JOURNAL OF BACTERIOLOGY, Aug. 1980, p. 1049-1053
0021-9193/80/08-1049/05$02.00/0

Vol. 143, No. 2

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

MICHIEL MEIJER[1]† AND WALTER MESSER[2]*

Department of Electron Microscopy and Molecular Cytology, University of Amsterdam, Amsterdam, The Netherlands,[1] and Max-Planck-Institut für molekulare Genetik, Berlin 33, Germany[2]

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 *Bam*HI recognition site and on the other side by a *Xho*I 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,

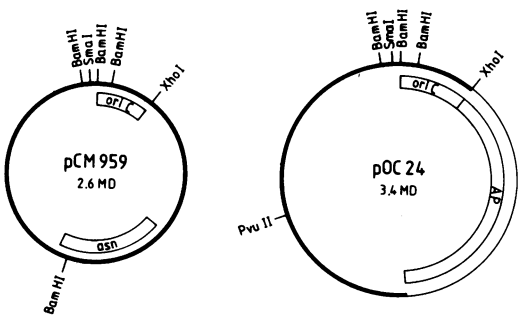

FIG. 1. *Restriction endonuclease cleavage map of the minichromosomes pOC24 and pCM959 (17). Only relevant restriction endonuclease recognition sites are shown. Thick lines represent chromosomal DNA. Map orientation is the same as that of the E. coli genetic map (1). oriC, Chromosomal replication origin of E. coli; asn, chromosomal gene for asparagine synthetase; AP, DNA fragment derived from the Staphylococcus aureus plasmid pI258 coding for ampicillin resistance (16).*

† Present address: Max-Planck-Institut für molekulare Genetik, D-1000 Berlin 33, Germany.

sequences adjoining the right (clockwise) boundary of *oriC*, the *Xho*I 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). Replicative 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: *Sma*I for pCM959 and *Pvu*II for pOC24 (Fig. 1). Unfortunately, all single recognition sites for pCM959

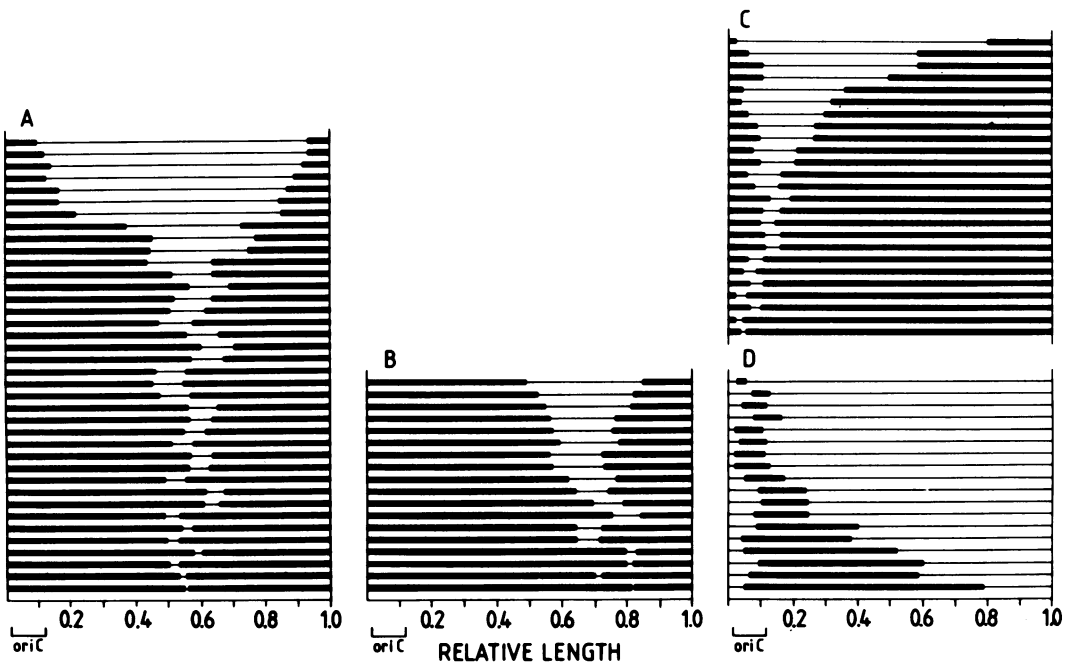

FIG. 2. *Replicative intermediates of pCM959 arranged in order of increasing extent of replication. Four groups of molecules are shown: (A) bidirectionally replicating molecules; (B) asymmetrically bidirectionally replicating molecules; (C) molecules replicating unidirectionally counterclockwise; (D) 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 CM993 (F^- thi asn 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. [^{3}H]thymidine (0.5 μCi/ml) was added at a cell density of about 5×10^7/ml. At a density of about 10^8 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 propidiumdiiodide-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 propidiumdiiodide, 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 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 λ (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

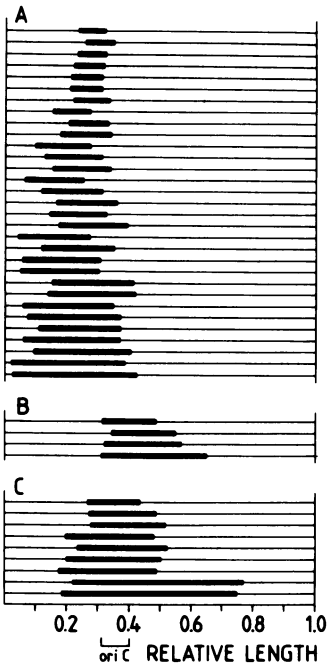

FIG. 3. *Replicative intermediates of minichromosome pOC24 arranged in order of increasing extent of replication. (A) Molecules replicating unidirectionally counterclockwise; (B) molecules replicating unidirectionally clockwise; (C) bidirectionally replicating molecules. Clockwise and counterclockwise refer to the maps in Fig. 1. Heavy bars of the normalized molecules represent the replicated region of each molecule. Replicating molecules were isolated as described in the legend to Fig. 2 with the following modifications. WM731 (F⁻ leu lac gal try his arg thyA drm rpsL met ilv recA1, pOC24) (16) was grown in tryptone yeast extract (Difco Laboratories) supplemented with 120 μg of penicillin G per ml (to select for the presence of pOC24). [³H]thymidine (1 μCi/ml) was added at a cell density of 10^8/ml. Cells were harvested at 2.5 × 10^8/ml and lysed by the cleared-lysate method (4). Replicative intermediates were linearized with restriction endonuclease PvuII. The position of oriC is indicated. Molecules were oriented such that the replicated region starts at or close to oriC (unidirectionally replicating molecules A and B) or oriC is within the replicated segment (bidirectionally replicating molecules, C).*

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[a]	Counterclockwise
pCM959	99	56 (57)[b]	38	18	43 (43)	18 (18)	25 (25)
pOC24	44	9 (20)			35 (80)	4 (9)	31 (71)

[a] 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).

[b] 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 pCM959 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. Lother, 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.

We gratefully acknowledge the expert technical assistance of M. A. deJong and the advice of G. Morelli and R. Lurz.

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
2. **Bird, R., J. Louarn, J. Martuscelli, and L. Caro.** 1972. Origin and sequence of chromosome replication in *Escherichia coli*. J. Mol. Biol. **70**:549–566.
3. **Blattner, F., and J. Dahlberg.** 1972. RNA synthesis startpoints in bacteriophage λ: are the promoter and operator transcribed? Nature (London) New Biol. **237**: 227–232.
4. **Cabello, F., K. Timmis, and S. N. Cohen.** 1976. Replication control in a composite plasmid constructed by *in vitro* linkage of two distinct replicons. Nature (London) **259**:285–290.
5. **Edlund, T., P. Gustafsson, and H. Wolf-Watz.** 1976. Effect of thymine concentration on the mode of chromosomal replication in *Escherichia coli* K-12. J. Mol. Biol. **108**:295–303.
6. **Eichenlaub, R., D. Figurski, and D. R. Helinski.** 1977. Bidirection replication from a unique origin in a mini-F plasmid. Proc. Natl. Acad. Sci. U.S.A. **74**:1138–1141.
7. **Hiraga, S.** 1976. Novel F prime factors able to replicate in *Escherichia coli* Hfr strains. Proc. Natl. Acad. Sci. U.S.A. **73**:198–202.
8. **Hirota, Y., S. Yasuda, M. Yamada, A. Nishimura, K. Sugimoto, H. Sugisaki, A. Oka, and M. Takanami.** 1979. Structural and functional properties of the *Escherichia coli* origin of DNA replication. Cold Spring Harbor Symp. Quant. Biol. **43**:129–138.
9. **Hobom, G., R. Grosschedl, M. Lusky, G. Scherer, E. Schwarz, and H. Kössel.** 1979. Functional analysis of the replicator structure of lambdoid bacteriophage DNAs. Cold Spring Harbor Symp. Quant. Biol. **43**:165–178.
10. **Jacq, A., and M. Kohiyama.** 1980. A DNA binding protein specific for the early replicated region of the chromosome obtained from *Escherichia coli* membrane fractions. Eur. J. Biochem. **105**:25–31.
11. **Kleinschmidt, A. K.** 1968. Monolayer techniques in electron microscopy of nucleic acid molecules. Methods Enzymol. **12B**:361–377.
12. **Masters, M.** 1975. Strains of *Escherichia coli* diploid for the chromosomal origin of DNA replication. Mol. Gen. Genet. **143**:105–111.
13. **Masters, M., and P. Broda.** 1971. Evidence for the bidirectional replication of the *Escherichia coli* chromosome. Nature (London) New Biol. **232**:137–140.
14. **McMacken, R., K. Ueda, and A. Kornberg.** 1977. Migration of *Escherichia coli dnaB* protein on the template DNA strand as a mechanism in initiating DNA replication. Proc. Natl. Acad. Sci. U.S.A. **74**:4190–4194.
15. **Meijer, M., E. Beck, F. G. Hansen, H. E. N. Bergmans, W. Messer, K. von Meyenburg, and H. Schaller.** 1979. Nucleotide sequence of the origin of replication of the *Escherichia coli* K-12 chromosome. Proc. Natl. Acad. Sci. U.S.A. **76**:580–584.
16. **Messer, W., H. E. N. Bergmans, M. Meijer, J. E. Womack, F. G. Hansen, and K. von Meyenburg.** 1978. Mini-chromosomes: plasmids which carry the *E. coli* replication origin. Mol. Gen. Genet. **162**:269–275.
17. **Messer, W., M. Meijer, H. E. N. Bergmans, F. G. Hansen, K. von Meyenburg, E. Beck, and H. Schaller.** 1979. Origin of replication, *oriC*, of the *Escherichia coli* K-12 chromosome: nucleotide sequence. Cold Spring Harbor Symp. Quant. Biol. **43**:139–145.
18. **Miki, T., S. Hiraga, T. Nagata, and T. Yura.** 1978. Bacteriophage λ carrying the *Escherichia coli* chromosomal region of the replication origin. Proc. Natl. Acad. Sci. U.S.A. **75**:5099–5103.
19. **Moore, D. D., K. Denniston-Thompson, K. E. Kruger, M. E. Furth, B. G. Williams, D. L. Daniels, and F. R. Blattner.** 1979. Dissection and comparative anatomy of the origins of replication of lambdoid phages. Cold Spring Harbor Symp. Quant. Biol. **43**:155–163.
20. **Oka, A., K. Sugimoto, M. Takanami, and Y. Hirota.** 1980. Replication origin of the *E. coli* K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. Mol. Gen. Genet. **178**:9–20.
21. **Prescott, D. M., and P. L. Kuempel.** 1972. Bidirectional replication of the chromosome in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **69**:2842–2845.
22. **Schnös, M., and R. B. Inman.** 1970. Position of branch points in replicating λ DNA. J. Mol. Biol. **51**:61–73.
23. **Sugimoto, K., A. Oka, H. Sugisaki, M. Takanami, A. Nishimura, Y. Yasuda, and Y. Hirota.** 1979. Nucleotide sequence of *Escherichia coli* K-12 replication origin. Proc. Natl. Acad. Sci. U.S.A. **76**:575–579.
24. **Szybalski, W.** 1977. Initiation and regulation of transcription and DNA replication in coliphage lambda, p. 2–45. *In* J. Copeland and G. Marzluff (ed.), Regulatory biology. Ohio State University Press, Columbus, Ohio.
25. **von Meyenburg, K., F. G. Hansen, L. D. Nielsen, and P. Jørgensen.** 1977. Origin of replication, *oriC*, of the *Escherichia coli* chromosome: mapping of genes relative to R·*Eco*RI cleavage sites in the *oriC* region. Mol. Gen. Genet. **158**:101–109.
26. **von Meyenburg, K., F. G. Hansen, L. D. Nielsen, and**

E. Riise. 1978. Origin of replication, *oriC*, of the *Escherichia coli* chromosome on specialized transducing phages λ *asn*. Mol. Gen. Genet. **160**:287–295.

27. **von Meyenburg, K., F. G. Hansen, E. Riise, H. E. N. Bergmans, M. Meijer, and W. Messer.** 1979. Origin of replication, *oriC*, of the *Escherichia coli* K-12 chromosome: genetic mapping and minichromosome replication. Cold Spring Harbor Symp. Quant. Biol. **43**:121–128.
28. **Yasuda, S., and Y. Hirota.** 1977. Cloning and mapping of the replication origin of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:5458–5462.