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The *rep* Mutation

III. Altered Structure of the Replicating *Escherichia coli* Chromosome

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The *rep* gene function of *Escherichia coli* is essential for the replication of P2 and φX174 double-stranded deoxyribonucleic acid (DNA). Compared with isogenic *rep*$^+$ strains, *rep* mutants show the following characteristics: larger cell size, more DNA per cell, and a slightly lower DNA/mass ratio. The replicating *rep* chromosomes show a steeper gradient of marker frequencies and contain more replicating forks per chromosome. The nucleoid body of *rep* mutants sediments faster and contains more DNA. We deduce that the *rep* function is required for the "normal" replication of the *E. coli* chromosome and that in its absence the *E. coli* chromosome replicates in an altered manner, perhaps involving slower-moving replicating forks.

Denhardt et al. (13) originally isolated *rep* mutants of *Escherichia coli* as strains unable to support growth of bacteriophage φX174. The infecting single-stranded deoxyribonucleic acid (DNA) is converted to the double-stranded replicative form (RF), but this "parental" RF is not replicated and progeny phage are not produced. All detectable viral proteins are made, and both closed circular (RF I) and open circular (RF II) duplex molecules are formed; the RF II can be found associated with a membrane fraction (14). Calendar et al. (6) showed that the *rep* mutation results in the complete inability to replicate phage P2 DNA, that it maps at 75 min, between *ilv* and *metE*, and that the wild-type allele is dominant. In addition, *rep* mutants fail to support the growth of phages M13, f1, fd, and 186 (unpublished data). The functional product of the *rep* gene appears to be a protein (14). *rep* mutants were also found to differ from wild-type cells in their increased sensitivity to thymine starvation, ultraviolet light and X-ray irradiation, and slightly reduced recombination frequency (6, 13, 14).

Because the available evidence indicates that the chromosomes of φX, P2, and 186 replicate unidirectionally (2, 10, 25), we investigated whether the *E. coli rep* chromosome replicates unidirectionally as would be the case if one of the growing forks in the bidirectionally replicating chromosome required the *rep* function. We found that although the *rep* chromosome is, in fact, replicated in a bidirectional manner from an origin in approximately the same position as in *rep*$^+$, there are some interesting alterations in the structure of the replicating chromosome. In this report we describe these alterations, as well as some aspects of cell composition known to change as a result of alterations in chromosome replication.

MATERIALS AND METHODS

Bacteria and bacteriophage. *E. coli* CR *thy*$^-$, *lac*$^-$, *Su*$^+$ and *E. coli* HF4704 *thy*$^-$ (at 37 C) *Su*$^-$ are standard φX^s strains used in this laboratory (12-14). *E. coli* CR34 F$^-$, *thr*$^-$, *leu*$^-$, *thy*$^-$, *lac*$^-$, a K-12 strain, was obtained from G. M. Crowley. Bacterial strains used in hybridization experiments are listed in Table 1. The *rep3* allele was introduced by P1 transduction as follows. Cells were put through two successive penicillin G (Calbiochem) selections to obtain spontaneous, low (non-zero)-reverting *ilv*$^-$ mutants. P1*kc*, grown on CR *rep* and *rep*$^+$, was used to transduce *ilv*$^+$ into the mutants, and *rep* and *rep*$^+$ co-transductants, were selected by screening with phages P2 and/or 186. The presence of the *rep3* allele in the transductants was confirmed by transducing it back into a CR *ilv rep*$^+$ strain; the CR *ilv*$^+$ *rep* transductants were indistinguishable from the original with respect to phage sensitivity, growth rate, and cell size.

Bacteriophages used to test the Rep phenotype were φX174ρ^-h8 (delayed lysis, extended host range strain), P2*vira*, and 186 (from R. B. Inman). P1*kc* was originally obtained from P. L. Bergquist, and Mu was obtained from a single plaque after spontaneous induction of Mx212 (provided by R. Bird and L. Caro).

Media and growth conditions. M9 salts (1), L broth (19), and mT3XD (12) have been described previously. M9 medium contained M9 salts plus 1 μg of thiamine per ml, 0.4% glucose, and 20 μg of thymine per ml; required amino acids were added to 50 μg/ml. M9 Cas medium contained, in addition to the above, 1% Casamino Acids (Difco). M9 glycerol was

TABLE 1. *Bacterial strains*[a]

Strain	Mu integrated at[b]	λ[c]
CB0129	—	+ ($cI_{857}S7$)
Mx16	*lac*	+
Mx156	*metA*	+
Mx173	*trp*	+
Mx195	*tyrA*	+
Mx212	*ilv*	−
Mx223	*thr*	+ (*ind*⁻)
Mx239	*malA*	+ (*ind*⁻)

[a] All strains are *E. coli* K-12 F⁻, suII⁺, *thy*, *leu*, P2[s] and were obtained from R. Bird and L. Caro.

[b] Integration of Mu at these sites results in the negative mutant phenotype.

[c] λ wild type, except where indicated otherwise.

M9 medium with 0.4% glycerol in place of glucose.

Cultures were grown in a 37 C room in Erlenmeyer flasks containing a magnetic stirring bar to provide aeration. Prior to an experiment, cultures containing 0.5×10^8 to 2×10^8 cells/ml were diluted twofold after each generation for at least four generations to approach steady-state growth conditions. To starve for amino acids, cells were filtered on a prewarmed membrane filter (Millipore Corp.) and washed with four to five volumes of warm M9 salts; the cells were then suspended by shaking the filter in medium without amino acids, and the filter was removed. This procedure usually took less than 2 min.

Preparation of phage DNA: (i) Mu [^{3}H]DNA. To an exponential L broth culture of CR34, Mu phage was added to a multiplicity of infection of 3 to 5 and $CaCl_2$ was added to 2 mM. At 4-min intervals, 100 μCi of [^{3}H]thymidine was added until 50 min after infection. The cells lysed 50 to 60 min after infection, and 10 to 15 min later debris and unlysed cells were centrifuged out (10,000 rpm for 10 min in a GSA rotor of a Sorvall RC-2 centrifuge). The supernatant was incubated with 2 to 3 μg of deoxyribonuclease I (Worthington Biochemical Corp. EC 3.1.4.5.) per ml at 37 C for 30 min, mixed with PEG 6000 and NaCl to give 10% and 0.5 M solutions, respectively, and kept in ice overnight. The precipitate was centrifuged (8,000 rpm, 10 min in a GSA rotor) and suspended in a solution containing 10 mM tris(hydroxymethyl) aminomethane (Tris; pH 7.6), 10 mM $MgCl_2$, and 10 mM NaCl (TMN), and the suspension was sedimented through a gradient of 5 to 20% sucrose in TMN in an SW27 rotor at 26,000 rpm for 50 min at 10 C. The sucrose gradient step was necessary to separate the phage from the free bacterial DNA; because Mu is very sensitive to CsCl, it is not advisable to use isopycnic centrifugation. The sucrose gradient fractions were dialyzed against TMN to remove sucrose and then against PEG 20,000 to reduce the volume. The dialysate was extracted with phenol at 0 to 4 C, and the DNA was precipitated with 0.1 volumes of 3 M sodium acetate (pH 5.5) and 2 volumes of isopropanol. The DNA was suspended in 0.1 × SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and banded in an equilibrium CsCl density gradient. The DNA from the gradient was precipitated with isopropanol and finally suspended in 2 × SSC. Samples were analyzed by neutral and alkaline sucrose gradient velocity sedimentation to check the integrity of the DNA; when prepared by the above procedure, the Mu DNA sedimented in neutral sucrose as one homogeneous species of about 25×10^6 molecular weigh (measured against φX RF II). Alkaline sucrose gradient sedimentation revealed that the DNA contained very few single-strand breaks. A 15- to 30-μg amount of Mu DNA (5.1×10^4 counts/min per μg) was obtained from 100 ml of culture.

(ii) λ [^{14}C]DNA. CB0129 was grown to 2×10^8 to 3×10^8 cells/ml at 34 C in M9 Cas medium. The cells were sedimented, suspended in 0.1 volume of medium containing 1 μg thymine per ml, and incubated at 43 to 44 C for 10 to 15 min with aeration. These cells were then added to fresh medium containing [^{14}C]thymine (0.5 μCi/1.3 μg per ml) and incubated at 39 C for 3 to 4 h. The cells were centrifuged, suspended in 0.01 M Tris (pH 7.6)-0.1 M KCl-0.1 mM ethylenediaminetetraacetate (EDTA), and subjected to freezing in acetone-carbon dioxide and thawing at 37 C. The viscous lysate was incubated with deoxyribonuclease I, and the debris was removed by centrifugation. The supernatant was centrifuged in a sucrose gradient in an SW27 rotor, as described above for Mu phage, and the fractions containing phage were layered on a CsCl step gradient and centrifuged for 1 h at 24,000 rpm in an SW27.1 rotor. The visible phage band was collected with a syringe inserted through the side of the tube, and dialyzed against TMN. The DNA was extracted from the phage by gently rocking the tube with borate-saturated phenol (13). All succeeding steps are as described for Mu DNA; 40 to 60 μg of λ DNA (2.6×10^4 counts/min per μg) was obtained from 100 ml of culture.

Preparation of E. coli DNA for hybridization. *rep*⁺ and *rep* derivatives of the Mx strains (Table 1) were inoculated from overnight cultures into L broth at about 5×10^8 cells/ml and incubated at 37 C. When the optical density (550 nm) of the cultures reached 0.3 to 0.4, they were diluted periodically with fresh medium to maintain approximately this optical density for six generations before harvesting. The cells were sedimented, suspended in 50 mM Tris (pH 8.1)-20 mM EDTA, and lysed with lysozyme, sodium dodecyl sulfate, and Pronase. The lysates were phenol-extracted twice, and the DNA was collected from the aqueous layer by spooling an isopropanol precipitate. The redissolved nucleic acid was digested with ribonuclease A (Sigma Chemical Co.; EC 2.7.7.16.; 10 μg/ml) and ribonuclease T1 (Worthington Biochemicals Corp.; EC 2.7.7.26.; 1 μg/ml) for 1 h at 37 C and then dialyzed against three changes of 2 M NaCl -50 mM Tris (pH 8.1)-20 mM EDTA. The DNA was purified further by isopycnic centrifugation in CsCl and finally dialyzed against 2 × SSC. The concentrations of DNA were estimated from the absorbance at 260 nm.

Hybridization. Schleicher and Schuell Bac-T-flex B-6 membrane filters (25 mm diameter), soaked in 3 × SSC, were each mounted on a filter assembly and washed with 2 to 3 ml of 3 × SSC; then 1 ml of

heat-denatured *E. coli* DNA (3 μg/ml) in 3 × SSC was slowly (45 to 60 s) filtered through. Each filter was slowly washed three times with 1 ml of 3 × SSC, dried in a vacuum desiccator, and then baked for 2 h at 80 C.

Mu [^{3}H]DNA and λ [^{14}C]DNA were mixed in 4 × SSC at 1.5 μg/ml each, sonically treated, denatured at 100 C for 10 min, quickly chilled, and added to an equal volume of charcoal-purified formamide.

Hybridization was performed by the method of Kourilsky et al. (17). The filters were placed in 0.5 ml of 2 × SSC-50% formamide in scintillation vials, and 0.2 ml of the labeled phage DNA was added. The mixtures were incubated at 40 C with gentle agitation in a New Brunswick gyratory water bath for 48 h. After incubation, the filters were washed twice by gentle stirring in 100 ml of 2 × SSC-50% formamide and then three times in 100 ml of 3 × SSC, dried, and counted in toluene-Omnifluor (New England Nuclear) scintillant.

Autoradiography. The autoradiography method used was based on that of Caro (7). A 5- to 10-μliter sample of each cell suspension was spread on an acid-cleaned slide and dried. The slides were washed three times with cold 5% trichloroacetic acid, once with absolute ethanol, and once with distilled water, air-dried, dipped in Ilford L-4 emulsion diluted 1:1 with water, and held at 45 C. After the slides had been drained and dried in an upright position, they were stored in sealed boxes at room temperature for 6 days to 6 weeks, depending on the experiment. Development was at 20 C for 5 min in Kodak D19, followed by a 10-s immersion in 1% acetic acid, 4 min in Kodak Rapid Fixer Solution A, 10 to 15 min in running, tap water, and 1 min in distilled water. The cells were stained with 0.02% methylene blue and viewed under oil immersion at ×1,000 in bright field.

Sedimentation of nucleoids. The procedure for nucleoid sedimentation was essentially that of Worcel and Burgi (27, 28). Cells in balanced growth were labeled with [^{14}C]thymine (*rep*$^+$) or [^{3}H]thymine (*rep*) for one generation, the growth was stopped by treatment with 2 mM EDTA-10 mM sodium azide, and the suspension was chilled. The two cultures were mixed, and the cells were collected by centrifugation and suspended at 10^{10} cells/ml in a solution containing in 0.01 M Tris (pH 8.1), 0.01 M NaN_3, 0.1 M NaCl, and 20% sucrose. A 0.2-ml sample of the suspension at 0 C was mixed with 0.05 ml of lysozyme (4 mg/ml in 0.12 M Tris [pH 8.1]-0.05 M EDTA) by gently rotating the tube for 1 to 1.5 min. The mixture was shifted to 25 C, and 5 μliters of 10% diethylpyrocarbonate and 0.25 ml of 1% Brij 58-0.4% sodium deoxycholate in 5 mM Tris (pH 8.1)-2M NaCl-0.01 M EDTA were added. After 3 to 5 min of incubation, the lysate was centrifuged at 5,500 rpm for 5 min at 2 C in an SS34 rotor of a Sorvall RC-2 centrifuge, and 0.1 to 0.2 ml of supernatant was layered on a high-salt, 10 to 30% neutral sucrose gradient. Sedimentation was at 4 C and 17,000 rpm for 20 to 30 min in an SW50.1 rotor. Fractions were collected from the bottom of the tube onto Whatman no. 5 filter squares, dried, and counted in toluene-Omnifluor scintillant.

Estimation of cell size and DNA content. Cell number was measured in a Coulter counter (model B; 30-μm orifice) after dilution of the cells into M9 salts-0.1% formaldehyde to 10^6 cells/ml or less. Cell size was measured on the same sample by reading the optical density of the undiluted sample at 450 nm in a Beckman Acta III spectrophotometer and dividing by the number of cells per milliliter. Size distributions were measured in a Coulter counter by manually setting the threshold levels and counting the number of cells in each interval.

DNA was estimated with diphenylamine reagent on duplicate, 20-ml culture samples after they had been washed and digested with perchloric acid (5). DNA/mass (optical density at 450 nm) ratios obtained in this way were the same as those estimated from the counts per minute per optical density (450 nm) unit of cultures continuously labeled with [^{14}C]thymine.

RESULTS

The rep mutant replicates bidirectionally. One of the most direct demonstrations of bidirectional replication in *E. coli* has been provided by Bird et al. (4). They extracted the DNA from exponential-phase cultures of a number of bacterial strains that differed in the location at which the bacteriophage Mu genome was inserted in the *E. coli* chromosome but that contained lambda at the normal att-λ site. The purified DNA was denatured, fixed to membrane filters, and incubated with sonically treated and denatured labeled lambda and Mu DNA. From the relative amounts of phage DNA that annealed to the bacterial DNA of the different strains, it was possible to construct a gradient of marker frequencies whose maximum was near *ilv* (74 min) and that decreased symmetrically to a terminus near *trp* (25 min).

We performed a similar analysis with *rep*$^+$ and *rep* transductants of various Mu, λ lysogens grown exponentially in L broth. The results are presented graphically in Fig. 1; in drawing the curves, we assumed the origin of replication to be at 74 min (4) since our data do not permit its precise location. The data for the *rep*$^+$ strains are in agreement with those of Bird et al. (4) showing bidirectional replication. For the *rep* mutants the results show clearly that replication is bidirectional with an origin and a terminus in positions approximately the same as for *rep*$^+$. However, the gradient of marker frequencies is steeper than for the *rep*$^+$ strains, suggesting that the *rep* chromosome possesses more replication forks than the *rep*$^+$ chromosome. (Throughout this paper, we use the terms replication fork and growing fork to refer to the pair of forks that is initiated at each origin on the bidirectionally replicated chromosome.) In the range of concentrations of phage DNA used, there is a linear relation between input and

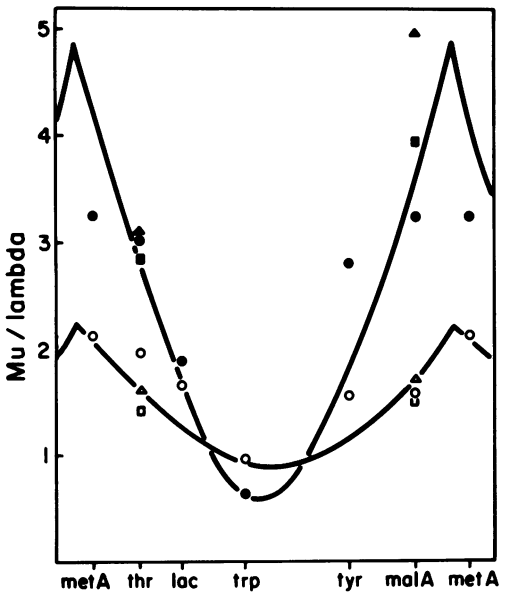

FIG. 1. *Gradients of marker frequency. Mu [^3H]DNA and λ [^{14}C]DNA were incubated with filters carrying DNA from various E. coli Mu, λ double lysogens. The ratios of $^3H/^{14}C$ bound to the filters (corrected for background, the ratio of input $^3H/^{14}C$ and the difference in molecular weights of Mu and λ DNAs [25 × 10^6 and 31 × 10^6, respectively]) are plotted against a linear projection of the genetic map. The background filters contained DNA from an exponential-phase culture of CR34 (Mu⁻, λ⁻). Each point represents an independent preparation of bacterial DNA and is the average of two or three filters. Open symbols, rep⁺; filled symbols, rep.*

annealed DNA (Fig. 2). The level of free phage due to spontaneous induction of the lysogens was no higher for *rep* strains than for *rep*⁺ strains.

There are more growing forks in the rep mutant. To confirm that there were more replicating forks in the *rep* chromosome than in the *rep*⁺ chromosome, and to measure the average number of growing forks per cell, we used an autoradiographic technique that determines the number of labeled cells as a function of the number of generations after a short pulse of [^3H]thymidine (8, 15). A culture in exponential growth was exposed to [^3H]thymidine for a small fraction of the generation time and then grown in nonradioactive medium for several generations to allow segregation of the strands labeled during the pulse. At intervals during this period, samples were taken and the cells were fixed to a slide, which was then coated with an emulsion. After exposure and development, the slides were examined to determine the proportion of the cells that had silver grains associated with them. The number of generations taken to achieve complete segregation of the labeled strands was proportional to the number of strands per cell initially labeled.

For our experiments, Mx239 *rep* and *rep*⁺ cells were grown for several generations at approximately 10^8 cells/ml in L broth or M9 Cas before pulse-labeling. In the experiment presented in Fig. 3, the slides were exposed long enough to ensure that there was an average of more than 10 grains per labeled cell. The fraction of unlabeled cells was then determined for the different samples and plotted as a function of the number of generations. Extrapolation of the plot of the percentage of labeled cells after segregation is complete back to the ordinate yields the number of strands labeled at the time of the pulse; since two strands are labeled by each growing fork, the number of growing forks is half of this number.

The results of an alternative method of analyzing the data are presented in Fig. 4. In this method the number of grains on each cell was determined, and the data were analyzed by plotting on a Poisson curve (8). Most cells with one associated grain presumably resulted from background as the P_1-i! values for one grain lie above the Poisson curves. The number of grains per labeled cell approached a constant value

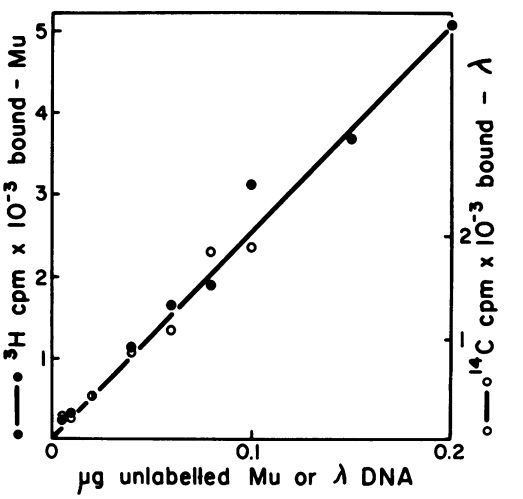

FIG. 2. *Relation between quantity Mu and λ DNA and amount hybridized. Various amounts of unlabeled Mu and λ DNAs, prepared as for the labeled phage DNAs, were immobilized on the filters with 3 μg of DNA from an ilv⁺ (Mu⁻) transductant of Mx212. Labeled Mu and λ DNAs were hybridized to the filters. Symbols:* O, λ [^{14}C]*DNA;* ●, *Mu* [^3H]*DNA.*

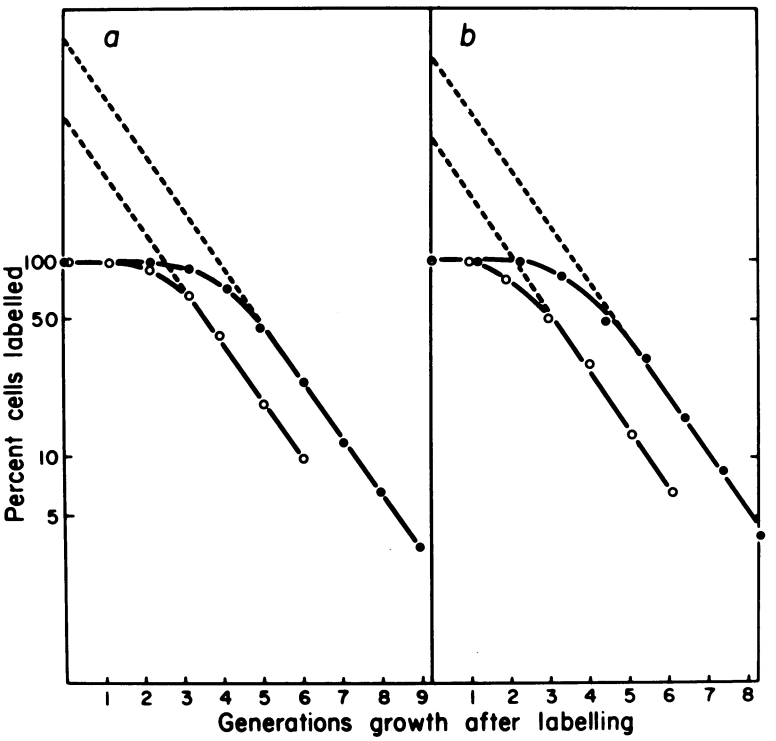

FIG. 3. *Segregation of unlabeled cells after pulse-labeling with [^{3}H]thymidine. Balanced growth cultures of Mx239 rep$^+$ and rep were pulse-labeled with [^{3}H]thymidine at (a) 20 μCi/ml (rep$^+$) and 40 μCi/ml (rep) for 2 min, or (b) 200 μCi/ml (rep$^+$) and 400 μCi/ml (rep) for 3 min (in a short pulse, rep strains incorporate roughly half as much [^{3}H]thymidine per cell as rep$^+$). The cells were washed free of [^{3}H]thymidine on a membrane filter (Millipore Corp.) with warm medium, and resuspended in warm medium at zero time. Samples were fixed with formaldehyde for estimation of cell number and for autoradiography. Cells were exposed for (a) 6 weeks or (b) 3 weeks prior to development and were scored as labeled (≥ 2 grains) or unlabeled (≤ 1 grain). Symbols: O, rep$^+$; ●, rep. (a) Cells grown in L broth; (b) cells grown in M9 Cas medium.*

(Fig. 4e), suggesting that there was not a large amount of sister-chromosome exchange. Both methods of analysis yielded the same result. For cells grown in L broth, the average number of growing forks in *rep*$^+$ cells was 2.65; in the *rep* mutant it was 7.4. In M9 Cas medium, *rep*$^+$ cells had an average of 2.2 growing forks; the *rep* mutant had 5.5. The values for *rep*$^+$ agree with those of Bird et al. (4); the values for the *rep* mutant confirm that there are more growing forks per chromosome in the mutant.

rep cells are larger and contain more DNA. It is a common observation that alterations in the normal pattern of DNA replication cause alterations in cell composition. For example, Pritchard and Zaritsky (24, 29) measured the changes in the cell size and DNA content of *E. coli* 15T$^-$ cells grown in media containing different concentrations of thymine. One of their observations was that a reduction in the "replication velocity" induced by a low thymine concentration caused an increase in cell size, a decrease in the DNA/mass ratio, and an increase in the amount of DNA per cell. We thought it of interest, therefore, to measure these quantities carefully in the *rep* mutant.

Mx223 *rep*$^+$ and *rep* cultures were grown in various media for several generations at the same cell concentration (0.4×10^8 to 2×10^8 cells/ml) to establish "balanced growth." Samples were taken at approximately one-generation intervals to measure cell number, optical density at 450 nm (proportional to cell mass) (24) and amount of DNA. These data are presented in Table 2 as the ratios of *rep* to *rep*$^+$ cell size and DNA content. The size distribution, measured with a Coulter counter, is presented in Fig. 5. It is apparent that the *rep* mutant cells are larger and contain more DNA per cell and less DNA per unit mass. The difference between the mutant and wild type is accentuated as the growth rate increases.

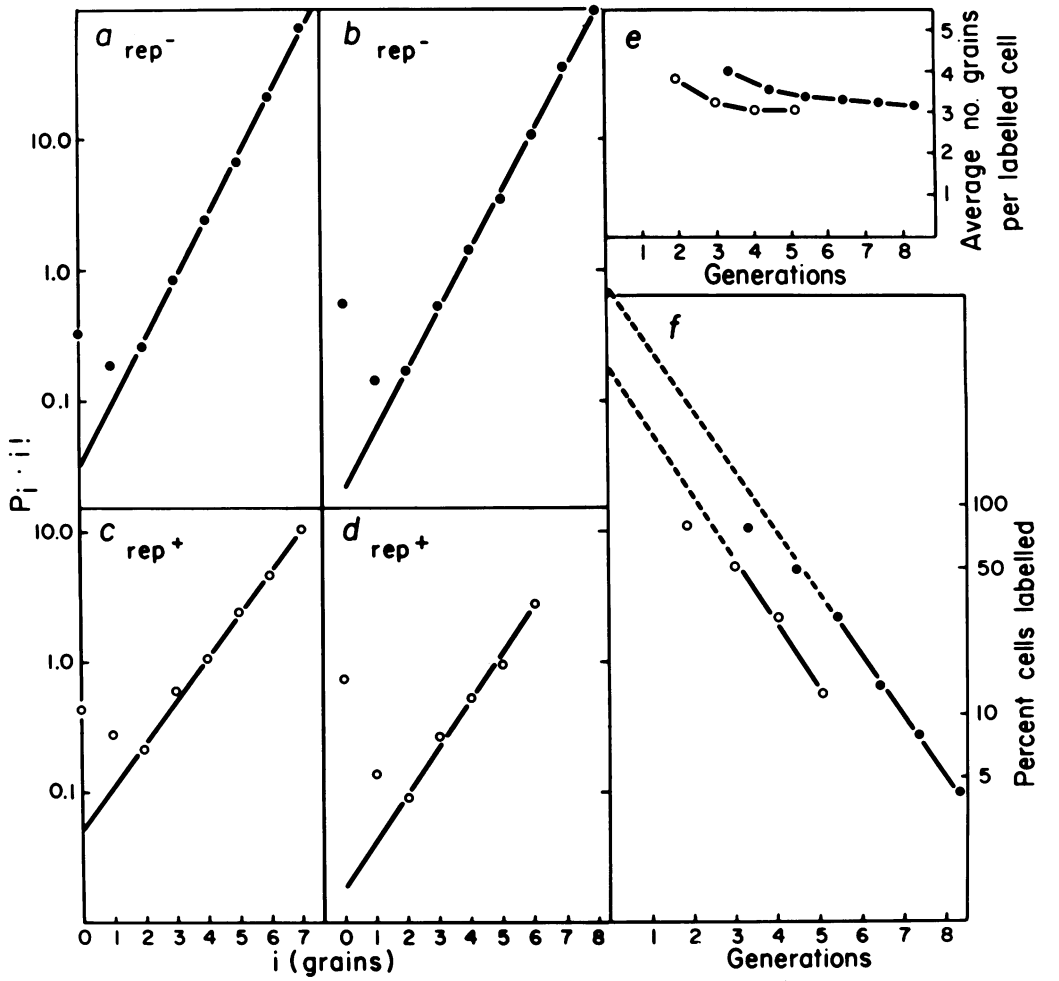

FIG. 4. *Poisson analyses of the segregation of labeled cells. Duplicate slides of those used to obtain the data for Fig. 3b were exposed for 5.5 days, and the number of grains over each cell was counted; 400 to 700 cells were scored for each sample. (a) Poisson distribution of grains for 592 rep cells after 4.4 generations; (b) rep, 415 cells, 5.4 generations; (c) rep⁺, 467 cells, 4.0 generations; (d) rep⁺, 506 cells, 5.1 generations; (e) average number of grains per labeled cell; (f) curve of segregation of unlabeled cells. Symbols: O, rep⁺; ●, rep.*

Because of the large number of growing forks, there should be a larger amount of residual DNA synthesis in the mutant when initiation of DNA replication rounds is blocked and ongoing rounds of replication are permitted to continue. A common method of preventing initiation of new rounds is to block protein synthesis by depriving amino acid auxotrophs of required amino acids (18, 20, 22, 23). The results of an experiment illustrating this effect are shown in Fig. 6. Mx223 *rep*⁺ DNA synthesis ceased after 60 min and an approximate 30% increase in DNA, whereas in *rep* it ceased after about 90 min and a 90% increase. Blockage of protein synthesis in CR *pro* (a *pro*⁻*lac*⁺ transductant of CR *lac*) by proline removal, and in CR by treatment with 200 μg of chloramphenicol per ml, was not complete but was sufficient to illustrate the higher residual DNA synthesis in the *rep* strains.

The nucleoid body is larger in the rep mutant. Because of the increased number of growing forks per replicating chromosome, we expected the nucleoid body of the *rep* mutant to be larger than that of *rep*⁺. The nucleoid body is believed to comprise the entire replicating chromosome of the cell, together with substantial amounts of ribonucleic acid and some protein, and was first isolated in an intact form after lysis in high salt by Stonington and Pettijohn (26). Using similar techniques, Worcel and Burgi (27) extended the characterization and

demonstrated that chromosomes that had completed replication in amino acid-starved cells sedimented more slowly than replicating chromosomes from exponentially growing cells, as would be expected from their relative DNA content.

The sedimentation profiles of nucleoid bodies isolated from exponentially growing *rep*$^+$ and *rep* cells are presented in Fig. 7. The nucleoid bodies isolated from the *rep* mutant have an increased sedimentation rate compared with *rep*$^+$, particularly from cells grown in richer media where the difference between *rep*$^+$ and *rep* is most pronounced (Table 2). This result is consistent with the conclusion that relative to the replicating *rep*$^+$ chromosome the replicating *rep* chromosome has more growing forks and more DNA.

DISCUSSION

Most of the experiments described here involved K-12 strains carrying the *rep3* mutation. The observed effects appear to be typical of *rep* strains in general, since other *rep* strains show the same properties, i.e., more DNA made during inhibition of protein synthesis (Fig. 6), more DNA per cell, and increased cell size (Fig. 5).

Our hybridization data (Fig. 1) indicate that the origin in the *rep* mutant is in the same region of the chromosome as the *rep*$^+$ origin, but perhaps closer to *malA* than to *metA*. Further work with additional strains is needed to settle this point. These results are sufficient, however, to rule out unidirectional replication in *rep* cells. In confirmation, the results of an experiment in which "origin" DNA was successively labeled with 5-bromouracil and [^{3}H]thymidine, extracted, irradiated with ultraviolet light, and sedimented through alkaline sucrose gradients (21) also led to the conclusion that replication in both *rep* and *rep*$^+$ strains is bidirectional (data not shown).

The important and unexpected feature of the hybridization experiment was the ste[illegible] gradi-

TABLE 2. *Comparison of rep*$^+$ *and rep strains of Mx223*

Medium	Generation time	Ratios[a]		
		Cell size	DNA/cell	DNA/mass
L broth	1.15 (27)	2.40 ± .22	2.25 ± .13	0.93 ± .04
M9 Cas	1.10 (39)	1.42 ± .04	1.27 ± .04	0.90 ± .03
M9 Glucose	1.06 (61)	1.26 ± .06	1.07 ± .05	0.87 ± .05
M9 Glycerol	1.02 (87)	1.24 ± .09	1.07 ± .07	0.85 ± .09

[a] All figures are ratios of *rep*/*rep*$^+$ plus or minus standard errors, except those in parentheses which are the generation times of the *rep*$^+$ strain in minutes.

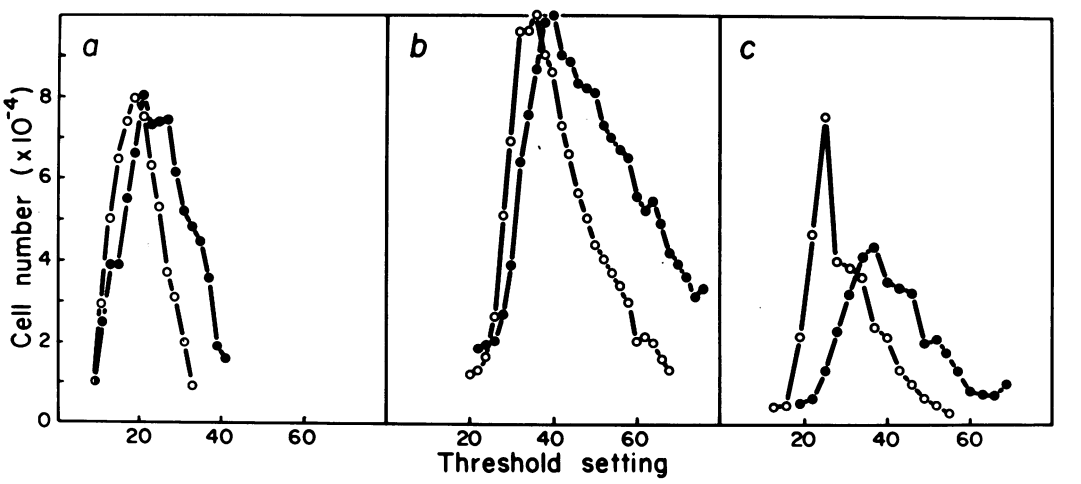

FIG. 5. *Size distributions of rep*$^+$ *and rep cells. Balanced growth cultues of (a) HF4704 rep*$^+$ *and rep*$_{am}$ *(D92, reference 14) in M9 medium containing 0.08% sodium pyruvate, (b) HF4704 rep*$^+$ *and rep*$_{am}$ *in L broth, and (c) Mx223 rep*$^+$ *and rep3 in L broth were sampled and diluted in M9-formalin. Each point represents the number of particles counted in a particular threshold setting interval and is plotted at the midpoint of the interval. Symbols: O, rep*$^+$*; ●, rep.*

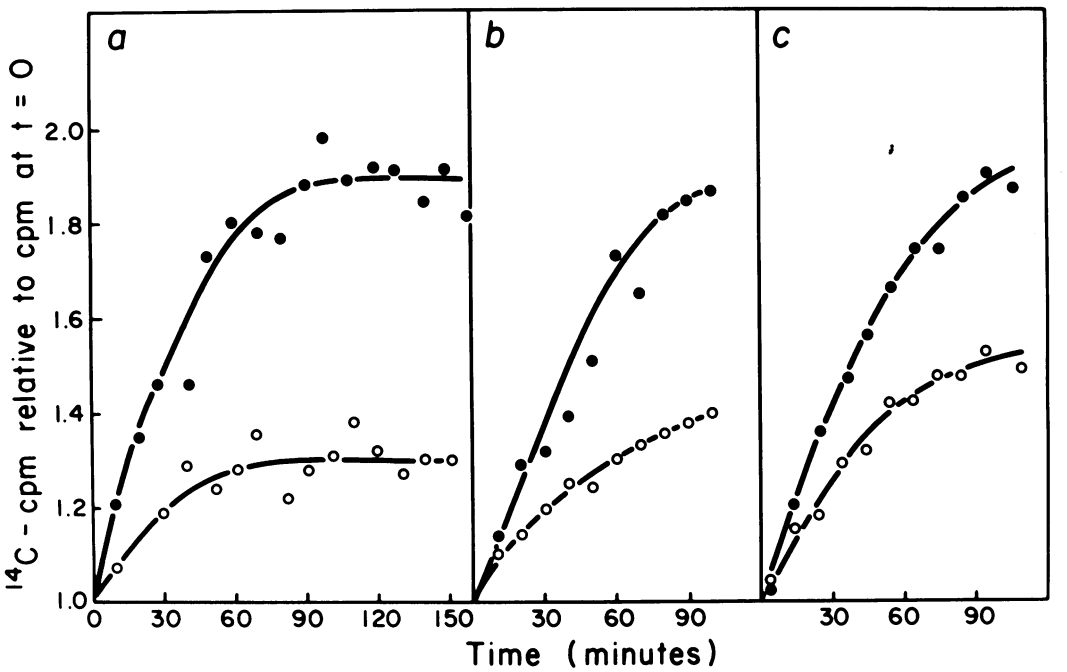

FIG. 6. *DNA synthesized during inhibition of protein synthesis. Isogenic rep$^+$ and rep strains were grown in exponential-phase (10^8 to 2×10^8 cells/ml) in a medium containing [^{14}C]thymine (0.2 μCi/20 μg per ml). Mx223 (a) and a proline-requiring derivative of CR (b) in M9 medium were filtered and washed, and resuspended in the same medium but without amino acids at zero time. To cultures of CR in mT3XD (c) chloramphenicol was added to 200 μg/ml at zero time. Samples were removed into ice-cold 5% trichloroacetic acid containing 1% thymine and thymidine, and kept on ice for at least 1 h. The samples were filtered on Whatman glass-fiber filters and washed with five 5-ml portions of water (95 to 100 C). The filters were dried, and the acid-insoluble radioactivity was determined in a scintillation counter. Data are shown as counts per minute relative to counts per minute at zero time. Symbols: O, rep$^+$; ●, rep.*

ent of marker frequencies in the *rep* mutant, for this strongly suggested that *rep* chromosomes contained more replication forks. The number of replicating forks per chromosome, N, is related to the ratio of origins to termini, O/T, by $N = O/T - 1$ (4). Taking O/T from the plots in Fig. 1, we have 2.6 for *rep*$^+$ and 8.5 for *rep*; these lead to estimates of 1.6 growing forks per chromosome in *rep*$^+$ and 7.5 in *rep* for cells grown in L broth.

The autoradiography experiments confirmed the increased number of growing forks in the *rep* mutant. Regardless of whether cells were scored individually for the numbers of grains and analyzed subsequently by the Poisson distribution, or simply as labeled or unlabeled, the same result was obtained. If we assume that the growing forks are shared equally by two chromosomes in each cell, L broth-grown cells contain 1.35 (*rep*$^+$) and 3.7 (*rep*) growing forks per chromosome. The corresponding figures for cells grown in M9 Cas are 1.1 and 2.8 for *rep*$^+$ and *rep*, respectively. We regard these values as more reliable than those derived from origin/terminus ratios because of the uncertainty concerning the position of the origin and the scatter of data seen especially at high Mu/λ ratios.

The presence of a larger number of replication forks in the *rep* mutant chromosomes implies a higher average amount of DNA per replicating chromosome and, as a consequence, a higher sedimentation rate for the *rep* nucleoid (Fig. 7). Alternative explanations for the faster sedimentation are that *rep* chromosomes possess an altered conformation or that they are associated with a larger amount of non-DNA material. We think these alternatives are unlikely. We do know that the sedimentation rates of both *rep*$^+$ and *rep* nucleoids are higher when the cells are lysed at low temperatures (28), suggesting that nucleoids obtained at 25 C do not have significant amounts of associated membrane material. We have no evidence for or against an altered conformation or composition of *rep* mutant nucleoids. We did verify that there was no sedimentation anomaly of the "cross-over" type

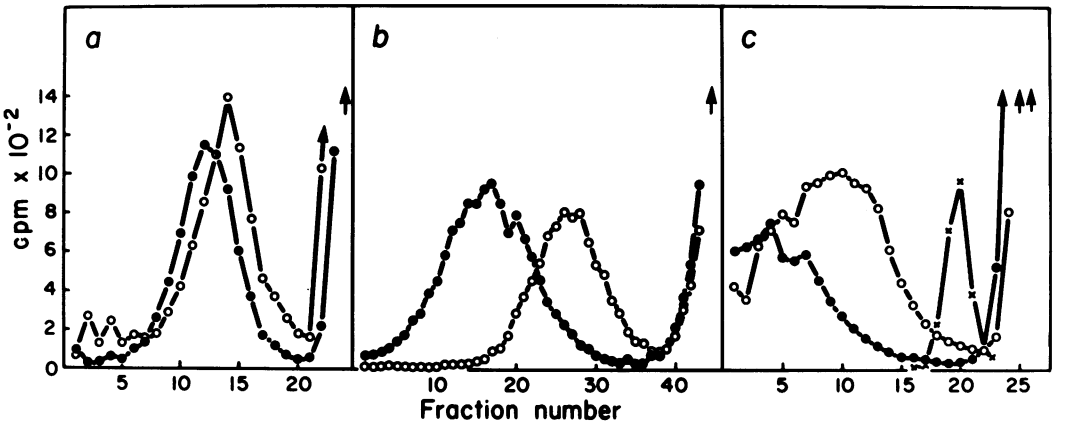

FIG. 7. *Sedimentation of rep⁺ and rep nucleoids. Lysates of rep⁺ and rep cells were sedimented through 5-ml sucrose gradients in an SW50.1 rotor at 17,000 rpm for the times given. Sedimentation is from right to left. The arrow (↑) indicates fractions containing more than 2,000 counts/min, presumably unincorporated precursors. Symbols: ×, ³²P-labeled T4 phage; O, rep⁺; ●, rep. (a) Mx223 grown in M9 glucose; centrifuged for 30 min. (b) Mx223 grown in L broth; centrifuged for 20 min. (c) CR34 grown in L broth; centrifuged for 25 min.*

seen by Chia and Schumaker (11) for large, linear, duplex DNA molecules at different rotor speeds; *rep* nucleoids always sedimented faster than *rep⁺* nucleoids.

An interesting aspect of this work is the similarity between the composition of *rep* cells and that of cells grown in glucose on limiting concentrations of thymine (24, 30). In glucose minimal medium, both *rep* and thymine-limited cells exhibit increased cell size and DNA per cell and a lower but constant DNA/mass ratio compared with *rep⁺* cells and cells grown in high thymine concentrations, respectively. In both cases they showed higher residual DNA synthesis during amino acid starvation and appeared to contain more replicating forks per chromosome. However, Zaritsky and Pritchard (30) found that *E. coli* 15 thymine auxotrophs fail to reach a steady state when grown in glucose minimal medium, even in high thymine concentrations, and their average cell size continuously increased. We found no indication of a continuous increase in cell size or DNA/cell for either *rep⁺* or *rep* strains of *E. coli* K-12 (Mx223) grown in "balanced-growth" conditions in glucose M9 containing 20 μg of thymine per ml. In lower thymine concentrations (0.25 μg/ml), cell size did increase continuously for *rep⁺* and *rep* (data not shown).

It appears, however, that thymine limitation and the *rep* mutation are not equivalent. CR*rep⁺thy⁻*, after growth for several generations on a medium containing 0.2 μg thymine per ml, still supported growth of φX174. In other words, it did not become phenotypically Rep⁻. Moreover the concentrations of thymine nucleotides in the *rep* mutant were similar to those in *rep⁺* cells (6; also unpublished data). For these reasons, we do not believe that the failure to replicate φX and P2 is due to competition for nucleotide pools or for replicative machinery.

A reasonable explanation of the phenomena reported in this paper is that in *rep* mutants the replication forks progress more slowly along the chromosome than in *rep⁺*. Although the results of a mutation in the *rep* gene in many respects mimic the effects of thymine limitation (3, 29), the means by which a reduction in velocity is brought about must be different, for slower replication forks cannot account for the complete failure of *rep* strains to replicate φX and P2 double-stranded DNA. One hypothesis is that the *rep* gene product interacts with the replicative machinery in some manner to make it more efficient; for some substrates, e.g., φX and P2, machinery lacking the *rep* gene product is completely inoperative, whereas for others, e.g., *E. coli*, the *rep*-deficient apparatus is only partially deranged. Experiments to test these ideas are in progress.

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