ORIGIN AND DIRECTION OF DNA SYNTHESIS IN E. COLI B/r*

By C. B. Ward† and D. A. Glaser

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated January 9, 1969

Abstract.—Replication of the chromosome of *E. coli* B/r has been studied by measuring the change with cell age of the rates of reversion to wild type of auxotrophic markers induced by nitrosoguanidine. Synchronous populations of bacteria were obtained by the procedure of Helmstetter and Cummings. The origin of DNA synthesis was determined to occur at 49 minutes ± 5 minutes on the genetic map, and the direction of synthesis was found to be clockwise.

Introduction.—The *E. coli* chromosome is thought to be a single circular double-stranded molecule of DNA which is replicated semi-conservatively by a Y-shaped growing point.[1–6] Several experimenters[5–16] have investigated the effect of integrated F-factors on the origin and direction of chromosomal synthesis without reaching general agreement on the results.

The replicon model of Jacob *et al.*[17] predicts that each F^- strain and Hfr strain has a unique origin and direction for chromosomal synthesis and that integration of an F-factor does not affect vegetative DNA synthesis. In contrast, Nagata's model[5] predicts that in Hfr strains, the origin is located at the site where the F-factor is integrated and that replication proceeds from the F-factor to the conjugation origin. In the Nagata model F^- strains have no unique chromosomal site for the initiation of DNA synthesis. There are data in the literature supporting both of these models. The work presented in this paper has been done in order to examine the prediction of the Nagata model for F^- strains using a technique which should avoid some possible artifacts of earlier methods.

Cerda-Olmeda *et al.*[13] have shown that the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) acts preferentially at the growing point on the chromosome, and hence a large increase in the mutation rate for a specific gene should occur at the cell age at which the gene in question is being replicated. Therefore, if one examines the back mutation rate of a given auxotrophic marker as a function of cell age, the cell age at which the gene is replicated may be determined.

Materials and Methods.—Bacterial strains: All strains used were derivatives of *E. coli* B/r, strain HB60 (from Herbert Boyer). These derivatives were: DG337 = HB60 Arg A^-, DG336 = HB60 His^-, DG339 = HB60 Met^-, and DG340 = HB60 Leu^-. All of these strains were obtained from HB60 by treatment with nitrosoguanidine.

Media: Cells were grown on a minimal salts medium that consisted of two solutions which were mixed shortly before use. Solution A contained 2.0 gm $(NH_4)_2SO_4$, 6.0 gm Na_2HPO_4, 3.0 gm KH_2PO_4, 3.9 gm NaCl, and 0.011 gm Na_2SO_4 dissolved in 200 ml of deionized H_2O. Solution B contained 0.2 gm $MgCl_2$, 0.1 gm $CaCl_2$, and 0.0005 gm $FeCl_3 \cdot 7H_2O$ dissolved in 800 ml of deionized H_2O. Glucose was added to a final concentration of 0.2%. This medium was supplemented with 50 μg/ml of DL-amino acids as needed for the growth of a particular strain.

Supplemented agar plates contained minimal salts medium; 0.2% glucose; 1.5% agar;

25 μg/ml of the L-amino acids arginine, proline, tryptophan, methionine, leucine, histidine; and 15 μg/ml thymidine. The appropriate growth factor was omitted in order to detect cells capable of growing without the growth factor in question.

Cell synchrony: The membrane collection technique of Helmstetter and Cummings[18] was used to synchronize the cells. In this procedure, one collects newly divided cells that have been eluted from a membrane filter to which a growing population of bacteria has been affixed. To prepare a large sample of synchronized cells, sufficient for the subsequent analyses, the newly divided cells in the synchronized eluent were collected in chilled flasks for 1 to 2 hr. These chilled cells were concentrated by filtration and resuspended in the desired volume of warm conditioned medium (minimal salts medium which had sustained growth to 3×10^7 cells/ml and had been filtered to remove the bacteria). When possible, the experiments were carried out both with and without this concentration step to assure that this step did not introduce artifacts into the results.

Nitrosoguanidine mutagenesis: Nitrosoguanidine was dissolved to a final concentration of 0.3% in pH 5.0 Tris-maleic acid buffer[19] and kept frozen until needed. Just prior to use, the nitrosoguanidine was melted and aerated to remove any diazomethane resulting from decomposition of the nitrosoguanidine.

The bacteria were removed from the culture medium by filtration through a 0.22-μ Millipore filter. After a washing with minimal salts medium lacking glucose, the cells were resuspended in pH 5.0 Tris-maleic buffer, and NG was added to a final concentration of 0.5 mg/ml. After a 15-minute period of incubation at 37°C, the cells were removed from the nitrosoguanidine solution by filtration and washings with minimal salts. The cells were resuspended in minimal salts and plated onto selective plates by spreading 0.1 ml of the desired dilution with a glass spreader.

Measurement of the rate of DNA synthesis: The rate of DNA synthesis was estimated by the rate of incorporation of H^3-thymidine, which was added to a 1-ml culture sample to a final concentration of 1 μg/ml. The sample was then incubated at 37°C for 2 min, at which time incorporation was stopped by the addition of 3 ml of ice-cold 7.5% trichloroacetic acid (TCA) containing 1 mg/ml of carrier thymidine. Each sample was filtered through a 0.22-μ Millipore membrane filter and washed with 5% TCA containing 1 mg/ml of carrier thymidine. After being dried, the filters were placed in the bottom of scintillation vials with 10 ml of Permaflur (Packard Instruments) and counted on a Packard Tri-Carb scintillation counter. H^3-thymidine of approximately 4 c/mmole was obtained from New England Nuclear Corporation.

Measurement of cell growth and synchrony: Cell numbers were determined with a 30-μ orifice Coulter counter that had been modified to allow the cell volume distribution to be displayed on a 256-channel pulse-height analyzer. This modification made possible rapid monitoring of the quality of the synchrony.

Results.—Cell age at which a new round of DNA synthesis starts: Since the start of rounds occurs at a time prior to division[20, 21] that may be different for different strains, it was necessary to determine the cell age at which it occurred for each of the strains used. This was accomplished by the measurement of the rate of DNA synthesis during the life cycle, the start of rounds being characterized by a doubling of the rate of DNA synthesis. The results of a typical experiment are shown in Figure 1. As can be seen from these data, the rate of H^3-thymidine incorporation increases by a factor of 2 about 15 minutes before division.

Synchronized mutagenesis: Since nitrosoguanidine acts preferentially at the growing point,[13] a large increase in the mutation rate of a given gene is expected at the cell age at which the gene in question is being replicated. Samples of synchronously growing auxotropic cells were removed at five-minute intervals, treated with nitrosoguanidine, and plated on selective plates to determine the

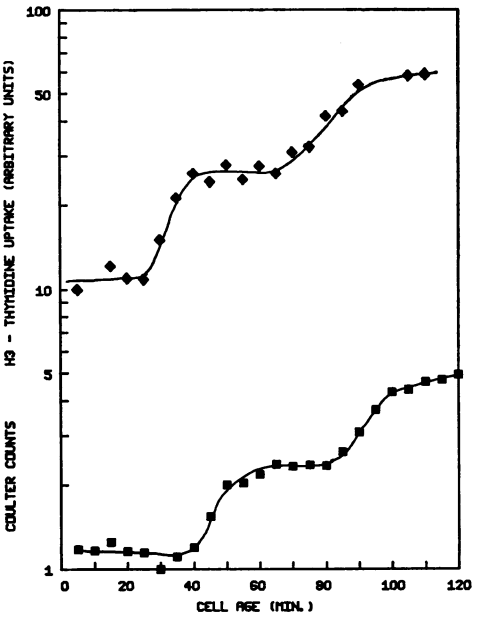

FIG. 1.—Measurement of the rate of incorporation of H^3-thymidine. Samples of 1 ml taken at 5-min intervals from a synchronized culture growing in glucose medium were mixed with H^3-thymidine to give a final concentration of 1 μg of thymidine/ml at a final specific activity of 50 mc/mmole. After a 2-min incubation period at 37°C, incorporation was stopped by the addition of 3 ml of ice-cold 7.5% trichloroacetic acid (TCA) containing 1 mg of carrier thymidine/ml. The samples were filtered through 0.22-μ Millipore filters and washed with 5% TCA containing 1 mg of carrier thymidine/ml. After being dried, the filters were counted on a Tri-Carb (Packard Instruments) scintillation counter.

back-mutation frequency as a function of cell age. The results of these experiments are shown in Figure 2. The maximum reversion rate for *arg* A^- is found at the cell age at which a new round of DNA synthesis is initiated, that of *met*$^-$ 13 minutes later, that of *leu*$^-$ 3 minutes after *met*$^-$, and that of *his*$^-$ 22 minutes after *leu*$^-$. If it is assumed that the genetic map of *E. coli* B/r is the same as that of *E. coli* K12, the above data indicate a clockwise direction of synthesis with an origin of replication at 49 minutes ± 5 minutes on the genetic map.

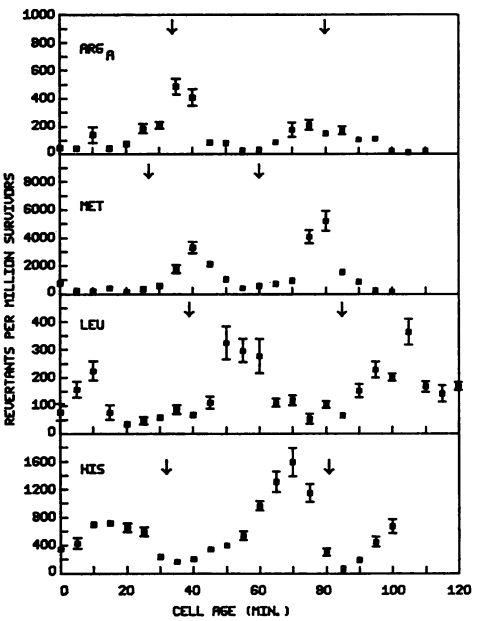

FIG. 2.—Synchronized mutagenesis of derivatives of *E. coli* B/r. Samples taken at 5-min intervals from a synchronized culture growing at 37°C in glucose medium were treated with nitrosoguanidine (500 μg/ml) in pH 5.0 tris-maleic buffer and incubated for 15 min. The samples were then filtered, washed to remove the nitrosoguanidine, and assayed for reversion of the markers indicated. The arrows indicate the cell ages at which a new round of DNA replication started, as measured by the quantity of H^3-thymidine incorporated into TCA-precipitable material during a 2-min pulse.

In Figure 3, the time at which the maximum reversion rate occurs after the start of rounds is plotted versus the position of the marker on the genetic map. The circular map has been opened at *leu* and is plotted with the clockwise direction going from right to left. The markers are plotted twice to indicate the circularity of the chromosome.

The above results depend on a knowledge of the location of each of the markers used on the genetic map. A requirement for leucine has been found to map at only one location on the *E. coli* K12 genome;[22] similarly, a requirement for histidine maps at only one location. However, both *arg* and *met* map at several locations, and since each of the strains used here was isolated specifically for these experiments, it was necessary to map the *arg* and *met* requirements of the strains. The position of the *arg*$^-$ strain was determined by cotransduction, using essentially the procedure of Lennox.[23] The *arg*$^-$ strain exhibited a 25 per cent linkage with *thy A*, and hence was assigned a map location at 54 minutes.

The *met* requirement was located by means of an interrupted mating technique similar to that of Taylor and Trotter.[22] This requirement mapped at about 76 minutes. This eliminated the possibility that the *met*$^-$ mutation was *met C*. Since all other *met*$^-$ mutations map between 74 and 77 minutes, a value of 76 minutes was assigned to the *met*$^-$ strain used here.

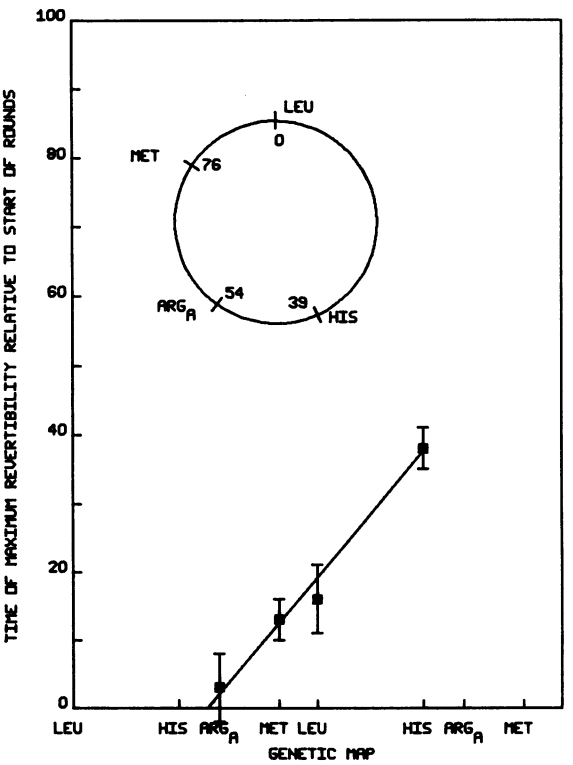

FIG. 3.—Summary of synchronized mutagenesis data for *E. coli* B/r.

In order to test for possible artifacts resulting from the concentration procedure, the *his*⁻ experiments were repeated using a procedure in which this step was eliminated. Samples of newly divided cells were collected at 37°C and allowed to grow to the desired cell age. The cells were then treated with nitrosoguanidine as described above. The results of this control were in excellent agreement with those obtained using the concentration procedure. Unfortunately, it was not possible to use this latter procedure on the other strains because the reversion rates of the other strains were too low.

Discussion.—The origin and direction of replication for *E. coli* B/r found in this work are in good agreement with that found by Pato and Glaser[14] and by Helmstetter,[15] both of whom employed the Helmstetter and Cummings synchrony technique and measured-enzyme inducibility to determine the time during the life cycle that a given gene is duplicated. These results also agree with those of Wolf *et al.*,[10] Abe and Tomizawa,[9] and Cerda-Olmeda *et al.*[13] The last three papers reported experiments which employed amino acid starvation and/or incorporation of bromouracil either to align the growing points or to prevent the initiation of a new round of DNA synthesis. Because of these nonphysiological treatments, it has been argued that the origin found may have been an artificial one and the correct model for vegetative growth is that of Nagata.[2] However, since the other experiments not involving amino acid starvation give the same results, this argument does not seem very strong, and one is inclined to look for possible artifacts in the experiments supporting the Nagata model.

With the exception of the experiments of Vielmetter and Messer,[16] all the experiments that support the Nagata model employed the same fractional filtration procedure to obtain synchronous growth. Cells synchronized by this procedure exhibit exponentially increasing DNA synthesis during the life cycle; cells synchronized by the Helmstetter and Cummings procedure exhibit a constant rate of DNA synthesis with a doubling of this rate about 15 minutes before division. This constancy of the rate of DNA synthesis between doublings is in agreement with Cairns[2] and Maaløe and Kjeldgaard.[24]

A possible way of reconciling these contradictory results has been suggested by Pato and Glaser.[14] These authors suggest that a vegetative origin exists as well as various possible alternative origins. These latter origins may be identical with the integration sites for F-factors. Nonphysiological treatment of cells could shift initiation to the mating origin in Hfr strains and cause initiation from one or more origins in strains that lack an integrated F-factor to determine a unique alternate site. There is some evidence to support this model. Wolf *et al.*[10] have presented data from one Hfr strain that was consistent with the existence of two origins. Irradiation is another example of a treatment known to affect both conjugational and vegetative systems in *E. coli*.[25–27]

The authors would like to thank Mrs. Erika Appe for her technical assistance.

* This work was supported in part by the U.S. Public Health Service through research grant GM12524 from the National Institute of General Medical Sciences.

† Supported by the Miller Research Foundation at the University of California, Berkeley.

[1] Meselson, M., and F. W. Stahl, these PROCEEDINGS, **44**, 671 (1958).
[2] Cairns, J., *J. Mol. Biol.*, **6**, 208 (1963).
[3] Bonhoeffer, F., and A. Gierer, *J. Mol. Biol.*, **7**, 534 (1963).
[4] Lark, K. G., T. Repko, and E. J. Hoffman, *Biochim. Biophys. Acta*, **76**, 9 (1963).
[5] Nagata, T., these PROCEEDINGS, **49**, 551 (1963).
[6] Keumpel, P., Ph.D. dissertation, Princeton University (1965).
[7] Nichi, A., and T. Horiuchi, *Japan. J. Biochem.*, **60**, 338 (1965).
[8] Rudner, R., E. Rejman, and E. Chargaff, these PROCEEDINGS, **58**, 1911 (1967).
[9] Abe, M., and J. Tomizawa, these PROCEEDINGS, **54**, 904 (1965).
[10] Wolf, B., A. Newman, and D. Glaser, *J. Mol. Biol.*, **32**, 611 (1968).
[11] Donachie, W., and M. Masters, *Genet. Res.*, **8**, 119 (1966).
[12] Berg, C., and L. Caro, *J. Mol. Biol.*, **29**, 419 (1967).
[13] Cerda-Olmeda, E., P. C. Hanawalt, and N. Guerola, *J. Mol. Biol.*, **33**, 701 (1968).
[14] Pato, M., and D. Glaser, these PROCEEDINGS, **60**, 1268 (1968).
[15] Helmstetter, C., *J. Bacteriol.*, **95**, 1634 (1968).
[16] Vielmetter and Messer, in *Cold Spring Harbor Symposia on Quantitative Biology*, in press.
[17] Jacob, F., S. Brenner, and F. Cuzin, in *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 28 (1963), p. 339.
[18] Helmstetter, C., and D. Cummings, these PROCEEDINGS, **50**, 767 (1963).
[19] Adelberg, E. A., M. Mandel, and G. C. C. Chen, *Biochem. Biophys. Res. Commun.*, **18**, 778 (1967).
[20] Clark, D. J., and O. Maaløe, *J. Mol. Biol.*, **23**, 99 (1967).
[21] Helmstetter, C. E., *J. Mol. Biol.*, **24**, 417 (1967).
[22] Taylor, A., and D. Trotter, *Bacteriol. Rev.*, **31**, 332 (1967).
[23] Lennox, E. S., *Virology*, **1**, 190 (1966).
[24] Maaløe, O., and N. O. Kjeldgaard, *Control of Macromolecular Synthesis* (New York: W. A. Benjamin, 1966).
[25] Low, B., *J. Bacteriol.*, **93**, 98 (1967).
[26] Hewitt, R., and O. Billan, *J. Mol. Biol.*, **13**, 140 (1965).
[27] Kohiyama, M., in *Cold Spring Harbor Symposia on Quantitative Biology*, in press.