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Replication of Deoxyribonucleic Acid in *Escherichia coli* C Mutants Temperature Sensitive in the Initiation of Chromosome Replication

HIROSHI SAKAI, SEIJI HASHIMOTO, AND TOHRU KOMANO

Laboratory of Biochemistry, Department of Agricultural Chemistry, Kyoto University, Kyoto, Japan

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An *Escherichia coli* HF4704S mutant temperature sensitive in deoxyribonucleic acid (DNA) synthesis and different from any previously characterized mutant was isolated. The mutated gene in this strain was designated *dnaH*. The mutant could grow normally at 27 C but not at 43 C, and DNA synthesis continued for an hour at a decreasing rate and then ceased. After temperature shift-up, the increased amount of DNA was 40 to 50%. When the culture was incubated at 43 C for 70 min and then transferred to 27 C, DNA synthesis resumed after about 50 min, initiating synchronously at a fixed region on the bacterial chromosome. The initiation step in DNA replication sensitive to 30 μg of chloramphenicol per ml occurs synchronously before the resumption of DNA replication after the temperature shift-down, being completed about 30 min before the start of DNA replication. When the cells incubated at 27 C in the presence of 30 μg of chloramphenicol per ml after the temperature shift-down to 27 C were transferred to 43 C with simultaneous removal of the antibiotic, no resumption of DNA replication was observed. When the culture was returned to 43 C after being released from high-temperature inhibition at 30 min before the start of DNA replication, no recovery replication was observed; whereas at 20 min, the recovery of replication was observed. These results indicated that HF4704S was temperature sensitive in the initiation of DNA replication. Analysis of HF4704S, by an interrupted conjugation experiment, indicated that gene *dnaH* was located at about 64 min on the *E. coli* C linkage map. In *E. coli* S1814 (a K-12 derivative), which was a *dnaH*^{ts} transductant from HF4704S (C strain) with phage P1, the mutated gene (*dnaH*) was demonstrated to be closely linked to the *thyA* marker by conjugation and P1 transduction experiments and to be distinct from genes *dnaA* through *dnaG*.

It has been determined that chromosome replication in bacterial cells proceeds sequentially from a fixed site in both directions (1, 4, 11, 26, 31). It has been also suggested that the initiation of chromosome replication required the simultaneous synthesis of certain proteins and that once replication starts the replication cycle will be completed in the absence of further protein synthesis (18, 24, 35). Thus, deoxyribonucleic acid (DNA) synthesis is controlled in vivo by regulating the frequency of the initiation of chromosome replication corresponding to cell division.

To investigate the regulatory mechanism of chromosome replication in *Escherichia coli* cells by characterizing conditional mutants altered in DNA synthesis, temperature-sensitive mutants have been isolated (2, 3, 6, 8, 12-15, 17). These mutations have been grouped into seven classes according to genetic analyses, *dnaA*, *-B*, *-C*, *-D*, *-E*, *-F*, and *-G* (32). The *dnaE* mutation corresponds to the temperature sensitivity of DNA polymerase III (10), and *dnaF* corresponds to that of ribonucleotide diphosphate reductase (9). Although it is not clear what steps are thermolabile in the case of other *dna* mutations, *dnaA* and *dnaC* are assumed to be responsible for the initiation process of DNA replication; *dnaA* and *dnaB* gene products, which are proteins, although the functions are not clear, have been partially purified, and are inactivated at higher temperatures in the in vitro assay that φX 174 system provides (30, 33). The products of *dnaA*, *-B*, *-C*, *-D*, *-E*, and *-G* genes are involved in the in vitro conversion of φX 174 single-stranded DNA to the replicative form

(30, 33), and the product of *dnaE* gene is involved in the conversion in the case of phage fd (33).

Thus, examination of the relationship between the functions of bacterial cells and the phage DNA replication will make it possible to identify the specific steps involved in the bacterial DNA replication and the dependency of phage DNA replication on the host cell functions.

To identify these specific steps, we isolated a new type of mutant temperature sensitive in the initiation of DNA replication, *E. coli* C (HF4704S), and tentatively designated the mutation *dnaH*. This report describes the basic properties of the *dnaH* mutation which affects the initiation of DNA replication.

MATERIALS AND METHODS

Bacterial strains. *E. coli* HF4704 (*hcr*$^-$, *thy*$^-$) was a derivative of strain C. HF4704S, a temperature-sensitive mutant, was derived from HF4704. HF4704S-11 was a streptomycin-resistant strain of HF4704S. C-129 (Hfr-4, *ura*$^-$, *str*^{s}) and C-1091 (Hfr-12, *ura*$^-$, *str*^{s}) were derivatives of strain C and used for the genetic analysis of HF4704S. (These Hfrs were kindly given by G. Bertani.) *E. coli* BT1000, a derivative of strain K-12, was a gift from R. L. Sinsheimer. S1814 (F$^+$, EndI$^-$, Sup$^-$, *str*^{r}, *thyA*$^-$, *polA1*$^-$, *dnaH*^{ts}) was constructed by transducing BT1000 (F$^+$, EndI$^-$, Sup$^-$, *str*^{r}, *thyA*$^-$, *polA1*$^-$) with a P1 lysate from HF4704S. *E. coli* KL16 (Hfr, *thi*$^-$) and PK191 (Hfr) were derivatives of strain K-12 and used for the crosses with S1814.

Media and buffers. TKB medium and starvation buffer (SB) were described by Denhardt and Sinsheimer (7). TPG-CA medium was described by Morishima et al. (25). Nutrient agar contained (per liter): tryptone (Difco Laboratories), 10 g; NaCl, 2.5 g; KCl, 2.5 g; agar, 10 g; and thymine, 20 mg. Liquid minimal medium, minimal agar, phosphate buffer, and minimal salts were described by Clowes and Hayes (5). Tryptone broth and crossing buffer were described by Sasaki and Bertani (29). CPM agar, modified UTA agar, contained (per liter): K_2HPO_4, 7 g; KH_2PO_4, 2 g; sodium citrate·$5H_2O$, 0.5 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; L-asparagine, 1 g; glucose, 5 g; agar, 14 g; Casamino Acids, 2.7 g; methionine, 0.01 g; phenylalanine, 0.01 g; arginine, 0.01 g; and tryptophan, 0.01 g. L broth contained (per liter): tryptone (Difco), 10 g; yeast extract, 5 g; NaCl, 5 g; glucose, 1 g; thymine, 0.02 g; and $CaCl_2$, 2.775 g. (pH was adjusted to 7.0 with 1 N NaOH.) L broth top agar is L broth containing 0.7% agar, and L broth bottom agar is L broth containing 1.2% agar.

Culture methods. Cell growth was followed spectrophotometrically at 610 nm or by viable counts. To change growth media or to remove supplements, the cells were pelleted by centrifugation at 3,000 × *g* for 10 min, washed once or twice with SB, and then resuspended in a fresh medium or SB.

Isolation of a temperature-sensitive mutant. A culture of *E. coli* HF4704, grown to 2 × 10^8 cells per ml in TKB medium containing 20 μg of thymine per ml, was transferred to fresh TKB medium supplemented with 20 μg of thymine per ml and mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (0.1 mg/ml) and incubated at 37 C for 15 min. After removal of NTG, the cells were incubated overnight at 27 C in the same medium without the mutagen. A portion of the culture was diluted 100-fold into TKB medium containing 20 μg of thymine per ml and 200 U of penicillin per ml, and incubated overnight at 43 C. After appropriate dilution with phosphate buffer, samples from the culture were spread on nutrient agar plates. Seven colonies, formed at 27 C, which did not grow at 43 C were picked, and their [^{3}H]thymine incorporation was investigated. A mutant temperature sensitive in DNA synthesis was found among these presumptive mutants.

Synthesis of macromolecules. DNA synthesis was estimated by the incorporation of [^{3}H]thymine into acid-precipitable materials. When *E. coli* HF4704S grown to 5 × 10^7 to 5 × 10^8 cells per ml was suspended in TPG-CA medium containing 2 μg of thymine per ml, [^{3}H]thymine was added at a final concentration of 0.01 to 5 μCi/ml. A portion was taken out and 10% ice-cold trichloroacetic acid was added. The mixture was allowed to stand on ice for at least 1 h. The precipitates produced were collected on Toyo-Roshi GB-100 or Whatman GF/C glass-paper disks, washed twice with 10 ml of 5% ice-cold trichloroacetic acid, and dried. Radioactivity was measured in a Beckman or Horiba liquid scintillation counter.

Extraction of DNA. To the cell suspension in 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1) was added lysozyme and ethylenediaminetetraacetic acid at final concentrations of 0.5 mg/ml and 10 mM, respectively. The mixture was allowed to stand on ice for 30 or 60 min. Sodium dodecyl sulfate was added to a concentration of 0.2%. An equal volume of distilled phenol saturated with the same buffer was added and stirred vigorously. After centrifugation at 800 × *g* for 10 min, the aqueous layer was taken and pooled. A ¼ volume of the same buffer was added to the phenol layer and vigorously stirred. After centrifugation, the aqueous layer was taken and added to the other solution. The residual phenol in the aqueous layer was extracted three times with ether, and the remaining ether was evaporated. Ribonucleic acid was added at a final concentration of 200 μg/ml. A 1/10 volume of 3 M potassium acetate and 2 volumes of 95% ethanol were added, and the mixture was kept at 4 C for 14 h. The precipitate produced was collected by centrifugation at 10,000 × *g* for 15 min, and dissolved in the buffer.

Centrifugation techniques. To analyze the density-labeled DNA, CsCl equilibrium centrifugation was carried out. To give a density of about 1.710 g/ml, 1.3 g of CsCl was added to the density-labeled DNA solution (1 ml) buffered to pH 8.1 with 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride containing 3 mM EDTA. Centrifugation was performed

at 35,000 rpm for 26 h or at 40,000 rpm for 22 h in an RPS-40 rotor of a Hitachi 65P ultracentrifuge. After fractionation, radioactivity was measured.

Pulse labeling. To the culture of HF4704S in TPG-CA medium containing 2 μg of thymine per ml was added 4 or 5 μCi of [^{3}H]thymine per ml. The culture was incubated at 27 or 43 C for the desired time and chilled in an ice-sodium chloride bath. The cells were collected, washed once with ice-cold SB, and resuspended in a fresh medium.

Density labeling. To the culture of HF4704S in TPG-CA low-phosphate medium (concentration of KH_2PO_4 was reduced to 23 mg/liter) were added 30 μg of 5-bromodeoxyuridine (BUdR) and 10 to 20 μCi of [^{32}P]phosphoric acid per ml. After incubation for appropriate time, cells were collected and lysed by the lysozyme-ethylenediaminetetraacetic acid treatment, and nucleic acid was extracted.

Genetic analysis. (i) HF4704S-11 cells grown overnight to 3.6×10^8 cells per ml in tryptone broth containing 10 μg of thymine and 10 μg of uracil per ml at 27 C were transferred to crossing buffer containing 20 μg of thymine and 20 μg of uracil per ml at a concentration of 7×10^8 cells per ml. Hfr-4 and Hfr-12 cells grown overnight to 10^8 cells per ml in the same medium as above without aeration were directly used for the conjugation. A 1-ml volume of the recipient culture and 0.5 ml of each donor culture were mixed, spread on a petri dish, and shaken occasionally and gently at 30 C. At the times indicated, a 0.05-ml sample was pipetted out into 10 ml of ice-cold SB and vigorously stirred with a Vortex mixer. A 0.1-ml volume of the suspension was spread on a CPM agar plate containing 20 μg of thymine and 50 μg of streptomycin per ml, and the plate was incubated at 43 C overnight. The number of colonies formed was counted.

(ii) *E. coli* S1814 was constructed by bacteriophage P1 transduction. *E. coli* BT 1000 was transduced with a P1 lysate from *E. coli* HF4704S as described by Miller (22). L Broth, L broth top agar and L broth bottom agar were used instead of LB broth, R top agar and R plates. A 10^5-fold enrichment for the mutant was performed as described by Miller (23). Six of twenty transductants defective in colony-forming ability at 43 C were clearly thymine requiring at 27 C, whereas BT 1000 was somewhat leaky at 27 C. Five out of six transductants were temperature sensitive in the initiation of DNA replication. One of them, S1814, was used in the present experiment.

(iii) The map position of *dnaH* in S1814 was determined by mating and bacteriophage P1 transduction. Mating experiments were carried out as follows. S1814 grown to stationary phase with aeration was diluted to give a concentration of 2×10^8 to 5×10^8 cells per ml with the same medium. KL16 and PK191 grown overnight to 2×10^8 to 3×10^8 cells per ml without aeration in L broth, which contained 10 μg of thiamine per ml if necessary, were used directly for the experiments. A 4-ml volume of the recipient culture and 0.5 ml of the KL16 or PK191 cultures were mixed in a flask. The mixture was shaken occasionally and gently at 27 C; 90 min later, a 0.1-ml sample was pipetted out, diluted 10^{-1}-, 10^{-2}-, and 10^{-3}-fold into ice-cold SB, and plated onto minimal agar plates. After incubation at 27 C for 48 h, the colonies formed were picked onto the fresh plate and incubated at 27 C for 36 h (master plate). Each master plate was replicated onto two fresh plates. One of them was incubated at 27 C, and the other was incubated at 43 C for 48 h. The number of colonies formed was counted.

Selection for *dnaH* and *thyA* markers and experiments in which *dnaH* marker was selected first were carried out by the methods described above.

Enzyme, chemicals and labeled materials. Lysozyme, nitrosoguanidine, BUdR, and CsCl were purchased from Sigma Chemical Co. [^{3}H]thymine (12.5 Ci/mmol) was purchased from the Daiichi Pure Chemical Co., Ltd. [^{32}P]phosphoric acid was obtained from the Japan Atomic Energy Research Institute.

RESULTS

Some properties of HF4704S. HF4704S grows normally at 27 C but not at 43 C. The turbidity, at 610 nm, of the mutant culture in TKB medium increased linearly at a lower rate than the wild-type strain at 43 C. Upon transfer from 27 to 43 C, the increase in viable counts in the mutant culture continued for 60 min and then ceased, during which time the viable counts increased approximately threefold. DNA synthesis, estimaed by the incorporation of [^{3}H]thymine into acid-insoluble materials, continued at 43 C at a decreasing rate for 60 min, then ceased (Fig. 1). Protein synthesis, esti-

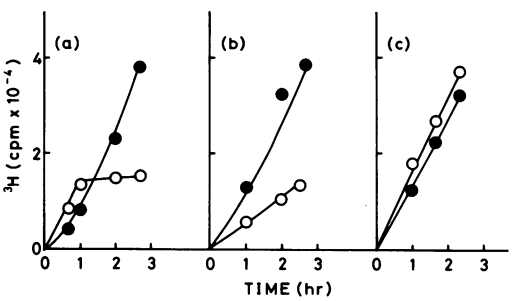

FIG. 1. *Macromolecular synthesis in E. coli HF4704S cells. When E. coli HF4704S grew to* 5×10^7 *cells per ml at 27 C in TPG-CA medium containing 20 μg of thymine per ml, the culture was divided into three equal portions and the cells were harvested by centrifugation. (a) One of the portions was transferred to the initial volume of TPG-CA medium containing 2 μg of thymine and 5 μCi of [*^{3}*H]thymine per ml; (b) another one was transferred to the initial volume of TPG-CA medium containing 20 μg of thymine, 1 μg of uracil, and 5 μCi of [*^{3}*H]uracil per ml; and (c) the third one was transferred to the initial volume of TPG-CA medium containing 20 μg of thymine per ml and 5 μCi of* DL-[^{3}*H]alanine, in which the Casamino Acids concentration was reduced to 0.54 mg/ml. Symbols: ●, 27 C; ○, 43 C.*

mated by the incorporation of DL-[^{3}H]alanine into acid-insoluble materials, continued linearly at almost the same rate at 43 as that at 27 C (Fig. 1). Although ribonucleic acid synthesis, estimated by the incorporation of [^{3}H]uracil into acid-insoluble materials, continued linearly at 43 C at a rate 50% of that at 27 C (Fig. 1), the β-galactosidase was normally induced at 43 C. The activities of DNA polymerases in HF4704S cells analyzed by the procedure of Komano et al. (16) were normal at 43 C in cell-free systems. The polynucleotide joining activity, estimated by the method of Okazaki et al. (28), was maintained at 43 C; the pulse-labeled short fragments of DNA could be joined to a longer DNA fragment. The precursor pool for DNA synthesis indicated that four deoxyribonucleotide 5'-triphosphates (27) existed even after the cessation of DNA synthesis at 43 C. Deoxyadenine, -thymidine, -guanosine, -cytosine triphosphate increased 230, 390, 240, and 340%, respectively, within 60 min after transfer from 27 to 43 C.

A point mutation could be responsible for the phenotypes of HF4704S and S1814, since revertants which could grow normally at 43 C appeared spontaneously with a frequency of approximately 10^{-7}.

DNA synthesis during the residual synthesis period. To examine whether DNA synthesis in the residual synthesis period proceeded in a semiconservative manner, the following experiment was carried out. HF4704S grown in the light medium at 27 C was transferred to a heavy medium containing BUdR at the time of temperature shift-up to 43 C. The simultaneous presence of the hybrid and light materials was observed, and the ratio of the size of the former peak to the latter was about 4:5 (Fig. 2a). The density-labeling experiment was also carried out on the cells 75 min after the temperature shift-up, when the residual DNA replication had already ceased. The bulk of DNA was found to be light in density (Fig. 2b).

At 43 C, DNA synthesis proceeded semiconservatively, and approximately 40% of the DNA was replicated after the temperature shift-up. Fifty percent of the DNA was replicated after the temperature shift-up (see Fig. 3). The amount of the residual DNA synthesis at 43 C agreed fairly well with the theoretical value of 39% which is expected if in an unsynchronized culture each chromosome contains one growing point and replication at 43 C proceeds to completion of every chromosome (24). Therefore, one of the most appropriate interpretations for the experimental results was that an initiation

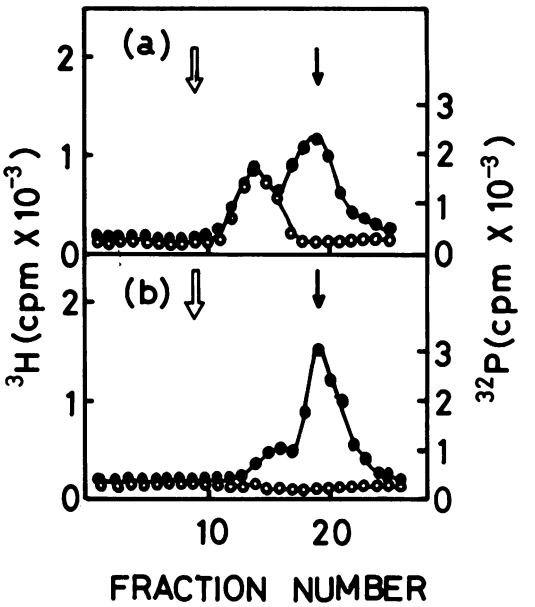

FIG. 2. *CsCl equilibrium centrifugation analysis of DNA density-labeled with BUdR. HF4704S cells were labeled with [^{3}H]thymine at a final concentration of 0.5 μCi/ml for more than three generations, grown to 1.5 $\times$ 10^8 cells per ml in TPG-CA medium containing 2 μg of thymine per ml, collected, washed, and suspended in TPG-CA low-phosphate medium containing 0.5 μg of thymine per ml. The culture was divided into two equal portions. (a) To one of them was added [^{32}P]phosphoric acid and BUdR at final concentrations of 10 μCi/ml and 30 μg/ml, respectively. The culture was transferred to 43 C and incubated for 100 min. (b) To the other were added the same materials as described in (a) after the incubation for 75 min at 43 C. DNA was extracted and dissolved in 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1) containing 3 mM ethylenediaminetetraacetic acid. To 1 ml of the DNA solution was added 1.3 g of CsCl to give a density of 1.710 g/ml. The solution was centrifuged for 26 h at 35,000 rpm at 20 C. After fractionation, radioactivity incorporated into acid-precipitable material was measured in a Horiba liquid scintillation counter. Symbols: ●, ^{3}H; ○, ^{32}P. The arrows (↓) indicate the density of light materials (1.703 g/ml). The arrows (⇩) indicate the position where heavy-heavy DNA is expected (1.790 g/ml).*

system in DNA replication did not function at 43 C and the ongoing cycle of DNA replication proceeded to completion.

Resumption of DNA synthesis and the effect of CM on it. When a culture of HF4704S incubated at 43 C for 70 min was transferred to 27 C, DNA synthesis resumed about 50 min after the temperature shift-down, but not in the presence of 30 μg of chloramphenicol (CM) per

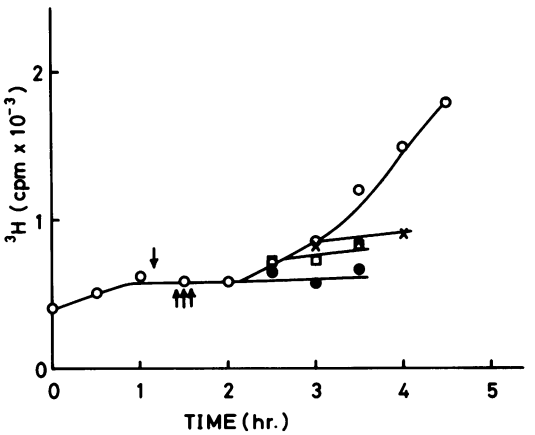

FIG. 3. *Recovery of DNA synthesis after the addition of 30 μg of CM/ml. HF4704S cells (1.2 × 10⁸ cells per ml) were uniformly labeled with [³H]thymine in TPG-CA medium containing 2 μg of thymine and 0.01 μCi of [³H]thymine per ml. The culture was incubated at 43 C for 70 min, and the temperature was shifted down to 27 C (↓; ○). At 15 min (●), 20 min (□), and 25 min (×) after the temperature shift-down, a portion (⅓ volume) of the culture was removed and CM was added (↑) to a final concentration of 30 μg/ml. At indicated times, a sample was withdrawn from each culture, and the radioactivity incorporated into acid-precipitable materials was measured.*

ml, and resumed at about 50 min after the removal of the antibiotic (data not shown). This suggested that the recovery of DNA synthesis required de novo synthesis of a CM-sensitive factor, presumably protein, and that the factor was irreversibly inactivated at 43 C.

To determine which process was sensitive to CM, a culture of HF4704S incubated at 43 C for 70 min was divided into equal portions and then transferred to 27 C. CM was added to each portion at a final concentration of 30 μg/ml, at 15, 20, and 25 min thereafter (Fig. 3). When CM was added 15 min after the temperature shift-down, the resumption of DNA synthesis was not observed. When CM was added at 25 min, DNA synthesis resumed about 50 min after the temperature shift-down, continued for 50 min at the same rate as the control, and then ceased. When CM was added at 20 min, the synthesis also resumed, continued for 30 min, and ceased. The slight DNA synthesis observed when CM was added at 20 min might be due to incomplete synchronization of initiation. When the culture was kept at 27 C without the addition of CM, synthesis resumed 50 min after the temperature shift-down. It continued to duplicate the amount of the DNA until 125 min, and simultaneously the rate of synthesis increased twofold. The time when the resumption of the DNA synthesis without CM increased twofold coincided with the time when the synchronous cell division took place after the high-temperature treatment. These facts indicated that the CM-sensitive process, completed between 25 and 35 min before the beginning of the resumption of DNA synthesis, permitted the initiation of the next cycle DNA replication. The results also suggested that the chromosomes aligned by the high-temperature treatment could not be replicated further in the presence of 30 μg of CM per ml at 27 C.

HF4704S cells incubated at 43 C for 70 min were transferred to 27 C, and 30 μg of CM per ml was added 10 min later. After incubation at 27 C for 70 min, the cells were again transferred to 43 C with simultaneous removal of the antibiotic. Reinitiation of DNA synthesis was not observed within 210 min. When a part of the culture was transferred to 27 C, DNA synthesis resumed 50 min later (Fig. 4a). The mutant cells were incubated at 27 C in the presence of 30 μg of CM/ml to stop the DNA synthesis and were transferred to 43 C with the simultaneous removal of the antibiotic (Fig. 4b). The results (Fig. 4b) suggested that the chromosomes aligned in the presence of 30 μg of CM/ml could not be replicated further at 43 C in the absence of the antibiotic. When a part of the culture was transferred back to 27 C, DNA synthesis resumed 45 min later.

The results shown in Fig. 4a and b suggested the existence of a thermolabile protein factor that could not be synthesized in the presence of 30 μg of CM/ml, and also suggested that the chromosome initiation, at the same fixed region on the chromosomes as when the cells were in the presence of 30 μg of CM/ml, was blocked at 43 C.

Effect of temperature reshift-up on the resumption of DNA synthesis. To determine which process was temperature sensitive, a temperature reshift-up experiment was performed (Fig. 5). HF4704S grown at 27 C was transferred to 43 C and incubated for 70 min to stop the DNA synthesis. The temperature was then shifted down to 27 C and, at the indicated times, reshifted-up to 43 C.

When temperature was reshifted-up at 10 min after the temperature shift-down, the synthesis was resumed. When temperature was reshifted-up at 20 min, the synthesis resumed and continued, for 70 min thereafter, to duplicate the amount of DNA indicated by the twofold increase in ³H counts incorporated into DNA (Fig. 5). Additional DNA synthesis, how-

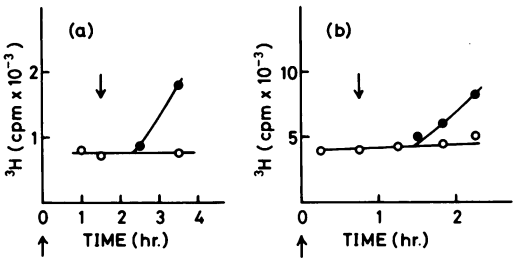

FIG. 4. *Effect of temperature shift-down on the recovery of DNA synthesis in the absence of 30 μg of CM/ml. (a) HF4704S cells (1.2 × 10⁸ cells per ml) were uniformly labeled with [³H]thymine in TPG-CA medium containing 2 μg of thymine and 0.01 μCi of [³H]thymine per ml. The culture was incubated at 43 C for 70 min and transferred to 27 C; 10 min later, CM was added at a final concentration of 30 μg/ml. At 80 min after the temperature shift-down (↑), the culture was diluted 100-fold into the same medium, which had been pre-warmed to 43 C, and was incubated at 43 C (○). At 90 min after the 100-fold dilution at 43 C, ½ volume of the culture was taken out and transferred back to 27 C (↓; ●). At the times indicated, a sample was withdrawn and chilled in an ice-sodium chloride bath. The cells were harvested at 0 C by centrifugation and suspended in 10% ice-cold trichloroacetic acid. The radioactivity incorporated into acid-precipitable materials was measured. (b) HF4704S cells (3 × 10⁸ cells per ml) were uniformly labeled with [³H]thymine in TPG-CA medium containing 2 μg of thymine and 0.07 μCi of [³H]thymine per ml. After the incubation for 180 min in the presence of 30 μg of CM/ml at 27 C, the culture was diluted 100-fold into the same medium (↑), which had been pre-warmed to 43 C, and incubated at 43 C (○). At 45 min after the dilution, ½ volume of the culture was taken out and transferred back to 27 C (↓; ●). At the indicated times, a sample was withdrawn and rapidly chilled in an ice-sodium chloride bath. The cells were harvested at 0 C by centrifugation and suspended in 10% ice-cold trichloroacetic acid. The radioactivity incorporated into acid-precipitable materials was measured.*

ever, did not occur, suggesting that the second cycle of DNA replication could not be initiated.

Therefore, the data from Fig. 5 indicated that the temperature-sensitive process was not completed during the first 10 min after return to 27 C. The operation of the temperature-sensitive process, which was achieved at 27 C between 30 and 40 min prior to the restart of the recovery synthesis, permitted new rounds of replication to start and be completed. The synchronous cell division in the mutant culture after the high-temperature treatment (data not shown) suggested that the resumption of DNA replication took place synchronously. The process sensitive to 30 μg of CM/ml occurred in the same time period (Fig. 3). Thus, the two processes (temperature sensitive and CM sensitive) seemed to coincide with each other.

Synchronous initiation and continuation of the recovery DNA replication. If HF4704S is defective in the initiation of DNA replication at 43 C, the recovery DNA replication cycle must be initiated at a fixed site on the chromosome at 27 C after incubation at 43 C for 70 min. To confirm the assumption, we carried out the following experiment. The region near the origin where DNA replication resumed in the mutant cells was pulse-labeled with [³H]thymine for 10 min at 27 C soon after DNA synthesis resumed (40 min after the temperature shift-down). The culture was grown in a medium containing cold thymine at 27 C for another 70 min, transferred to 43 C, and incubated for 70 min to complete the DNA replication cycle. The two halves of the culture were density-labeled for 10 min with BUdR in the presence of [³²P]phosphoric acid at 40 and at 75 min, respectively, after the temper-

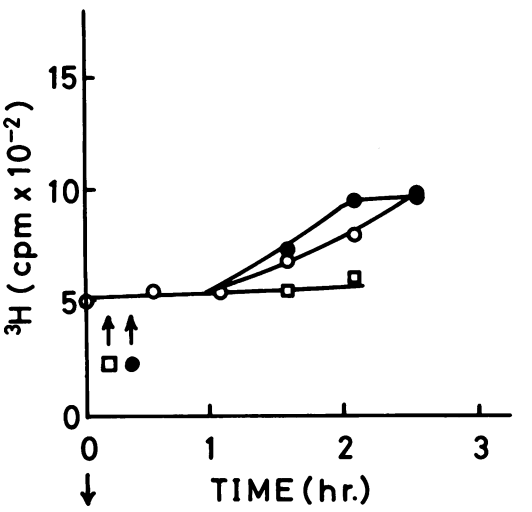

FIG. 5. *Effect of reshift-up of the temperature on the recovery of DNA synthesis. HF4704S cells (1.2 × 10⁸ cells per ml) uniformly labeled with [³H]thymine in TPG-CA medium containing 2 μg of thymine and 0.02 μCi of [³H]thymine per ml were collected, washed, and resuspended in the same medium containing 2 μg of thymine and 0.01 μCi of [³H]thymine per ml. The culture was incubated at 43 C for 70 min, and the temperature was shifted down at 27 C (↓; ○). At 10 min (□) and 20 min (●) after the temperature shift-down, a portion (⅓ volume) of the culture was removed, transferred again to 43 C, and incubated at the temperature. The arrow (↑) indicates the time when reshift-up of the temperature was carried out. At indicated times, a sample was withdrawn from each culture, and radioactivity incorporated into acid-precipitable material was measured.*

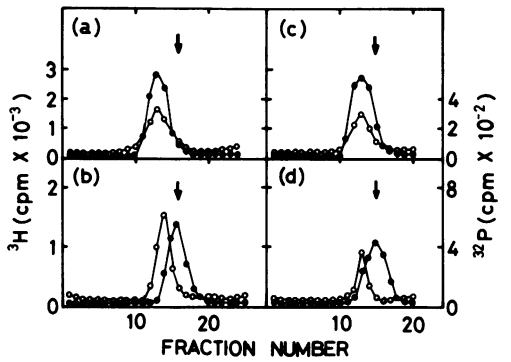

FIG. 6. *Synchronous initiation and continuation of the recovered DNA replication. HF4704S cells were grown to 2 × 10⁸ cells per ml at 27 C in TPG-CA medium containing 20 μg of thymine per ml, and then were transferred to the same fresh medium. The temperature was shifted up to 43 C, and the culture was incubated for 75 min. The temperature was then shifted down to 27 C, and at 40 min after the temperature shift down the cells were pulse-labeled with 5 μCi of [^{3}H]thymine per ml for 10 min, chilled rapidly in an ice-sodium chloride bath, and washed once with ice-cold SB. The cells were suspended in TPG-CA low-phosphate medium containing 1 μg of thymine per ml and grown at 27 C for 70 min (intermediate incubation). The temperature was shifted up to 43 C, and the cells were incubated for 75 min and again shifted down to 27 C. The culture was divided into two equal portions, each of which was density-labeled with 30 μg of BUdR per ml in the presence of 20 μCi of [^{32}P]phosphoric acid for 10 min (a) at 40 min and (b) at 75 min, respectively, after incubation. When the time period during which the intermediate incubation at 27 C was carried out after the pulse labeling with [^{3}H]thymine was reduced to 40 min, density pulse labeling was carried out (c) at 40 min and (d) at 75 min after incubation at 43 C for 75 min. The cells were chilled, washed once with ice-cold 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1), suspended in the same ice-cold buffer containing 10 mM ethylenediaminetetraacetic acid and 0.5 mg of lysozyme per ml, and allowed to stand on ice for 30 min. After freezing and thawing three times in an acetone-solid CO_2 bath, heat-treated pancreatic ribonuclease was added at a final concentration of 0.1 mg/ml, and the mixture was allowed to stand on ice for 30 min. The DNA was extracted by the phenol method, sheared by vigorous shaking, and dissolved in 0.5 ml of 0.03 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1) containing 3 mM ethylenediaminetetraacetic acid. To 0.3 ml of the DNA solution were added 0.7 ml of the same buffer and 1.3 g of CsCl to give a density of 1.710 g/ml. Centrifugation was at 40,000 rpm for 22 h at 20 C. After fractionation, to each fraction was added 0.5 ml of 0.6 N NaOH. The mixture was incubated for 18 h at 37 C and neutralized with 0.05 ml of 6 N HCl. To the mixture was added 1 ml of ice-cold 10% trichloroacetic acid in the presence of 300 μg of ribonucleic acid as a carrier, and the precipitate produced was collected on a glass paper disk and washed three times with 8 ml of ice-cold 5% trichloroacetic acid. Symbols: ○, ^{32}P; ●, ^{3}H. The arrows indicate the density of light materials (1.703 g/ml).*

ature shift-down to 27 C. Extracted DNA was sheared and analyzed by CsCl equilibrium centrifugation.

When the DNA was density-labeled after resumption of synthesis (at 40 min for 10 min), the ^{3}H-labeled peak coincided with the ^{32}P-labeled peak and appeared at a hybrid density (Fig. 6a). For the late period labeling (at 75 min for 10 min), the ^{32}P-labeled peak appeared at the hybrid density, but the ^{3}H-labeled peak appeared at a light density (Fig. 6b).

In another experiment the culture was pulse-labeled with [^{3}H]thymine, as above, and grown in a medium containing cold thymine at 27 C for another 40 min. When the culture was density-labeled with BUdR at 40 and 75 min, the same results were obtained (Fig. 6c, d).

These results indicated that the reinitiation of DNA replication proceeded synchronously during at least three successive generations and that HF4704S was defective in the initiation of DNA replication at 43 C.

Genetic analyses. The mutation conferring temperature sensitivity in DNA synthesis was mapped by crossing HF4704S-11 (F^-) at 30 C with Hfr-4 and Hfr-12 which carried the wild-type allele (29, 34). Hfr-4 and Hfr-12 injected their chromosome into the F^- cells counterclockwise and clockwise, respectively. Temperature-resistant recombinants began to appear 30 min after the start of conjugation when HF4704S-11 was crossed with Hfr-4, and at 60 min when the recipient was crossed with Hfr-12 (Fig. 7). In both the experiments, 83% (with Hfr-4) or 89% (with Hfr-12) of the wild-type recombinants were *thy*$^+$. The mutated gene, tentatively designated *dnaH*, was demonstrated to be located in 64-min position, near the *thy* marker, on the linkage map of *E. coli* C, although precise and quantitative evaluation of the results by this technique (interrupted conjugation) was not obtained (Fig. 8).

To determine the position of the *dnaH* locus in *E. coli* K-12, the mutation was transduced into *E. coli* BT1000. The transductant S1814 showed exactly the same properties as HF4704S in residual DNA synthesis and the effects of CM or reshift-up of temperature on the recovery of DNA synthesis.

S1814, phenocopied as F^-, was crossed with KL16 (Hfr) and PK191 (Hfr), which injected their chromosomes clockwise and counterclockwise, respectively. *thyA*$^+$ and *dnaH*$^+$ recombin-

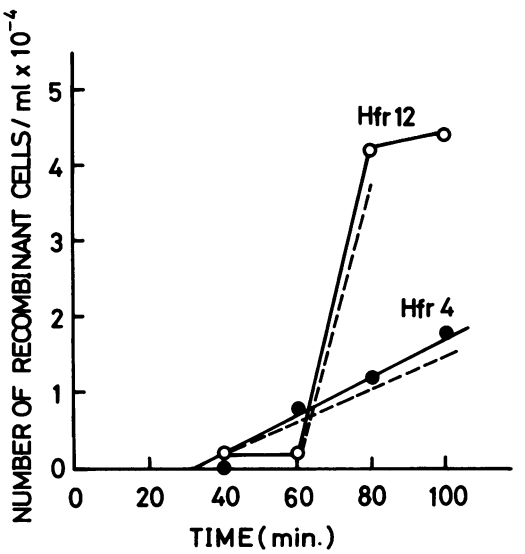

FIG. 7. *Interrupted transfer experiment of HF4704S-11 with the Hfrs. Symbols:* ○, *HF4704S-11 crossed with C-1091 (Hfr-12);* ●, *HF4704S-11 crossed with C-129 (Hfr-4);* -----, *number of recombinants converted from thy⁻ to thy⁺.*

ants were selected and scored for *dnaH*⁺ and *thyA*⁺ markers, respectively. The results (Table 1) suggest that *dnaH* is located near the *thyA* marker.

More precise mapping of *dnaH* on the chromosome of strain K-12 was done by transducing S1814 with bacteriophage P1 lysate from BT1000 (Table 2). *dnaH* and *thyA* markers were co-transduced at frequencies of 9.0 and 30%, respectively. One should note that a polarity was observed in the transduction experiments.

According to the results described above, *dnaH* marker was demonstrated to be 0.5 to 1.0 min distant from *thyA* marker on the map of the K-12 chromosome.

DISCUSSION

The isolated temperature-sensitive mutant HF4704S can grow normally at 27 C but not at 43 C. However, sufficient physiological functions are maintained for at least 40 h at 43 C so that colonies can be formed when the cells are transferred to 27 C. DNA synthesizing functions such as DNA polymerase activities, precursor pool of deoxyribonucleoside 5'-triphosphates, and polynucleotide joining activity (except initiation) are normal.

In the residual DNA synthesis period at 43 C, DNA synthesis proceeds in a semiconservative manner, and the amount of DNA synthesized is 50% as estimated from the amount of [^{3}H]thymine incorporated (Fig. 3) or is 40 to 50% as estimated from the density-labeling experiment (Fig. 2). From these facts, it can be concluded that the net increase in the amount of DNA during the residual synthesis period is 40 to 50%, which is in good agreement with the theoretical expectation (35).

In the initiation of a new round of DNA replication, the following processes have been considered to be involved: (i) protein syntheses (18, 21), and (ii) synthesis of ribonucleic acid (19). In (i), processes sensitive to (a) 30 μg of CM and (b) 150 μg of CM/ml are involved. The former is found to occur at about 30 min before the start of rounds for a doubling time of 40 min at 37 C (21). In *E. coli* C, a possibly analogous process is completed between 25 and 35 min before the beginning of initiation at 27 C (Fig. 3). Process (i-b), which occurs after the former process (i-a) is completed, is found to be completed just before the beginning of rounds of DNA replication (21). The process sensitive to 0.2% of phenethyl alcohol, which may be analogous to the latter process (20), is observed to be completed within 5 min before the beginning of reinitiation of DNA replication at 27 C (data not shown). The completion of these processes are necessary to reinitiate the next cycle of DNA replication. The temperature reshift-up experi-

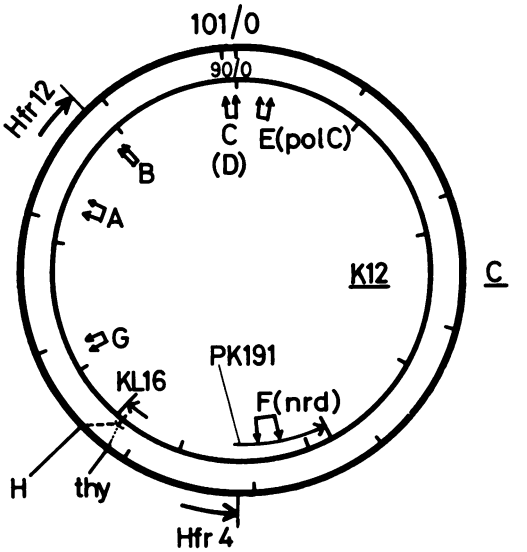

FIG. 8. *Genetic map of E. coli C compared with that of E. coli K-12. The letters A, B, C, D, E, F, G, and H indicate the positions of the dna genes. The inner circle and the outer circle indicate the genetic maps of E. coli K-12 and C, respectively. An arc-shaped arrow indicates the direction of the chromosome transfer.*

TABLE 1. *Linkage of dnaH locus with thyA*

Donor	Recipient	Selected marker (no. tested)	Percent marker frequency (no. tested)	
			thyA$^+$	*dnaH*$^+$
KL16, Str^s *thyA*$^+$ *dnaH*$^+$	Str^r *thyA*$^-$*dnaH*^{ts}	Str^r *thyA*$^+$ (119)	100 (119)	17.6 (21)
		Str^r *dnaH*$^+$ (50)	32.0 (16)	100 (50)
PK191, Str^s *thyA*$^+$ *dnaH*$^+$	Str^r *thyA*$^-$*dnaH*^{ts}	Str^r *thyA*$^+$ (90)	100 (90)	12.2 (11)
		Str^r *dnaH*$^+$ (92)	30.4 (28)	100 (92)

TABLE 2. *Co-transduction linkage of dnaH allele*

(A) Donor	(B) Recipient	(C) Selected marker (no. tested)	(D) Unselected marker (no. tested)	(E) Percent Linkage [(D)/(C)] × 100
thyA$^+$*dnaH*$^+$	*thyA*$^-$*dnaH*^{ts}	*thyA*$^+$ (256)	*dnaH*$^+$ (23)	9.0
thyA$^+$*dnaH*$^+$	*thyA*$^-$*dnaH*^{ts}	*dnaH*$^+$ (214)	*thyA*$^+$ (64)	30

ment (Fig. 5) indicates that in HF4704S the temperature-sensitive process is considered to be the same process as that sensitive to 30 μg of CM/ml if the 30 μg of CM/ml-sensitive process is a single one, or to be one of the 30 μg of CM/ml-sensitive processes if more than one process is sensitive to 30 μg of CM/ml.

The recovery of DNA replication after the temperature shift-down to 27 C is demonstrated to be initiated synchronously in a fixed region on the chromosome (Fig. 6). And the fixed region is evidently the normal one where an ordinary initiation event, which is blocked in the presence of 30 μg of CM/ml or by amino acid starvation, takes place (Fig. 4a and b). The results obtained above give good support to the assumption that mutant HF4704S is defective in the initiation of DNA replication at 43 C and that the thermolabile factor, which may be accumulated to function, is a protein and may be irreversibly inactivated at 43 C. These characteristics are similar to those of *dnaA* and *dnaC* (3, 12).

Gene *dnaH* was preliminarily mapped on the *E. coli* C linkage map at about 64 min, close to the *thy* marker, by crossing HF4704S with Hfr-4 and Hfr-12 (Fig. 8) (34). This position is considered to correspond to about 58 min, near the *thyA* marker (54 min), on the *E. coli* K-12 linkage map (Fig. 8). Mapping of *dnaH* on the *E. coli* K-12 chromosome was also performed. The two loci are revealed to be separated by 0.5 to 1.0 min on the K-12 linkage map according to the results of P1 transduction experiments (Table 2).

We may conclude that *dnaH* is distinct from *dnaA* and *dnaC* which are essential to the initiation of DNA replication in *E. coli*. It is located near *dnaF* and *dnaG*, both of which, however, are not initiation defective. *dnaH* is considered to be the third gene essential to the initiation of DNA replication.

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