CHROMOSOME REPLICATION IN *ESCHERICHIA COLI* K12 MUTANT AFFECTED IN THE PROCESS OF DNA INITIATION

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THE regulation of replication of DNA plays a key role in cell multiplication. JACOB, BRENNER and CUZIN (1963) postulated that any genetic element capable of autonomous replication must possess an origin of replication (replicator), and the feasibility of this model seems to be increasing.

Many workers have studied the origin and the order of replication of the *Escherichia coli* chromosome. Some of them suggested that the site of the origin is influenced by the insertion of F, but is random in F^- cells (NAGATA 1963, NISHI and HORIUCHI 1966, VIELMETTER, MESSER and SCHUTTER 1968). The existence of a unique site for the origin has been indicated by others (ABE and TOMIZAWA 1967, WOLF, NEWMAN and GLASER 1968, CARO and BERG 1968, CERDA-OLMEDO and HANAWALT 1968), but the location of the origin is still in slight disagreement.

Mutants of bacteria with temperature sensitive defects in initiation could help to analyze the mechanism of regulation of chromosome replication. Mutants blocked in DNA synthesis at a restrictive temperature have been found and some of them appear to be blocked in initiation (MENDELSON and GROSS 1967, HIROTA, RYTER and JACOB 1968, KUEMPEL 1969). A selective procedure was devised to isolate mutants which are able to finish the round of replication of the chromosome in progress, but not to initiate a new one at an elevated temperature. We describe the experiments performed with one of the thermosensitive mutants isolated by the procedure, and present evidence that the mutation affects the initiation of DNA synthesis. The replication of prophage genomes on the chromosome of the mutant was determined by DNA-DNA hybridization techniques, and the genetic site of initiation and the order of replication are proposed.

MATERIALS AND METHODS

Bacterial and phage strains: E. coli K12 HMT; a thymine and methionine requiring derivative of Hfr Hayes was used for the isolation of temperature sensitive mutants. N167 is one of the mutants isolated by the procedure described below. The bacteria were lysogenized with one of the following phages: λ*sus*08, φ80, 424 and 186 (from Dr. F. JACOB). λc, φ80c, 424c and 186c were clear plaque mutants, and were used for preparation of phage DNA. *E. coli* C600 was used for preparation and titration of these phages. H677 *his,tyrA,purC* (Phabagen Collection), AT2092 *arg*H,*his,phe*A,*pur*F (Coli Genetic Stock Center #3579), AT2457 *gly*A (CGSC #4507),

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AB2277 *ilv,met*E,*his,trp,pro* (EGGERTSSON and ADELBERG 1965) were recipients in P1 transduction (The gene symbols used are those given by TAYLOR and TROTTER 1967).

Media: Mineral medium C (Roberts *et al.* 1957) was supplemented with 0.25% glucose, 0.05% Casamino acid (Difco), 20 μg/ml of tryptophane, 2 μg/ml of thiamine and 2 μg/ml of thymine. This is called CCT. $^{13}C^{15}N$-medium used for the density label is C medium in which NH_4Cl was replaced by 10^{-2}M $^{15}NH_4Cl$ (98% enrichment, Japan Radio Isotope Assoc.) and which was supplemented with 2% of an acid hydrolysate of algae grown with $^{13}CO_2$ (from $Ba^{13}CO_2$, 50% enrichment, Isomet) and $^{15}NH_4Cl$. (Ankistrodesmus was provided by Dr. MESELSON: MESELSON and WEIGLE 1961). In $^{12}C^{14}N$-medium, NH_4Cl and a hydrolysate of algae grown with CO_2 and NH_4Cl were used instead of the heavy materials. Two μg/ml of thymine was added to the media. Peptone broth contained 5g of polypeptone, 2.5g of NaCl per l of water. For peptone agar, peptone broth was solidified with 1.5% of agar. For Penassay broth, Bacto-Penassay broth (Difco) was used. SSC contained 0.15 moles of NaCl and 0.015 moles of sodium citrate adjusted to pH 7.2 with NaOH. Two hundred μg/ml of chloramphenicol was used to inhibit protein synthesis.

Determination of bacterial count: Bacteria were counted by colony count on peptone agar plate containing thymine, after incubation for 24 hr at 30°C.

Measurement of DNA synthesis: Bacteria were grown in CCT medium containing 0.05 μc/ml of ^{14}C-thymine to determine the total amount of DNA. An aliquot was withdrawn at an indicated time and DNA was precipitated with 5% TCA (Trichloroacetic acid). The precipitate was collected on a glass fiber filter and washed with 5% TCA. After drying, the filter was counted in a liquid scintillation counter.

Labeling of DNA for density centrifugation: The bacteria were grown in $^{12}C^{14}N$-medium for several generations. 0.1 μc/ml of ^{14}C-thymine was added when the bacterial DNA was to be labeled continuously. A portion of the growing chromosome was labeled by a pulse of ^{3}H-thymine (20 μc/ml) for 5 min, followed by a chase with 200 μg/ml of thymine, filtration on membrane filter and resuspension in fresh medium. The cells to be labeled with the heavy isotopes were filtered onto a membrane filter, washed with C medium without NH_4Cl and resuspended in $^{13}C^{15}N$-medium, with or without ^{14}C-thymine. They were then incubated at an indicated temperature.

End labeling of DNA for the hybridization: The bacteria to be tested were grown at 30°C for several generations in the CCT medium containing ^{14}C-thymine (1 μc/ml) to about 1×10^8 cells/ml. The cells were filtered, washing thoroughly in CCT medium, suspended in a fresh medium pre-warmed at 42°C, and ^{3}H-thymine (20 μc/ml) was added. The incubation at 42°C was continued for 60 min. The cells were chilled and harvested.

Pulse labeling of DNA for hybridization: Bacteria were grown for several generations in CCT medium which contained ^{14}C-thymine of low specific activity (0.005 μc/ml) to determine the total amount of DNA. Two ml of the culture was taken at an indicated time into a pre-warmed tube containing 20 μc of ^{3}H-thymine and incubated at the indicated temperature. At the end of the pulse labeling period, the tube was chilled in ice. 0.1 ml of sample was added to 1 ml of 5% TCA to determine the incorporation of ^{3}H-thymine into DNA. To the rest of the culture, crushed ice containing 10^{-3} M KCN and thymine (400 μg/tube) was added.

Bacterial DNA preparation: The labeled bacteria were harvested by centrifugation and suspended in 0.5 ml of SSC. The cells were lysed in the presence of EDTA (0.01M) and lysozyme (500 μg/ml) and by freezing-thawing in a dry ice acetone bath. The lysate was treated with trypsin (100 μg/ml) at 37°C for 30 min, followed by treatment with 0.1% of sodium dodecyl sulfate, and shaken by hand with SSC-saturated phenol. The aqueous layer was dialysed against SSC. Non-labeled bacterial DNA used for hybridization was extracted from *E. coli* cells by the same procedure and purified by ribonuclease digestion and repeated precipitation with alcohol. The purified DNA was dissolved in SSC.

CsCl density centrifugation: The dialysed DNA was mixed with 2.5 ml of SSC and CsCl to give a final density of 1.71 g/cm^3 and centrifuged at 35000 rpm for 40 hr in the 65 rotor of a Spinco ultracentrifuge. Fractions were collected from the bottom of the centrifuge tube, and the radioactivity of each fraction was counted.

Phage and phage DNA preparation: Phages were prepared by the confluent plate lysis method. Phages in the lysate were precipitated by polyethylene glycol (SALSER, personal communication). ^{32}P-phages (10 μc/μgP) were prepared by the procedure described by OGAWA and TOMIZAWA (1967). The phages were purified by CsCl density gradient centrifugation. After dialysis of the phages against SSC, DNA was extracted by the SSC-phenol method and dialyzed against SSC.

DNA-DNA hybridization: The hybridization was accomplished by the method described by DENHARDT (1966). Each membrane filter (Millipore, HA) was loaded with 1 μg of denatured phage DNA. The filters were preincubated in a mixture containing Ficoll, polyvinylpyrrolidone and albumin for 6 hr at 63°C. The labeled bacterial DNA was mixed with non-labeled *coli* DNA (10 μg/membrane). For the pulse labeled DNA, ^{32}P-phages were added to measure the efficiency of hybridization of homologous DNA on each membrane. The DNA mixture was denatured and fragmented by heating to 100°C for 7 min in 0.5N NaOH and neutralized with 1N HCl (TOMIZAWA and OGAWA 1968). One ml of DNA mixture in 3 × SSC and a membrane filter loaded with phage DNA were incubated at 63°C for 20 hr. The filters were washed with 3mM Tris buffer pH 9.4 and dried, and radioactivity was measured by a liquid scintillation counter. Duplicate or triplicate membranes were used for each sample.

Isolation of mutants: Cells of a logarithmic phase culture of *E. coli* HMT at 30°C in Penassay broth containing thymine were collected and treated with 700 μg/ml of nitrosoguanidine for 60 min in 0.2 M acetate buffer (pH 5.0) (ADELBERG *et al.* 1965). They were then washed, diluted 1 to 100 in peptone broth containing thymine and grown overnight at 30°C. The mutagenized culture was diluted in CCT to about 2×10^7 cells/ml and incubated at 30°C. When the culture attained about 2×10^8 cells/ml, it was transferred to 42°C and incubated for 60 min. The cells were collected by filtration on a membrane filter and resuspended in prewarmed CCT medium to which 10 μg/ml of 5-bromouracil was added. The incubation of the cells at 42°C was continued for 2 hr. The culture was illuminated with a mercury lamp (Toshiba, SHL, 100V) through a glass plate, at a distance of 7 cm for 2 hr at 0°C. The surviving fraction of cells was usually about 0.1%. After repeating the procedures of the temperature treatment, 5-bromouracil-labeling and the illumination, the surviving cells were spread on peptone agar plates containing thymine and incubated at 30°C. The developed colonies were replica plated on peptone agar containing thymine and incubated at 42°C. About 2–5% of the surviving colonies could not grow at 42°C. The clones of thermosensitive colonies thus obtained were examined for their growth in CCT medium at 30°C. The clones which showed normal growth were further examined for their DNA synthesis by measuring incorporation of ^{14}C-thymine when the exponential cultures were shifted from 30° to 42°C. About 40% of the thermosensitive clones tested showed a residual increase of DNA as if the initiation of a new round of DNA replication was inhibited by exposure to 42°C. The mutant N167 is one of them. Most of the mutants which immediately stop DNA synthesis at 42°C were selected out by the procedure, presumably because they were not able to survive incubation at 42°C without DNA synthesis for 3 hr.

RESULTS

DNA synthesis of mutant N167: When the culture of N167 grown in CCT medium at 30°C was transferred to 42°C, DNA synthesis gradually ceased, reaching, after 40 to 50 min, roughly 150% of the level present at the time of the temperature shift. If the temperature was shifted back to 30°C, DNA synthesis resumed almost immediately (Figure 1). The rate of the resumed synthesis was greater than that of exponentially growing cells at 30°C. One doubling time was 55 min in the exponentially growing culture at 30°C and 45 min for the resumed synthesis. The resumed synthesis stopped after a lag when the temperature was raised again to 42°C. The residual increase depends on the time when the temperature was raised (Figure 1). If the temperature was shifted up

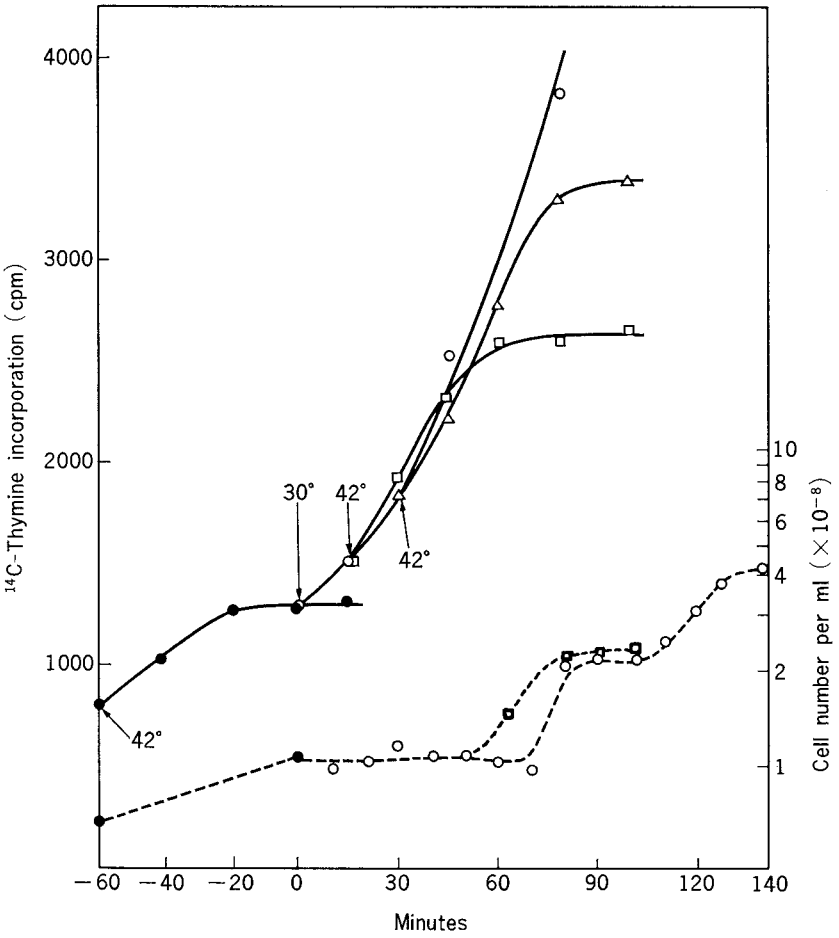

FIGURE 1.—DNA synthesis and cell division at different temperatures. An exponential culture of N167 grown at 30°C in CCT containing ^{14}C-thymine was incubated at 42°C (●). After 60 min at 42°C, the culture was shifted back to 30°C (○). It was again exposed to 42°C at 15 min (□) or 30 min (△) at 30°C. —— for DNA, - - - - for cell number.

again 15 min after the shift down, the DNA synthesis stopped when DNA had doubled. With a shift up at 30 min, DNA synthesis proceeded further, and stopped, as seen in Figure 1. Figure 1 also shows the increase of cell number measured by colony count during the temperature treatment of the culture. The cell number doubled during the first exposure to 42°C, and after the shift down to 30°C, cell division occurred in a stepwise fashion. The first division occurred 80 to 90 min after the initiation. If the temperature was raised 15 min after the shift down, stepwise division occurred once, after the termination of DNA synthesis.

Chloramphenicol added 10 min before resumption blocked the synthesis (Figure 2). If the drug was added at the time of the shift down to 30°C, synthesis

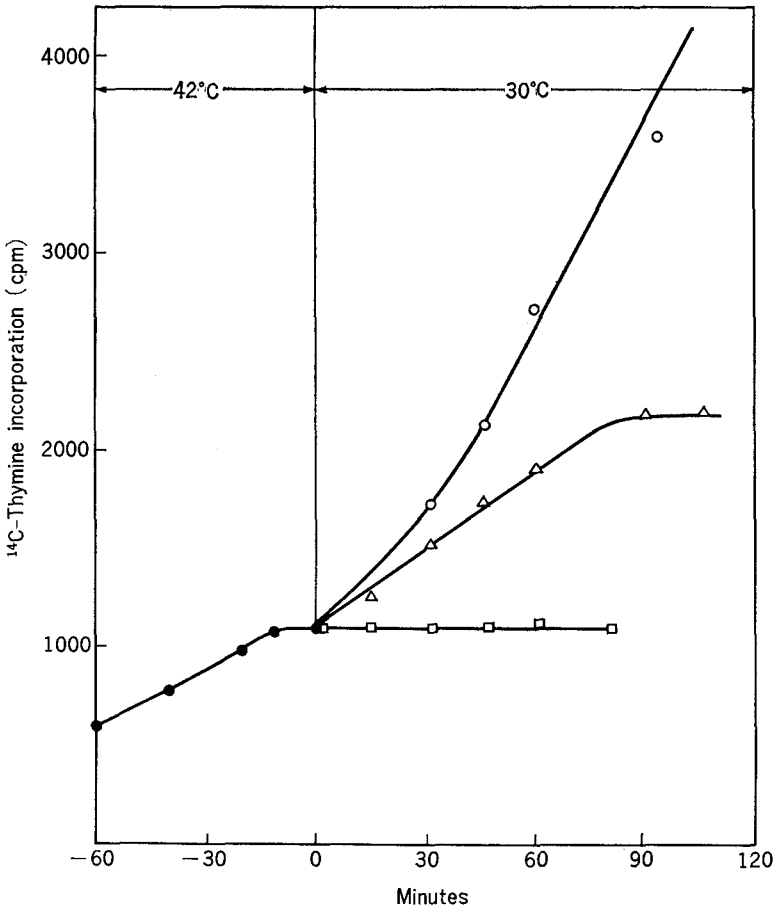

FIGURE 2.—Effect of chloramphenicol on the resumed synthesis of DNA at 30°C. An exponential culture of N167 grown at 30°C in CCT containing ^{14}C-thymine was incubated at 42°C for 60 min (-●—●-) and shifted back to 30°C (-O—O-). Chloramphenicol was added to 200 μg/ml at 10 min before the shift down to 30°C (-□—□-) or at the time of the shift down (-△—△-).

occurred but came to a halt at 80 to 90 min, when DNA had doubled. These properties of the mutant suggested that DNA synthesis in N167 at 42°C continues till a round of synthesis is finished and then stops, and new initiation occurs synchronously upon shift down to 30°C. The initiation depends upon protein synthesis.

To prove the synchronous initiation of replication of the bacterial chromosome at a fixed point after the temperature shift of N167 culture, the following experiments were performed.

The cells in an exponential culture of N167 grown in $^{12}C^{14}N$-medium containing ^{14}C-thymine were pulse labeled with ^{3}H-thymine. The cells were suspended in $^{12}C^{14}N$-medium with ^{14}C-thymine and exposed to 42°C for 60 min.

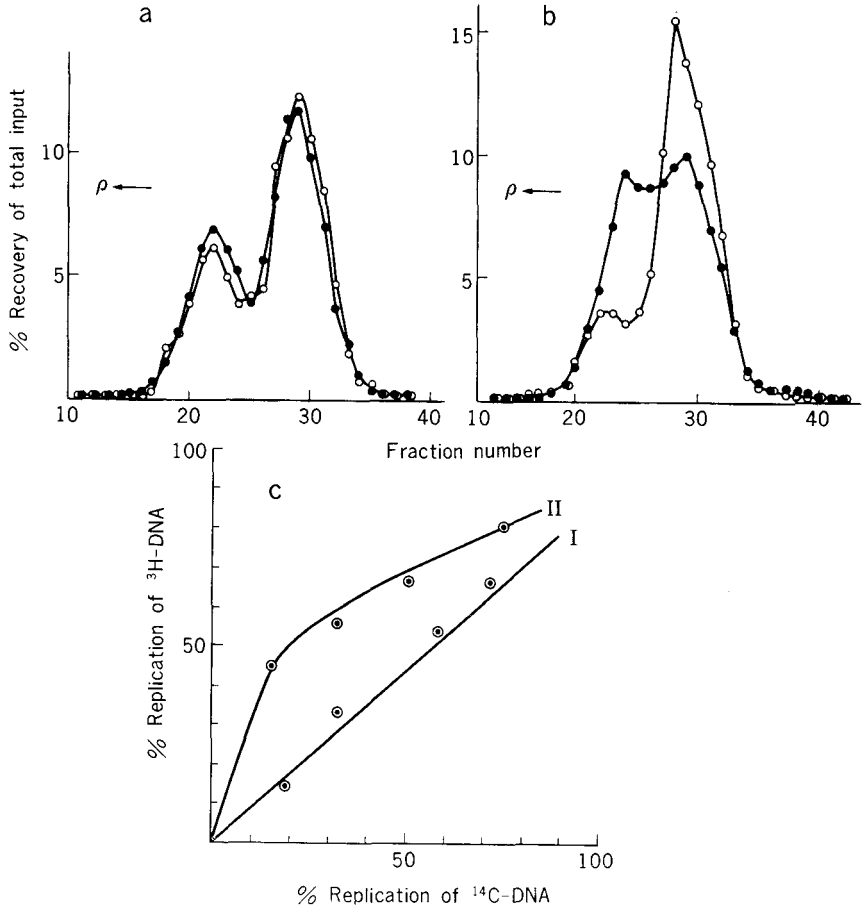

FIGURE 3.—CsCl density centrifugation of DNA pulse labeled in the exponential phase or at the initiation of resumed synthesis. (a) An exponential culture of N167 grown at 30°C in $^{12}C^{14}N$-medium containing ^{14}C-thymine was pulse labeled with ^{3}H-thymine for 5 min, immediately exposed to 42°C for 60 min, and then transferred to $^{13}C^{15}N$-medium and incubated at 30°C. DNA was extracted from the cells every 15 min and centrifuged in CsCl density gradients. The figure shows radioactivity profile of the DNA sample at 30 min. Total input of radioactivity was: $^{3}H = 4800$, $^{14}C = 1800$ (b) An exponential culture of N167 grown as (a) was exposed to 42°C for 60 min, returned to 30°C and immediately pulse labeled with ^{3}H-thymine for 5 min. After 15 min at 30°C, it was again replaced to 42°C and incubated for 45 min. The cells of the culture were then transferred to $^{13}C^{15}N$-medium and incubated at 30°C. DNA samples were prepared at 20, 30, 40, 60 min and centrifuged as above. The figure shows the radioactivity profile of the sample at 20 min. Total input of radioactivity was: $^{3}H = 4500$, $^{14}C = 2650$ (c) From the density distribution of ^{3}H-DNA and ^{14}C-DNA, the percent of replicated DNA (in hybrid density fraction) to the total labeled DNA was calculated for every DNA sample. The values for ^{3}H-DNA were plotted *versus* those for ^{14}C-DNA. Curve I shows the results of experiments (a) and curve II shows those of experiment (b). (-O—O- for ^{14}C, -●—●- for ^{3}H).

They were then transferred to $^{13}C^{15}N$-medium and incubated at 30°C. DNA was extracted from a portion of the cells every 15 min during the incubation, and centrifuged in CsCl density gradients. By measuring the density distribution of the radioactivity, the replication of the ^{3}H-pulse-labeled region and ^{14}C-labeled DNA was followed. The ^{3}H-labeled region proceeded to replicate with the *same rate* as the resumed synthesis of the total DNA (Figure 3a and curve I in c). This indicates the alteration of the replication sequence of the chromosome, as seen after amino acid starvation or 5-bromouracil replacement (LARK, REPKO and HOFFMAN 1963, ABE and TOMIZAWA 1967). The alteration was such that the preexisting growing point ceased during the growth at 42°C and replication was initiated at fixed point(s) or at randomly distributed point(s) on the chromosome. To test whether the altered replication started at fixed point(s), the following experiment was carried out. A culture of N167 grown in $^{12}C^{14}N$-medium in the presence of ^{14}C-thymine at 30°C was transferred to 42°C and incubated for 60 min. The culture was replaced to 30°C and pulse labeled with ^{3}H-thymine for 7 min. After another 8 min at 30°C, the cells were again exposed to 42°C for 45 min, collected on a membrane filter, suspended in $^{13}C^{15}N$-medium and incubated at 30°C. DNA samples were prepared from the cells taken at intervals during the incubation in the heavy medium and centrifuged in CsCl solutions. The replication pattern of ^{3}H *versus* ^{14}C shows that a large fraction of ^{3}H-labeled DNA replicated earlier than overall DNA (Figure 3b, and curve II in c). This means that the reinitiation of DNA synthesis after shift up and subsequent shift down occurred at fixed point(s) on the chromosome.

The observation that the rate of the resumed DNA synthesis at 30°C was greater than that in an exponential culture suggested an increase in numbers of the replication point and this was proven by the following experiment. The cells of an exponential culture in $^{12}C^{14}N$-medium were pulse labeled with ^{3}H-thymine and incubated at 42°C for 60 min. They were then transferred to $^{13}C^{15}N$-medium containing ^{14}C-thymine and incubated at 30°C. DNA samples were prepared from the cells at 20 min, 40 min and 60 min, and analyzed by CsCl density gradient centrifugation. The ^{14}C-labeled DNA appeared in the full heavy region even though the portion which was pulse labeled with ^{3}H-thymine was still in the light fraction (Figure 4a). This indicates that DNA synthesis was initiated prematurely. When the temperature was raised again at 15 min during the resumed synthesis in the heavy medium containing ^{14}C-thymine, the ^{14}C-labeled DNA did not appear in the full heavy fraction indicating that the dichotomous reinitiation is also thermosensitive (Figure 4b).

The origin and the direction of the replication of the chromosome: The above experiments showed that preexisting growing points of the chromosomes of N167 ceased during the growth at 42°C and after subsequent shift down of the temperature, and the replication initiated synchronously from fixed area(s) of the chromosomes. These properties of the mutant seemed likely to make possible an investigation of the genetic locus of the initiation and the order of replication of the chromosome of *E. coli*. The following experiments were performed to determine the order of replication of genetic markers on the chromosome of N167,

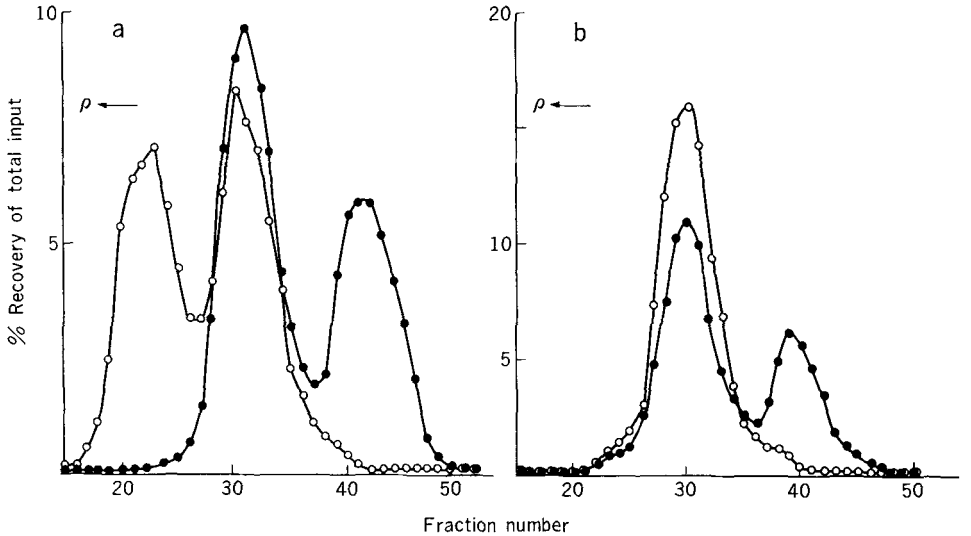

FIGURE 4.—Premature initiation after temperature shift from 42°C to 30°C. An exponential culture of N167 grown in $^{12}C^{14}N$-medium at 30°C was pulse labeled with ^{3}H-thymine for 5 min, and incubated at 42°C for 60 min. The cells of the culture were then transferred to $^{13}C^{15}N$-medium containing ^{14}C-thymine and incubated at 30°C. They remained at 30°C all the time (a), or were transferred to 42°C after 15 min at 30°C (b). The figures show the radioactivity profile in CsCl density centrifugation of DNA prepared from the cells incubated for 60 min in the heavy medium. Total input of radioactivity was: (a) $^{3}H = 4500$, $^{14}C = 2100$ (b) $^{3}H = 5900$, $^{14}C = 2900$ (-○—○- for ^{14}C; -●—●- for ^{3}H).

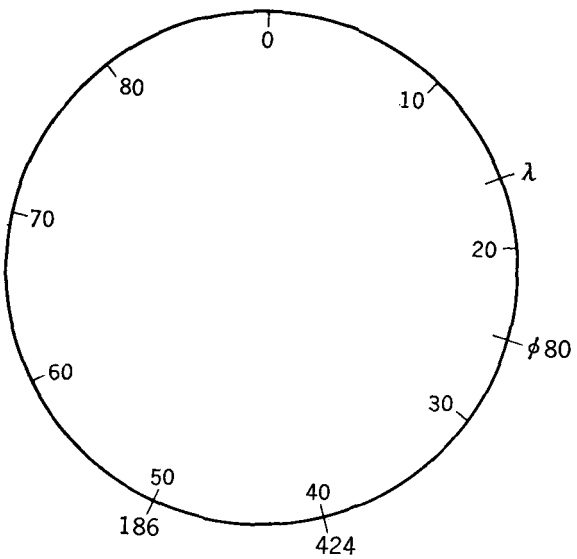

FIGURE 5.—Map of the prophage loci. The attachment sites of the prophages used are shown on the K12 chromosome map (TAYLOR and TROTTER 1967).

and the results were analyzed assuming that DNA synthesis reached the *terminus* during exposure to 42°C and initiated synchronously at the *origin* by shift down to 30°C.

DNA of the lysogenic N167 was labeled with radioactive thymine during or after the temperature treatment as described below, DNA was extracted and annealed to phage DNA fixed on membrane filters. From the radioactivity bound to the membrane, the appearance of the prophage chromosome in the labeled region of the bacterial chromosome was measured and the order of the replication of various prophages was estimated. The relevant prophage loci are shown in Figure 5. The exact genetic locus of the 186 attachment site had never been reported. It was found that prophage 186 is cotransducible with *phe*A(50′), *tyr*A(50′), and *gly*A(49′) at a frequency of 14% (16/116), 20% (39/190) and 2% (3/157), respectively. (The numbers which follow gene symbols indicate the positions on the genetic map according to TAYLOR and TROTTER 1967). The 186 attachment site must be very close to the 50′ position.

(1) *End labeling of DNA at 42°C:* The lysogenic bacteria grown in CCT medium containing ^{14}C-thymine at 30°C were transferred to the medium containing ^{3}H-thymine at 42°C. After 60 min, DNA was extracted. In this way, DNA of the replicating chromosome in the exponential state was labeled with ^{14}C and the replicating end of DNA was labeled with ^{3}H. Non-lysogenic N167 was also labeled by the same procedure and DNA was extracted. Each DNA preparation was annealed to phage DNA homologous to the prophage present. The percentage of the radioactivity specifically bound to phage DNA compared to the total input radioactivity was obtained, and the ratio of the percent of end-label (^{3}H) to exponential label (^{14}C) for each phage was calculated (see the legend of Figure 6). Since the age distribution function of the chromosome in an exponentially growing population can be expressed by equation (1) (SUEOKA and YOSHIKAWA 1965), the theoretical value of the ratio of ^{3}H label in a prophage positioned at x to the total ^{3}H label, and that of ^{14}C label in the prophage to the total ^{14}C label can be expressed by equations (2) and (3), respectively.

$$f(x) = (\ln 2)\, 2^{1-x} \tag{1}$$

where x is the position of replication point on the chromosome.

$$2\xi\int_0^x f(x)dx \Big/ 2\int_0^1 (1-x)\, f(x)\, dx = -2\xi\,(\ln 2)\,(2^{-x}-1) \Big/ 2\ln 2 - 1 \tag{2}$$

$$\xi\left\{\int_0^x f(x)dx + 2\int_x^1 f(x)dx\right\} \Big/ \int_0^1 (1+x)\, f(x)dx = (\xi \ln 2)\, 2^{1-x} \tag{3}$$

where ξ is the size of a prophage genome relative to a chromosome of bacteria ($0<\xi<1$). From the equations (2) and (3), the following relation can be derived.

$$\frac{\text{percent of hybridized } ^{3}\text{H in end label}}{\text{percent of hybridized } ^{14}\text{C in exponential label}} = \frac{2^x - 1}{2\ln 2 - 1} \tag{4}$$

This ratio becomes smaller as the prophage is located closer to the origin. From the experimental values for each phage, it can be deduced that the position of 186 is closest to the origin among the phages used, and the order of replication is 186—λ—φ80—424. Tentatively plotting 424 at x=1 on a chromosome replicating clockwise, we obtain the value of x for each phage from the map position of the prophage, as in Figure 6. The experimental values of the ratio of per-

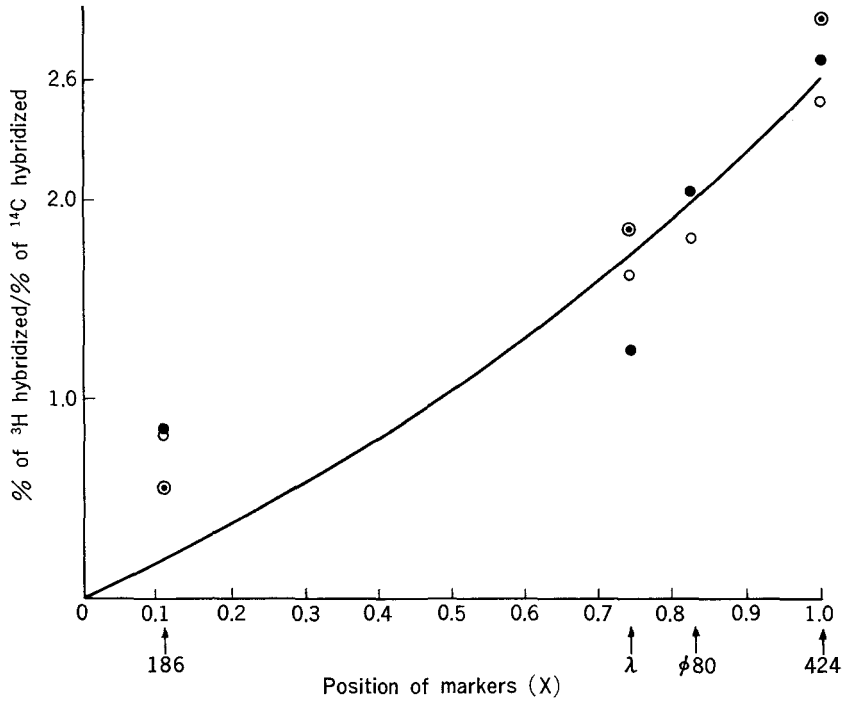

FIGURE 6.—Hybridization of end-labeled DNA. The ratio of the percentage of the hybridization of end-labeled DNA to that of exponential labeled DNA for each phage was calculated. An example is shown below.

Source of DNA	Percent of labeled DNA hybridized to phage 424 DNA: End label ³H-DNA	Exponential label ¹⁴C-DNA
N167(424)	1.51	0.69
N167	0.26	0.19
Specific binding	1.25	0.50

$$\text{Ratio of end to exponential} = \frac{1.25}{0.50} = 2.5$$

The solid line shows the theoretical value of the ratio for position x on the bacterial chromosome. 424 was tentatively plotted at $x=1$ and x for the other prophages was obtained according to the map in Figure 5 with clockwise replication.

centage of hybridization of ^{3}H and ^{14}C are plotted *versus* x for each phage on the figure. As revealed in Figure 6, the experimental values agree fairly well with the theoretical values except for 186. From these results, the origin would be located between 424 (40′) and 186 (50′).

(2) *Pulse-labeling of DNA during synchronous replication:* DNA synthesis of N167 (λ*sus*08) grown in CCT medium containing ^{14}C-thymine was synchronized by incubating cells at 42°C and then bringing them back to 30°C. Fifteen min after the shift back to 30°C, the temperature was again raised to 42°C to allow one round of replication but no further initiation. A portion of the culture was

labeled with ^{3}H-thymine at every interval during the synchronous replication. The intervals were so arranged that about one eighth or one-tenth of a chromosome is labeled during the period. From the amount of ^{14}C-thymine incorporation determined at appropriate intervals, we could estimate the time when the DNA had doubled. It took about 40–50 min after the shift down to 30°. DNA was extracted from the pulse labeled cells and annealed to phage DNA on membrane filters. The percent value of ^{3}H-activity specifically bound to phage DNA was corrected by the hybridization efficiency of the control of ^{32}P-phage DNA. The amounts of the replicated prophage genomes in each pulse labeled segment of DNA were obtained by multiplying the fractional content of phage specific DNA by the total amount of ^{3}H-thymine incorporated during the period of each pulse labeling. The results are shown in Figure 7. The pattern of the replication of the

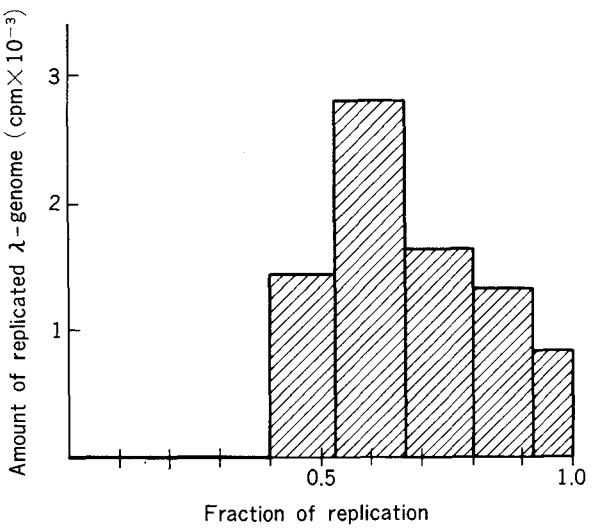

FIGURE 7.—Hybridization of pulse labeled DNA of N167(λ*sus*08). DNA synthesis of N167 (λ*sus*08) was synchronized by temperature treatment as described in the text. During the round of DNA replication, 2 ml each of the culture was taken and pulse labeled with ^{3}H-thymine. The first sample was taken at 0 min of the resumed synthesis at 30°C, the interval to the second sample was 15 min, and later samples were taken each 5 min. They were labeled during the interval without overlap. The incorporation of ^{3}H-thymine into DNA was determined. DNA was extracted and annealed, in the presence of ^{32}P-phage DNA, to phage DNA. The calculation was done as follows:

	Percent of hybridization of ^{3}H-DNA (a)	Percent of hybridization of ^{32}P-phage DNA (b)	Percent of hybridization corrected by efficiency a/b×100	Percent of specific binding (c)
lysogenic bacteria DNA	1.36	62.7	2.17	1.78
non-lysogenic bacteria DNA	0.25	63.3	0.39	...

The amount of the replicated λ-genome = radioactivity of incorporated ^{3}H-thymine × (c)
$= 1.6 \times 10^{5}$ cpm $\times$ 1.78 $= 2.8 \times 10^{3}$ cpm

The ratio of the amount of ^{3}H-thymine incorporation in a pulse to the sum total of ^{3}H-thymine incorporation in every pulse was calculated. The ratios of all pulses were sequentially added and plotted on the abscissa to show the fraction of the replication of DNA.

prophage genome agrees with one that might be expected from the origin and the order of the replication deduced from the results of the experiment with the end-labeled DNA.

DISCUSSION

DNA synthesis in cells of strain N167 stopped at 42°C after a residual increase, and resumed upon shift down to 30°C. Chloramphenicol added before the shift down completely abolished the resumption of the synthesis. The density label experiments showed that premature initiation occurred after the initiation of the resumed synthesis. The premature initiation did not occur when the culture was again exposed to 42°C at 15 min after the shift down. Both the first initiation of the resumed synthesis and the premature initiation may depend upon a protein which is thermosensitive and affected by the mutation. Chloramphenicol added at the time of the temperature shift down did not prevent the resumed synthesis of DNA, but prevented the premature initiation. It seems that the synthesis of the protein is immediate and the delay of the effect of chloramphenicol permitted the first initiation of DNA synthesis.

The occurrence of synchronous initiation of DNA replication in N167 at fixed point(s), probably at the origin, upon shift down of temperature subsequent to shift up was clearly shown by the results of the experiments (Figure 3). But it is not obvious whether, at the high temperature, DNA synthesis continues till a round of replication is finished or stops at a random location. However, the result of the density label experiment shown in Figure 3a indicates that preexisting growing points did not proceed upon shift down to 30°C, and the blocked growing point did not interfere with the propagation of a newly formed growing point at 30°C. Moreover, during the residual synthesis at 42°C, cell number doubled, or increased a little more than two fold, indicating that cell division occurred in all the cells. If cell division depends upon termination of chromosome replication but not upon initiation (CLARK 1968), then chromosome replication must terminate in all the cells at 42°C. These results strongly suggest that during growth at 42°C, DNA replicated till a round of synthesis was finished. Furthermore, a reasonable interpretation would be difficult to give for the result of the hybridization experiment of the end-labeled DNA if we assumed that the chromosome replication is arrested at a random location.

The coordination of DNA synthesis and division in *E. coli* cells has been investigated and models of the division cycle have been presented. The initiation of chromosome replication has been thought to be regulated by the increase of cell mass or the accumulation of regulatory substance(s) (HELMSTETTER 1968, CLARK 1968). When the DNA synthesis in N167 was synchronized by incubating at 42°C and reinitiating at 30°C, the division occurred stepwise (Figure 1). The phasing of the cell division cycle might be considered as the consequence of the synchronization of DNA replication. The sequential event of cell division and DNA replication in N167 is explained in the following way. After the temperature shift of the exponential culture of N167 to 42°C, all the cells divided

during the residual synthesis of DNA, as described above. Therefore, after 60 min at 42°C, the cells may be in a phase which follows the completion of a round of DNA replication and of cell division. In fact, the size of the cells was microscopically uniformly small. The first stepwise division after the shift down to 30°C would be a consequence of the sequential reaction: initiation, termination and division process, occurring synchronously. We explain that the premature initiation observed after the initiation is caused by excess accumulation of the regulatory substance(s) during the growth at 42°C. The second division would then be thought of as the consequence of the termination of the prematurely initiated replication. The interval between the first and the second division was shorter, as expected.

The hybridization experiments were designed to determine the frequency of a prophage genome on the bacterial chromosome. If autonomous replication of a phage genome was induced, even in a small population, the interpretation of the results would not be correct. For the lysogenization of λphage, we used λ*sus*08 which is defective in DNA replication in *su*⁻ bacteria, like N167 (JOYNER *et al.* 1966, OGAWA and TOMIZAWA 1967). Although no such markers are known for the other phages, the rate of the spontaneous induction of these prophages did not increase in our experimental conditions (data not shown). Furthermore, the function for φ80 replication is thermosensitive (Dr. OZEKI, personal communication). Therefore, an unfavorable effect of induction on the experiments is not expected.

From the results of hybridization, and assuming unidirectional replication of the chromosome with one replication point and one origin, the order of 186–λ–φ80–424 was suggested. It is consistent with our previous results that the replication of the chromosome proceeds clockwise from the origin between *lys* and *his*. The ratio of $^{3}H/^{14}C$ in the end-labeling of the 186 lysogen is higher than the postulated theoretical value, in spite of the coincidence of the values for the other lysogens with the theoretical curve. In the derivation of the equations, it was assumed that all the cells in the population had one replicating point and no resting period, and that every cell finished the round of replication but did not initiate a new one after shift up of the temperature. If any of the assumptions are not satisfied in the experimental conditions, the experimental value will deviate from the calculated value. In fact, the residual increase of DNA synthesis at 42°C in N167 was usually 50%, higher than the 39% value calculated by SUEOKA and YOSHIKAWA 1965 for DNA replication at one point without a resting period. The pattern of the deviation of ratio of 186 would be explained if 1) a part of the population has a resting period after formation of the temperature sensitive protein; or 2) a part of the population could initiate at 42°C. Even with this reservation, the order of replication, and consequently the location of the origin postulated from the results, would not be changed.

During the preparation of this report, it was reported that the temperature sensitive mutation at *dna*A involved in the process of the initiation of DNA synthesis was cotransduced with *ilv* by P1 phage (HIROTA, MORDOH and JACOB

1970). We examined our mutant N167 and found that the thermosensitivity is cotransducible with *ilv* to AB2277 at a frequency of 18% (50/280). The genetic locus of the mutation of N167 may be the same or very close to *dna*A.

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SUMMARY

A thermosensitive mutant of *E. coli* K12 was isolated by a selective procedure. DNA replication of the mutant ceases at the termination of the round at 42°C, and initiates from fixed points on return to 30°C. Chloramphenicol added before the temperature shift inhibited the initiation. The premature initiation occurred dichotomously after the initiation at 30°C, and was prevented by exposure to 42°C or by chloramphencol. By DNA-DNA hybridization technique, the replication of prophage genomes on the chromosome of the mutant cells was revealed to be synchronized with the temperature shift. An order of replication of 186–λ–φ80–424, and the location of the origin between 424 and 186 are proposed.

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