Functional Analysis of Minichromosome Replication: Bidirectional and Unidirectional Replication from the Escherichia coli Replication Origin, oriC

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Replicating molecules of minichromosomes pCM959 and pOC24 were analyzed by electron microscopy. Replication of pCM959 proceeded bidirectionally from the replication origin, oriC, in about 60% of the molecules; the rest of the molecules replicated unidirectionally in either direction. pOC24, in which deoxyribonucleic acid to the right (clockwise) of the oriC segment is deleted, seemed to replicate predominantly unidirectionally counterclockwise from oriC.

Chromosomal replication of Escherichia coli K-12 starts from a fixed site on the chromosome, the replication origin oriC, and proceeds bidirectionally toward the terminus (2, 13, 21). This replication origin has been cloned on minichromosomes, plasmids which contain oriC as their only origin of replication (15, 17, 27, 28), on λ asn transducing phages (18, 26), and on F′ plasmids (7, 12, 25).

The replication origin was localized within the minichromosomes by deletion analysis (15, 17). A DNA segment of 422 base pairs, defined on one side by a BamHI recognition site and on the other side by a XhoI recognition site (see Fig. 1) was found to be necessary and sufficient for the replication of adjacent DNA. The nucleotide sequence of this oriC segment was determined (15, 23). Recently, the minimum DNA segment required for minichromosome replication has been determined more precisely by analysis of deletions introduced from either end of the 422-base pair fragment (20). This minimum segment constitutes 232 to 245 base pairs. Deletion of all or part of this segment (8, 17, 20) or mutation within oriC (20) abolishes origin function. This demonstrates that oriC is the only origin present in minichromosomes and that no secondary origins exist.

Initiation of replication in minichromosomes is under a control similar to that of the initiation of the host chromosome. Particularly, protein synthesis and the products of genes dnaA, dnaC, and rpoB are required (27), as well as the products of dnaB and dnaI genes (B. Heimann and W. Messer, unpublished data). However, it is not known whether replication in minichromosomes proceeds bidirectionally as in the E. coli chromosome or whether the sequence in the 422-base pair (or the 232- to 245-base pair) oriC segment contains enough information to initiate bidirectional replication.

To answer this question, we determined the replication pattern of minichromosomes which contain different segments of DNA around oriC (Fig. 1). The minichromosome pCM959 has been derived from λ asn by in vivo deletion and is a plasmid which contains exclusively chromosomal DNA (15, 27). The sequences in and around both boundaries of oriC are identical to those of the oriC region in the chromosome. pOC24 was derived from the larger minichromosome pOC2 (16) by restriction endonuclease-mediated in vitro deletion (17). In this plasmid,

![Fig. 1. Restriction endonuclease cleavage map of the minichromosomes pOC24 and pCM959 (17). Only relevant restriction endonuclease recognition sites are shown. Thick lines represent chromosomal DNA. Map orientation is the same as that of the E. coli genetic map (1). oriC, Chromosomal replication origin of E. coli; asn, chromosomal gene for asparagine synthetase; AP, DNA fragment derived from the Staphylococcus aureus plasmid p1258 coding for ampicillin resistance (16).](http://jb.asm.org/)

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sequences adjoining the right (clockwise) boundary of oriC, the XhoI recognition site, were deleted. This placed the DNA fragment determining ampicillin resistance in pOC2 next to oriC (Fig. 1).

Replication intermediates of pCM959 and pOC24 were isolated as described previously (4, 6), except that thymine starvation was omitted to avoid any possible effects of thymine limitation on the replication pattern (5). Exponentially growing cells were shock arrested and lysed, and the DNA was purified by dye-CsCl gradient centrifugation (4, 6). Replicating intermediates were isolated from the gradients from a position between closed circular and linear DNA. Replicative intermediates had been shown to band at that density in a pilot experiment using pulse-labeled DNA (data not shown). They were digested with restriction endonucleases having single recognition sites for each plasmid: SmaI for pCM959 and PvuII for pOC24 (Fig. 1). Unfortunately, all single recognition sites for pCM959

Fig. 2. Replicative intermediates of pCM959 arranged in order of increasing extent of replication. Four groups of molecules are shown: (A) bidirectionally replicating molecules; (B) asymmetrically bidirectionally replicating molecules; (C) molecules replicating unidirectionally counterclockwise; (D) molecules replicating unidirectionally clockwise. The position of oriC is indicated. Clockwise and counterclockwise refer to the maps in Fig. 1. Heavy bars represent the replicated region of each molecule. Replicating molecules were isolated from a 3-liter culture of CM959 (F' thi his recA, pCM959) (15) grown at 37°C in minimal medium supplemented with 0.2% glucose-5 µg of thiamine per ml-0.5% Casamino Acids. [3H]thymidine (0.5 µCi/ml) was added at a cell density of about 5 × 10⁶/ml. At a density of about 10⁶ cells per ml, growth was arrested by adding 0.1 M sodium azide, and the culture was rapidly cooled in dry ice-methanol. Cells were harvested and lysed by the lysozyme-sodium dodecyl sulfate-salt method (6). Replicating molecules were purified by two successive centrifugations in a propidiumdiodide-cesium chloride gradient at 40,000 rpm for 60 h at 15°C. Gradients were fractionated, and small samples were counted. DNA from the region in between covalently closed circular DNA and linear DNA was pooled, extracted with CsCl-saturated isopropanol to remove the propidiumdiodide, and precipitated with ethanol. The pellet was dissolved, and part of the material was linearized with restriction endonuclease SmaI and prepared for electron microscopy. Open circular pCM959 DNA was used as an internal length marker. Only molecules in which both halves of the replication loops were identical in length (±5%) and for which the total length was within ±10% of the length marker used were used for analysis. Individual molecules were normalized to unit length and classified by using the following rationale. Molecules in which one end of the replicated region was within or very close to the position of oriC were assigned to classes C and D. Molecules in which the ends of the replicated regions have progressed beyond that point must replicate bidirectionally. They were assigned to class A (symmetrically bidirectional) if the midpoint of the nonreplicated part was between 0.5 and 0.65 of the total length. Obviously, this grouping is ambiguous for molecules with very short replicated regions. These were included in group D because this assignment is unfavorable for the conclusions drawn from the experiment.
are close to oriC.

Restricted molecules were mounted on Parlodion-coated grids by using the aqueous spreading technique (11), rotary shadowed with Pt-Pd at a low angle, and examined by electron microscopy. The frequency of replicative intermediates detected among all molecules inspected was about $10^{-4}$.

The position of oriC, and thus of the start of replication within the minichromosomes, is known (Fig. 1) (17). This obviates the need for using a second restriction enzyme to determine the location of the origin. In agreement with the position of oriC is the observation that replicative intermediates with short replicated regions show these small "eyes" always at a position which can be matched with the position of oriC. This was found for 8 molecules of pCM959 (see Fig. 2D), 7 molecules of pOC24 (see Fig. 3A), and 25 molecules of pOC2 (data not shown).

Replicative intermediates of pCM959 are shown in Fig. 2. They can be grouped into four classes: molecules replicating bidirectionally and symmetrically from oriC (Fig. 2A), asymmetric bidirectional molecules in which one replicated part has progressed further from oriC than the other (Fig. 2B), and molecules replicating unidirectionally in counterclockwise (Fig. 2C) or clockwise (Fig. 2D) direction. This pattern is very similar to that observed for replicating molecules of phage $\lambda$ (22) and F plasmids (6).

More than half of the molecules were in the symmetric bidirectional class (Table 1). This demonstrates that the replication origin present in pCM959 promoted bidirectional replication and that both replication directions were frequently initiated simultaneously. In some molecules, initiation of one replication fork seemed to be delayed relative to the other (or one fork stopped replication prematurely), thus yielding the asymmetric bidirectional class. Unidirectional replication of molecules might also be due to such a delay: small plasmid molecules might have finished replication before initiation of the other replication direction could occur. This also demonstrates the high level of resolution obtained in these small minichromosomes with

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**Table 1. Bidirectional and unidirectional replication of replicative intermediates of minichromosomes pCM959 and pOC24**

| Minichromosome | Total no. examined | Bidirectional replication | | Unidirectional replication |
|----------------|--------------------|---------------------------|---------------------------|
|                |                    | Total          | Symmetrical | Asymmetrical | Total          | Clockwise* | Counterclockwise |
| pCM959         | 99                 | 56 (57)        | 38          | 18          | 43 (43)        | 18 (18)    | 25 (25) |
| pOC24          | 44                 | 9 (20)         |             |             | 35 (80)        | 4 (9)      | 31 (71) |

* Clockwise and counterclockwise refer to the orientation of the maps shown in Fig. 1, which are drawn in the same orientation as the E. coli genetic map (1).

* Number within parentheses indicates the percentage of the total.
respect to the analysis of the timing of bidirectional replication.

The observation that unidirectionally replicating molecules can do so in either direction (Table 1) suggests that there are active start signals for both replication directions on both DNA strands, as has been proposed and disputed for phage λ (3, 9, 14, 19, 24).

The pattern observed for replicative intermediates of pOC24 appears to be different. Although only a few replicating molecules were found, and most of these showed only relatively small loops of replicated DNA, the results (Fig. 3) indicate that the majority of molecules replicated unidirectionally, counterclockwise from the origin (Table 1). This suggests that for efficient bidirectional replication, sequences to the right (clockwise) of the 422-base pair oriC segment are required. If the low efficiency of initiation of clockwise replication in pOC24 is due to a (partially) missing start signal, this sequence must be very close to the clockwise boundary of oriC, as exemplified by the pCM950 molecules which replicate unidirectionally clockwise.

The observation that a DNA-binding protein isolated from the membrane of E. coli (10) binds specifically to a site within the 422-base pair oriC segment and to a second site which lies within 72 base pairs to the right of its clockwise boundary (A. Jacq, H. Loather, W. Messer, and M. Kohiyama, ICN-UCLA Symposium on Molecular and Cell Biology, in press) might suggest that this second recognition site constitutes part of the apparently missing start signal in pOC24.

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