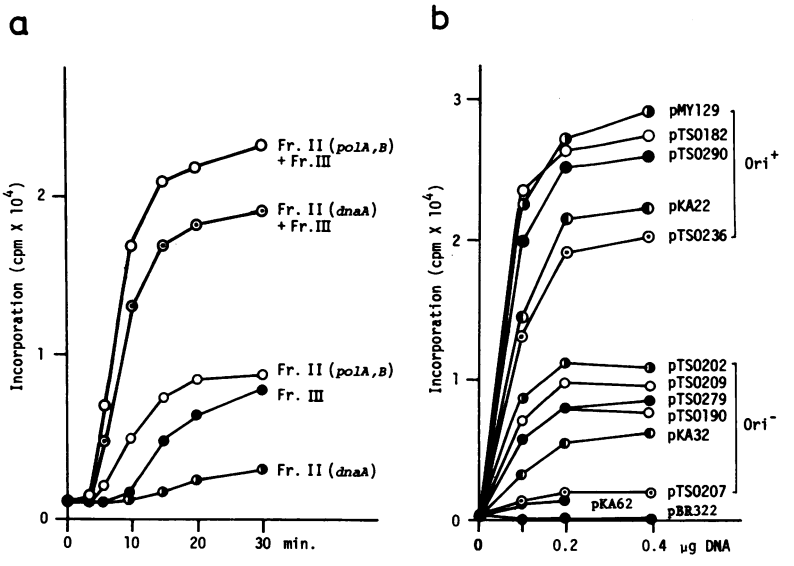
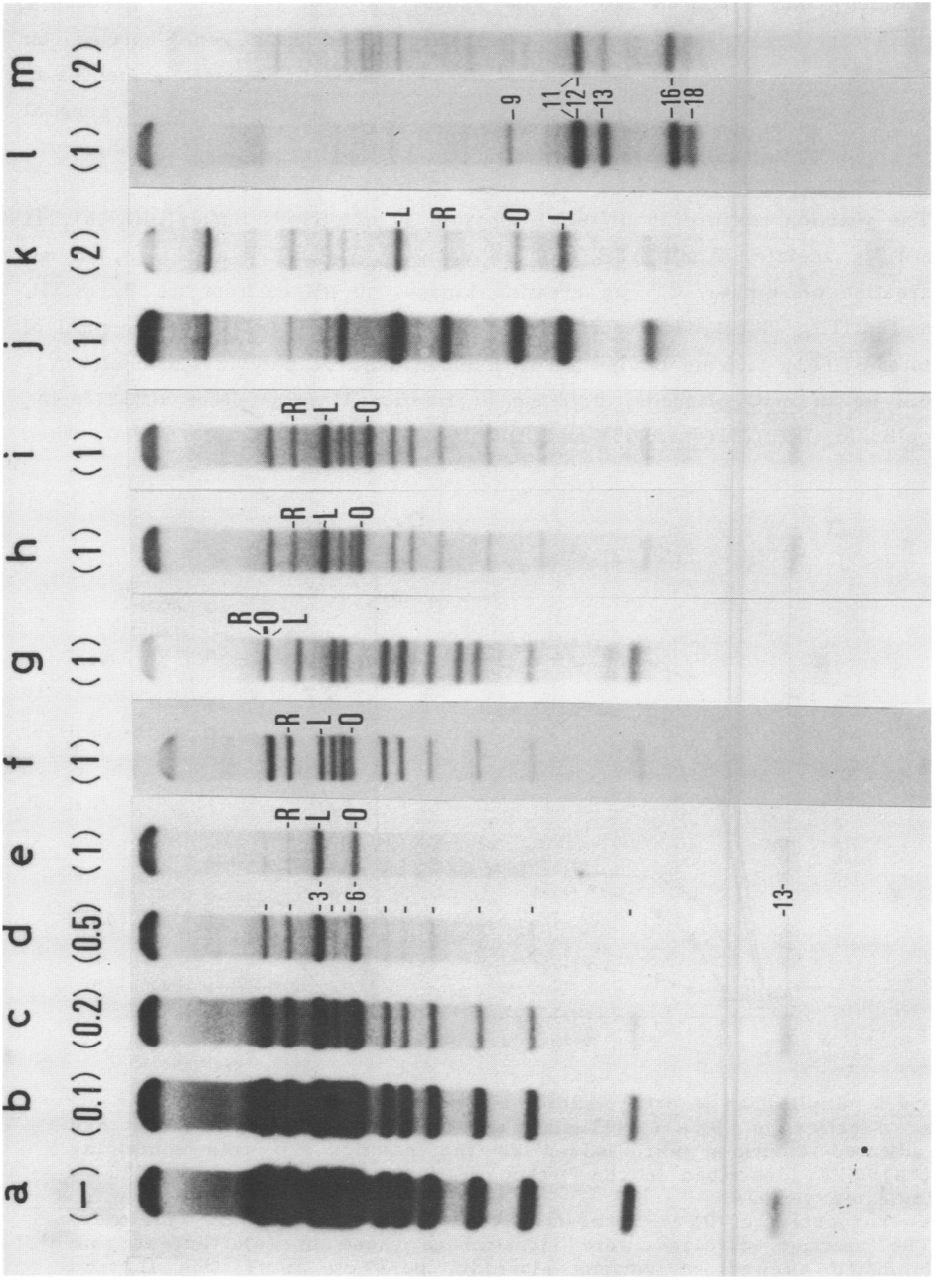


Fig.2 Replication of oriC plasmids in the in vitro system.
a. Kinetics of DNA synthesis. pTS0182(OriC⁺)(0.2 µg each) and indicated fractions were added to the reaction mixtures containing (^{3}H)dTTP, described in the Method section, and incubated at 30°C at indicated periods.
b. The extent of DNA synthesis directed by OriC⁺ and OriC⁻ plasmids. The reaction mixtures were identical to those in (a), except that indicated amounts of various plasmids, 0.12 mg of fraction II from HMS83(polA polB) and 0.05 mg of fraction III were added. Incubation was carried out for 30 min. at 30°C.


a b c d e f g h i j k l m
(—) (0.1) (0.2) (0.5) (1) (1) (1) (1) (1) (1) (2) (1) (2)
-R
-L
-O
-3-
-6-
-13-
-9
11
-12-
-13
-16-
-18

fraction II from the dnaA strain was used, dnaA protein was inactivated by incubation for 10 min at 37°C in the presence of 5 mM ATP, 50 mM creatine phosphate and 0.2 mg/ml creatine kinase. Incubation was carried out at 30°C for indicated periods. Reaction was terminated by 5% TCA, precipitates were mounted on glass filters, and radioactivity was determined.

Analysis of reaction products

dNTPs in the above reaction mixtures were replaced by 10 μM each of four (α^{32}P)dNTPs, and 1 μM to 20 μM ddTTP were added. Incubation was made for 10 min at 30°C, unless otherwise noted. Reaction was terminated by adding SDS to 1%, and after shaking with 80% phenol, aqueous layers were passed through Agarose A5m columns(0.6 cm x 20 cm). The DNA fraction was collected, and aliquots were digested with restriction enzymes. Resulting fragments were resolved by 5% polyacrylamide gel electrophoresis, and identified by autoradiography.

RESULTS

The extent and time course of DNA synthesis directed by various oriC plasmids are shown in Fig.2. As has been observed by Fuller et al.(10), incorporation by OriC$^+$ plasmids steeply increased after a few minutes lag, and reached to a plateau of which the level was about 50-70% of the template added(Fig.2 a). In contrast, the extent of DNA synthesis with mutant plasmids was much lower than that of the wild type, although their levels depended on the type and positions of mutations(Fig2 b). The result is consistent with that of the in vivo OriC assay(6-8). By omitting fraction III, the extent of DNA synthesis directed by OriC$^+$ and OriC$^-$ plasmids was markedly reduced, respectively.

Fig.3 DNA synthesis directed by oriC plasmids in the presence of a chain terminator ddTTP. The composition of reaction mixtures and assay conditions were described in the Method section(Analysis of reaction products). (a - e): EcoRI-XhoI-HinfI digests of DNA formed on pTS0182. (f): EcoRI-XhoI-HinfI digest of DNA formed on pTS0182 without fraction III. (g): SalI-XhoI-HinfI-PstI digest of DNA formed on pTS0279. (h): EcoRI-XhoI-HinfI digest of pTS0202. (i): EcoRI-XhoI-HinfI digest of pTS0209. (j,k): PstI-HindIII-BglII-BamHI digests of DNA formed on pMY129. (l,m): HaeIII-SalI digests of DNA formed on pTS0182. The ddTTP/dTTP ratios in reaction mixtures were indicated in parentheses above the columns. Labels R,L and O by the side of columns indicate fragments generated from the right and left of oriC, and those containing oriC, respectively. Numbers between columns d & e and l & m respectively correspond to those of restriction fragments given at inner and outer rings of circular restriction maps in Fig.5.

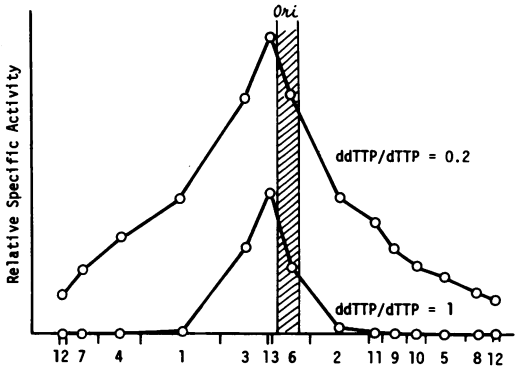


Fig.4 The starting point of bidirectional replication. Relative specific activities of fragments shown in Fig.3 c & e were estimated from the label and chain length, and plotted along the linear map of pTSO182, opened at fragment 12. Shaded boxes represent oriC. Fragment Numbers in horizontal axis correspond to those on inner ring in Fig.5.

To identify the site and direction of replication, incorporation was limited by adding a chain terminator ddTTP together with (α-^{32}P)dNTPs. The products were digested with appropriate restriction enzymes, and labelled fragments were identified by polyacrylamide gel electrophoresis, followed by autoradiography. Restriction patterns generated from pTSO182 at increasing levels of ddTTP are shown in Fig.3 a-e. At higher ratios of ddTTP/dTTP, only a few fragments were heavily labelled. The specific activities of fragments estimated from the label and length were plotted along the sequence of pTSO182, as shown in Fig.4. It is clear that the replication starts from the left side of oriC and proceeds symmetrically in both directions. Essentially identical labelling patterns were obtained without ddTTP, but by a brief incubation at reduced substrate concentrations. The bidirectional replication at the left side of oriC was also observed with other OriC$^+$ plasmids, pTSO236, pTSO290, pKA22 and pMY129. As an example, labelling patterns obtained from pMY129 are shown in Fig.3 j,k. Since these OriC$^+$ plasmids carry different sequences in the flanking regions of oriC(see Fig.1) and the extent of DNA synthesis was reduced only by mutations introduced in oriC, it is obvious that information directing bidirectional replication at its adjacent region is contained within the oriC sequence.

To investigate the initial incorporation site more precisely, the products synthesized with pTSO182 at higher ddTTP concentrations were digested into more small pieces. Typical autoradiograms are shown in Fig.3 l,m.

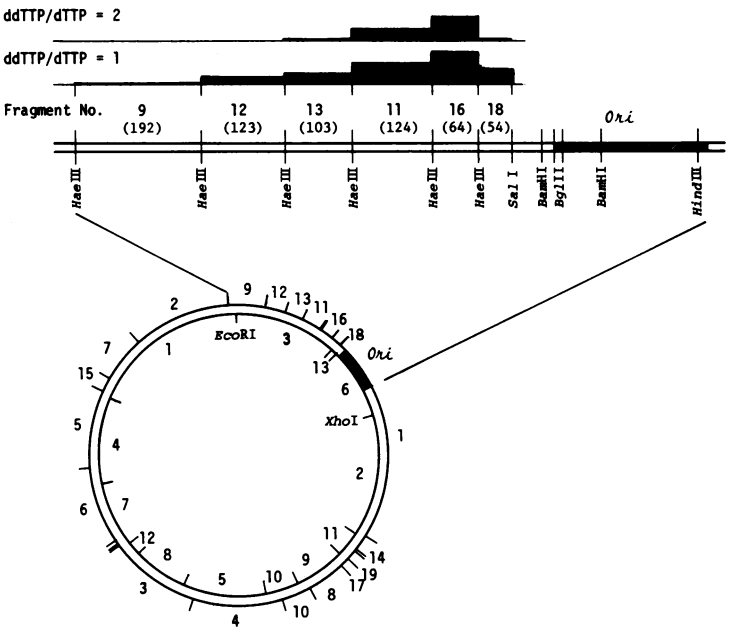


Fig.5 Circular restriction maps of pTS0182, and labelled fragments at ddTTP/dTTP ratios=1 & 2. Fragments generated by digestions of EcoRI+XhoI+HinfI and SalI+HaeIII were numbered in order of decreasing sizes, and indicated on inner and outer rings, respectively. Relative specific activities of labelled fragments shown in Fig.3 l & m were estimated and indicated by histograms on the restriction map. Height of histograms represents relative specific activity. Numbers in parentheses are chain length(bp) of fragments.

Labels were mainly identified in five bands at ddTTP/dTTP=1, and in two bands at ddTTP/dTTP=2. The labelling patterns were not changed by prolonged incubation, and identical results were obtained by using fraction II from either the HMS83(polA polB) or dnaA strain. Taking into account the map positions of fragments in bands, the relative specific activities of fragments roughly estimated are shown by histograms on the restriction map of Fig.5. The specific activity was the highest in fragment 16 and decreased toward left. At ddTTP/dTTP=2, little radioactivity was detected in fragment 18. It is therefore most likely that the initial DNA incorporation predominantly begins from the right of fragment 16, possibly in the fragment 18 area(67 to 122 bp outside of the oriC sequence), and proceeds toward left. This area is not the E.coli chromosomal, but the pBR322 sequence. Note that in the above analysis, newly formed chains are

identified as a proper band only when traversed a certain restriction area, and those initiating or terminating within the area are not identified. The initiation site can be localized precisely by digesting into smaller pieces.

Fraction II alone from the HMS83 strain has a considerable activity to generate DNA synthesis(see Fig.2 a). When the products were analysed, however, a striking difference was found in the labelling pattern. As shown in Fig.3 f, all the regions of plasmids had been labelled rather uniformly even at higher ddTTP concentrations. Under the conditions, the level of incorporation by OriC$^-$ plasmids decreased further and pBR322 vector does not promote any incorporation, indicating that the labelling of DNA by fraction II alone depends on the oriC sequence. It is therefore evident that DNA initiation is restricted to a specific region by the component(s) supplemented by fraction III. Since the addition of fraction III increases the level of DNA synthesis, we must assume that the component(s) contained in fraction III has at least two functions; one stimulating the overall initiation of DNA replication and the other restricting the DNA initiation site. The mostly likely component supplemented by fraction III is dnaA protein. If this is the case, the latter function appears to require a relatively large quantity of dnaA protein, for fraction II of the HMS83 strain contains a certain level of dnaA protein.

As three OriC$^-$ plasmids, pTSO202, pTSO209, and pTSO279, each containing mutation at different part of oriC, directed significant levels of DNA synthesis(see Fig.2 b), synthesized products were analysed as above. The labelling patterns were rather similar to those generated by OriC$^+$ plasmids but without fraction III, although the labels in fragments generated from the left of oriC to oriC area appeared to be slightly heavy(Fig.3 g-i). The result implies that the oriC sequence is associated with the function of the component which restricts the DNA initiation site.

DISCUSSIONS

Analysis of labelling patterns of oriC plasmids in the in vitro replication system clearly indicated that replication started at a region near, but outside, the 245 bp oriC sequence and proceeded bidirectionally. Furthermore, similar labelling patterns were obtained with three Ori$^+$ plasmids, pTSO182, pTSO236, and pMY129, differing in the sequences at the actual initiation sites, and the replicating function was reduced by mutations introduced only within oriC. We thus concluded that the 245 bp oriC sequence contained information enough to direct bidirectional replication at its adjacent region.

Participation of rifampicin-sensitive RNA polymerase in the replication of oriC plasmids has been demonstrated(11). It is therefore reasonable to assume that the leftward DNA synthesis is preceded by primer RNA synthesis initiated at an upstream region(at the 5'-side), most likely within oriC, and switched to DNA at the outside of oriC by the action of the component supplemented by fraction III. As mentioned previously, fraction II alone from the HMS83 strain had the ability to promote oriC-dependent DNA synthesis, but lacked the activity initiating DNA synthesis at the specific region. At a low level of the supplemented component, the RNA-DNA transition may take place randomly. The most likely candidate of the supplemented component is dnaA protein. Since the apparent RNA to DNA transition site is outside oriC, some mechanism must be present by which the dnaA gene product determines the distance from the oriC sequence. A more precise interpretation of the result should be made by detecting primer RNA and the exact site of RNA to DNA transition. Experiments along this line are in progress.

Several genes have been identified as essential for initiation of DNA replication, but little is known about the functional roles of these gene products. Our sequence modification experiments(6-9) have demonstrated that oriC contains multiple important sites in which even a single base substitution affects OriC function and silent sites in which insertion or deletion of short sequences, but not base substitution, destroys OriC function. We correlated these sites to recognition sequences and spacer sequences, respectively, and speculated that multiple recognition sequences have precisely been arranged on oriC(6-9). Our model for the bidirectional replication directed by oriC is as follows. An initiation complex is constructed from multiple components under the direction of the oriC sequence, and initiates leftward transcription probably within oriC. The resulting primer RNA is switched to DNA at an adjacent region of oriC. The formation of such a leading strand would result in opening of the duplex DNA and induce synthesis of a lagging strand in the reverse direction. The lagging strand may become the leading strand for the rightward replication. This model is supported by our preliminary experiment in which labelled strands at higher ddTTP concentrations were fractionated by separated strands of template. We assume that long specific sequences are not involved in the switching of RNA to DNA and the initiation of the lagging strand, as the similar labelling patterns were obtained with three Ori^+ plasmids differing in the sequences adjacent to oriC.

If primer RNA is indeed initiated within oriC by rifampicin-sensitive RNA polymerase, promoter must be present in oriC. Our attempts, however, to identify promoter within oriC by using a promoter-cloning vector(14) and by an in vitro transcription system were unsuccessful, in contrast to the report by Lother & Messer(15) who assigned two promoters in the opposite directions in oriC by in vitro transcription experiments. The initiation of primer RNA synthesis in oriC is assumed to be highly regulated, so that such a promoter could be identified only in a very specified system. According to the result in this paper, the RNA-DNA transition occurs at the outside of oriC. Two RNA-DNA transition sites, however, had been assigned within oriC by Okazaki et al.(16) based on analysis of in vivo products. These sites could be those of lagging strands. Another possibility will be that the initiation site in the in vitro system is different from that of the in vivo system. It would be necessary to re-investigate the transition sites in the in vitro system.

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