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Reinitiation of Chromosome Replication in the Presence of Chloramphenicol Under an Integratively Suppressed State by R6K

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The autonomous replication of an R plasmid, R6K (*amp, str*), was shown not to be affected by chloramphenicol. It provoked integrative suppression and gave rise to Hfr strains when integrated into the chromosome of a strain of *Escherichia coli* K-12 with a temperature-sensitive mutation in the gene, *dnaA*. An Hfr strain designated as Hfr(R6K) no. 1 was thus obtained and characterized. It was not completely stable as shown by a plating efficiency of 0.6 at 42 C relative to that at 30 C. The density labeling and the ultracentrifugation analysis suggested that the deoxyribonucleic acid replication in this Hfr strain did not stop immediately after completion of the round already started before temperature shift-up and the addition of chloramphenicol. These observations are discussed in relation to a possibility that the chromosome replication of this Hfr strain is under the control of the integrated plasmid at a nonpermissive temperature.

Temperature-sensitive *dnaA* mutants become temperature-resistant phenotypically by integrative suppression with the F plasmid (9) or some R plasmids (8, 10, 12, 13). The use of some specific plasmids whose replication control system is different someway from that of the host chromosome would be of great advantage in clarifying the mechanism by which the replication of the chromosome is controlled.

Clewell (2) reported that the replication of Col E_1 was not affected by chloramphenicol (abbreviated as CM), although the replication of the host chromosome was inhibited. This plasmid does not provoke integrative suppression. However, having been encouraged by this report (2), we tried to find a plasmid whose replication is not affected by CM but which can provoke integrative suppression. We found that an R plasmid, R6K (previously termed R_{TEM}) (3, 6), could be a suitable candidate. This report deals with the evidence obtained by the use of this plasmid suggesting that the chromosome replication is under the control of the integrated plasmid at a nonpermissive temperature.

MATERIALS AND METHODS

Bacterial strains used. Substrains of *Escherichia coli* K-12, CR34 (F^-, *thi, thr, leu, thy, lac*, λ^-), and its *dnaA* temperature-sensitive mutant, CRT46 (F^-, *thi, thr, leu, ilv, thy, lac, mal*, λ^-, *dnaA*^{ts}), were kindly given by Y. Nishimura. A derivative of the latter strain to which R6K had been transferred from RC85 (F^-, *met*, λ^r, λ^-, $ColE_1^r$, R6K), kindly given by T. Arai, was designated as CRT46(R6K). This R plasmid determines resistance to streptomycin and ampicillin. It was previously designated R_{TEM} (3) and now termed R6K. It belongs to the incompatibility group of X (4). In the mating experiments, CSH 2 (F^-, *met, pro*) was used as a recipient. In the density labeling experiments, deoxyribonucleic acid from *Proteus mirabilis* PM 17 was used as an internal standard DNA.

Media and chemicals. Penassay broth (Difco) supplemented with 10 μg of thymine per ml was used as the complete liquid medium, and eosine methylene blue agar (Kyokuto, Tokyo) (5, 7) with added sugar at 1% and supplemented with 10 μg of thymine per ml was used as the complete agar medium. Synthetic EM sugar agar (5, 7), supplemented with appropriate nutritional requirements and antibiotics, was used for selection in mating and for scoring nutritional deficiencies of transconjugants. M9 medium (1) substituted with 0.1% glycerol instead of glucose and supplemented with thymine, threonine, leucine, isoleucine, and valine, each at 10 μg/ml, and thiamine hydrochloride, at 2 μg/ml, was used and designated as M9 glycerol T10. Thymine was added at 2 μg/ml to M9 glycerol when thymine uptake was measured. This medium was designated as M9 glycerol T2. ^{3}H-labeled thymine (49.03 mCi/mM) purchased from Japan Isotope Kyokai was used for the density labeling. Heavy-labeled acid hydrolysate from *E. coli* was obtained by the method of Rownd (11). Streptomycin, ampicillin, and CM were purchased commercially.

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Residual thymine uptake after the addition of CM. Cells were fully labeled by growing overnight in M9 glycerol T2 containing 1 μCi of [^{3}H]thymine per ml and the culture was diluted 10 times with the same labeling medium. After incubation to make logarithmic phase cultures, 100 μg of CM per ml was added and 0.5-ml samples were withdrawn at indicated intervals. Three milliliters of 4% perchloric acid was added to each sample and kept in ice for 15 min. It was then filtered through a Whatman GF83 glass filter and washed with 3 ml of 0.4% perchloric acid two times followed by two-time washings with 3 ml of ethanol. The filters were then dried, steeped into a vial containing 5 ml of toluene-DPO-1,4-bis-(5-phenyloxazolyl)-benzene mixture (4.0 and 0.1 g per liter, respectively) and counted by a Beckman scintillation counter, model LS-250.

Comparative thymine uptake in the presence and absence of CM. An overnight culture in the medium M9 glycerol T10 at 30 C was centrifuged and resuspended in M9 glycerol T2 to give optical density (OD) value of 0.05 by using a Shimazu-Bausch-Lomb Spectronic 20. Then the culture was grown at 30 C until the OD was equal to 0.07. At that time [^{3}H]thymine was added to a final concentration of 1 μCi per ml. After 2 h of incubation the culture was divided into four samples each consisting of 2 ml. To two samples, CM was added and then were incubated at 30 or 40 C as indicated. The other two were incubated at 30 and 40 C without CM. One-fourth milliliter of sample was withdrawn at indicated intervals. The same counting procedure as described in the previous section was followed.

Mating and tests for the genetic constitution of the recombinants. Essentially the same procedures were used as described in the previous report (13) except for the difference in antibiotic resistance tested.

Density labeling experiments. An overnight culture in M9 glycerol T10 at 30 C was centrifuged and resuspended into the same medium to give an OD value of 0.05. When the culture reached OD 0.07, the cells were harvested and resuspended into M9 glycerol substituted with $^{15}NH_4Cl$ for $^{14}NH_4Cl$. At the same time, 100 μg of CM per ml and ^{15}N-labeled acid hydrolysate of *E. coli* (11) were added and incubated at 30 or 40 C for 6 or 18 h. Isolation of DNA and CsCl density gradient centrifugation were performed as described by Rownd (11).

RESULTS

Reinitiation of R6K replication in the presence of CM. CM, an inhibitor of protein synthesis in bacteria, inhibits a new round of DNA replication and the round already initiated at the time of its addition is not affected by it. The relative thymine incorporation into acid-insoluble material in the presence of 100 μg of CM per ml at 30 C was examined. As shown in Fig. 1, only the residual replication (about 40% increment) could be seen in CR34 and in CRT46. In contrast, thymine uptake over residual replica-

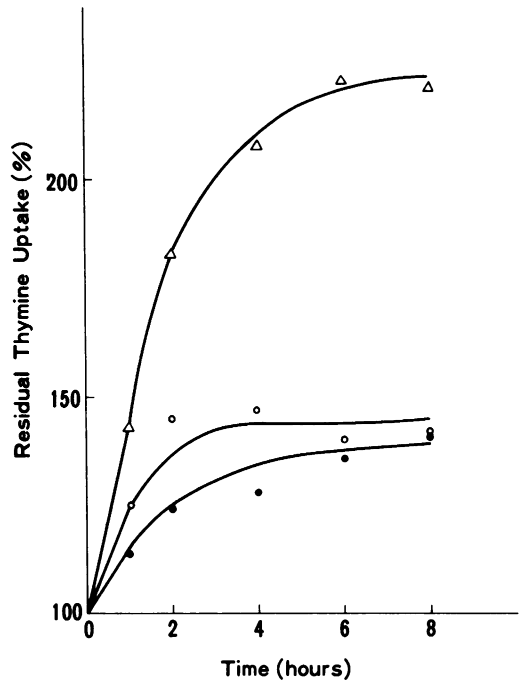


FIG. 1. *Fully prelabeled logarithmic phase cultures at 30 C were supplemented with 100 μg of CM per ml and samples were withdrawn at indicated intervals and counted. The values were expressed as percentages of those at time zero. Initial counts at time zero of CR34, CRT46, and CRT46(R6K) were 36,562, 9,560, and 12,532 counts/min, respectively. Symbols:* (O) *CR34;* (●) *CRT46;* (Δ) *CRT46(R6K).*

tion comprising more than two times as high as that of the initial content was observed in CRT46(R6K). The density-labeled profile with CRT46(R6K) also revealed that the R plasmid did not stop replicating at 30 C after completion of the already initiated replication round in the presence of CM (Fig. 2). These results suggest that the initiation of the replication of R6K is insensitive to CM at least for a few rounds of replication.

Isolation and characterization of an Hfr made by integration of R6K. The reversion frequency to temperature resistance was compared between CRT46 and CRT46(R6K). CRT46(R6K) produced temperature-resistant revertants at a frequency far higher than CRT46 did (Table 1). Temperature-resistant revertants derived from CRT46(R6K) were isolated, purified by successive single colony isolations, and examined for the ability to give rise to *met*$^+$, *thr*$^+$, *leu*$^+$ recombinants by mating with CSH 2. One of them, designated Hfr(R6K) no. 1, was chosen for further study. Table 2 shows the frequency of recombination of Hfr(R6K) no. 1

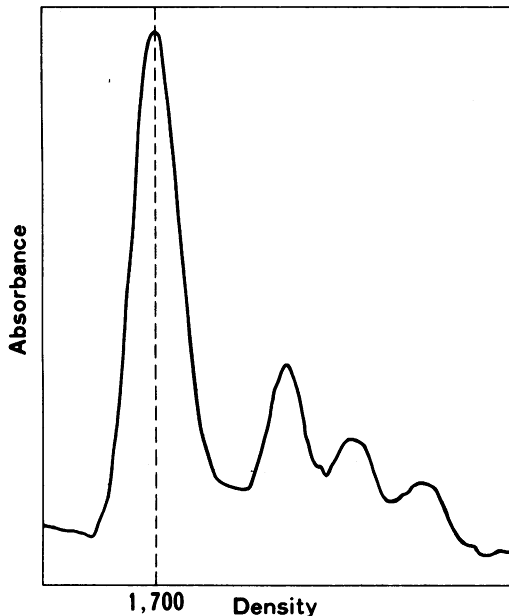


FIG. 2. *Density profile of DNA from CRT46(R6K) grown overnight in ^{15}N-labeling medium in the presence of 100 µg of CM per ml at 30 C. DNA from P. mirabilis grown in a light medium without CM was used as an internal standard (1.700). The peaks from right to left correspond to heavy-heavy, hybrid, light-light, and internal standard DNA, respectively.*

TABLE 1. *Reversion frequency to temperature resistance*[a]

Strain	Temp		Reversion frequency
	30 C	42 C	
CRT46(R6K)	4.7×10^8	1.7×10^4	3.6×10^{-5}
CRT46	4.1×10^8	$<1 \times 10$	$<2.4 \times 10^{-8}$

[a] Appropriately diluted logarithmic phase cultures of each strains in Penassay broth (Difco) grown at 30 C were plated on eosine methylene blue glucose agar supplemented with 10 µg of thymine per ml and incubated at 30 and 42 C. Numbers of temperature-resistant revertants were read after 3 days.

and CRT(R6K) when mated with CSH. The frequency of recombination of the chromosomal marker, *metA*$^+$, was higher in the mating cross of the Hfr than in that of the R$^+$ parent. However, the number of transconjugants obtained when selected with the drug resistance markers derived from the R plasmid was not significantly different in the two crosses. Table 3 shows the genetic constitution of the recombinants obtained in Table 2. Chromosomal markers of the donor alleles were recovered in the recombinants derived from the Hfr but not in those from the R$^+$ parent. Among the recombinants derived from the Hfr cross, one temperature-sensitive recombinant was obtained. This indicates that the temperature-sensitive mutation in the gene, *dnaA*, is still preserved genotypically in this Hfr strain. The plating efficiency of this Hfr was about 0.6 at 42 C relative to that at 30 C, but all of the colonies formed at 30 C could grow at 42 C when replica plated.

TABLE 2. *Frequency of recombination in the mating of Hfr(R6K) no. 1 or CRT46(R6K) and CSH2*[a]

Cross	Selection markers		Frequency of recombination
	Donor	Recipient	
Hfr(R6K) no. 1	*met*$^+$	*thr*$^+$, *leu*$^+$	4.0×10^{-8}
× CSH2	*str*^{r}	*thr*$^+$, *leu*$^+$	3.3×10^{-7}
	amp^r	*thr*$^+$, *leu*$^+$	7.1×10^{-7}
CRT46(R6K)	*met*$^+$	*thr*$^+$, *leu*$^+$	$<2 \times 10^{-9}$
× CSH2	*str*^{r}	*thr*$^+$, *leu*$^+$	4.5×10^{-7}
	amp^r	*thr*$^+$, *leu*$^+$	1.1×10^{-6}

[a] Mating was interrupted at 75 min.

Thymine incorporation into the Hfr DNA in the presence of CM. Thymine incorporation was examined in the presence and absence of CM at 30 and 40 C. A slight increase was observed in Hfr(R6K) no. 1 after temperature shift-up and the addition of CM (Fig. 3). When these data were compared by calculating the relative inhibition by CM, it was shown that the Hfr strain at 40 C is the least affected by CM (Fig. 4).

Density profile of the DNA synthesized after temperature shift-up and the addition of CM. The density labeling experiments were performed to make sure that reinitiation of chromosome replication had occurred in the presence of CM after temperature shift-up in this Hfr. The culture medium incubated at 30 C was changed from $^{14}NH_4Cl$ to $^{15}NH_4Cl$, CM, and ^{15}N-labeled acid hydrolysate were added, and the temperature was shifted up to 40 C or was left at 30 C. After 6 or 18 h of incubation at 30 or 40 C, the DNA was collected and subjected to Spinco model E analytical ultracentrifugation. As shown in Fig. 5, the peak of heavy-heavy DNA was observed only when Hfr(R6K) no. 1 was grown at 40 C but not at 30 C. The wild-type CR34 did not produce heavy-heavy DNA even at 40 C. These results indicate that the replication of Hfr(R6K) no. 1 in the presence of CM continues after the completion of residual replication only at a nonpermissive temperature but not at a permissive temperature. It is possible that the initiation mechanism of integrated R6K does not operate at a permissive temperature.

TABLE 3. *Genetic constitution of the recombinants*[a]

Cross	Donor markers										
	mal	*thr*	*leu*	*lac*	*pro*	*thy*	*dnaA*	*ilv*	*met*	(*amp*	*str*)
Hfr(R6K) no. 1 × CSH2	16	0	0	0	0	0	1	0	100	96	95
	0	0	0	0	0	0	0	1	0	100	100
	0	0	0	0	0	0	0	0	0	100	85
CRT46(R6K) × CSH2	0	0	0	0	0	0	0	0	0	100	100
	0	0	0	0	0	0	0	0	0	100	79

[a] The recombinants obtained in the mating described in Table 2 were used. The markers used for selection and counterselection were shown by 100 and 0 (underlined), respectively, and the results were expressed as percentages.

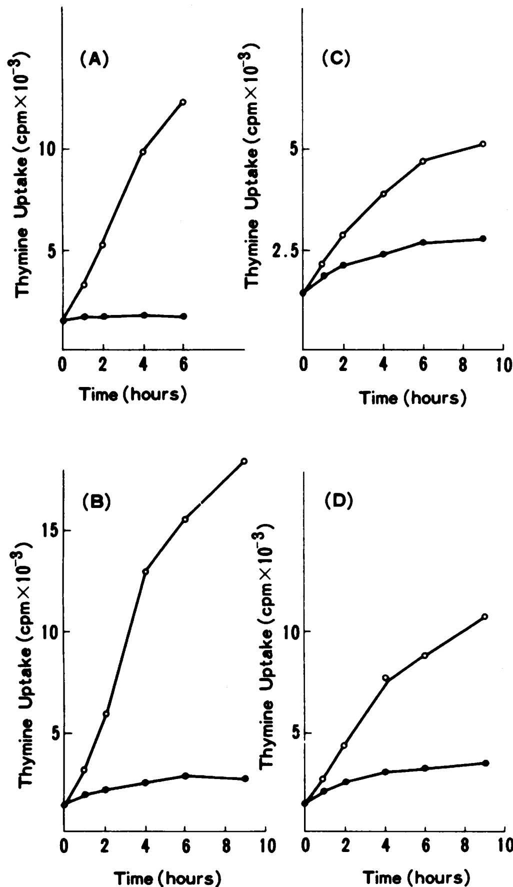


FIG. 3. *An early logarithmic phase culture in M9 glycerol T2 at 30 C was added [³H]thymine at 1 μCi per ml and grown for 2 more h. Then the culture was divided into four samples (time zero) and further grown with or without CM at 30 or 40 C. Samples were withdrawn at indicated intervals and counted. The thymine uptakes without CM were plotted by open circles and those with CM closed circles. (A) CR34 at 40 C, (B) CR34 at 30 C, (C) Hfr(R6K) no. 1 at 40 C, (D) Hfr(R6K) no. 1 at 30 C.*

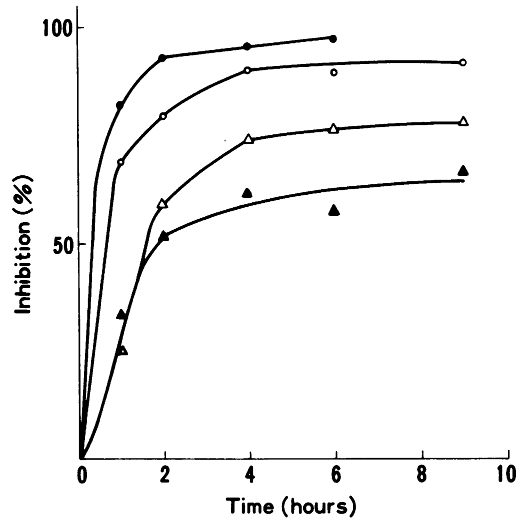
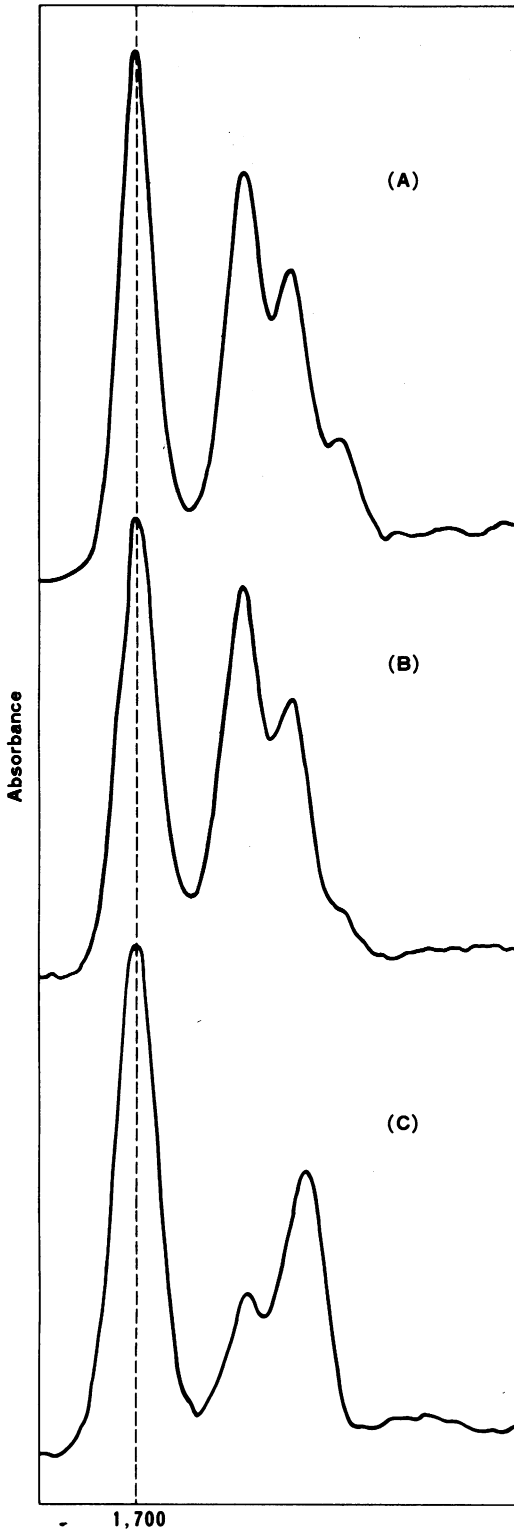


FIG. 4. *Calculation of percentage of inhibition was done using the data in Fig. 3. The increments from initial counts with CM were divided by those without CM. Symbols: (●) CR34 at 40 C; (○) CR34 at 30 C; (▲) Hfr(R6K) no. 1 at 40 C; (△) Hfr(R6K) no. 1 at 30 C.*

DISCUSSION

The F plasmid (8), some R plasmids including R100 (8, 10, 12, 13), and ColV (10) were shown to provoke integrative suppression when integrated into the host chromosome with a temperature-sensitive mutation in a gene, *dnaA*. Nishimura et al. (9) interpreted this phenomenon as showing that the defective chromosome has become a part of the plasmid replicon and is replicated under the control of the integrated plasmid. This interpretation was justified by a finding that the integratively suppressed strain returns to temperature sensitive in the presence of agents inhibitory to the replication of the plasmid (9, 12). It is of interest to what extent and in which biochemical reactions the control



mechanism of the chromosome replication can be replaced by that of the plasmid. We initially intended to show the replacement of the control system of the chromosome replication by $ColE_1$ because the replication of this plasmid was shown not to be affected by CM even after completion of the round of replication already started at the time of its addition (2). However, it was later abandoned since it was shown not to provoke integrative suppression. Then we tried to find a plasmid whose replication is not affected by CM but which can provoke integrative suppression. In this report we show that another plasmid, R6K, may be a suitable candidate because the initiation of the replication of R6K was insensitive to CM at least for a few rounds after its addition and it produced a fairly stable Hfr by integrative suppression. This Hfr strain was not completely stable as judged by a plating efficiency of 0.6 at 42 C relative to that at 30 C. However, it was stable enough to permit complete positive growth at 42 C when the colonies formed at 30 C were replica plated. Moreover, it did not lose its Hfr nature after repeated subcultures for over 1 year.

The [^{3}H]thymine incorporation and the result of the density labeling seem to be consistent with a conclusion that the initiation of chromosome replication is not immediately affected by CM and hence is under the control of the integrated R6K at a nonpermissive temperature. In the density labeling experiment the DNA of CR34 was collected at 6 h of incubation at 40 C in the presence of CM, whereas that of the Hfr was collected at 18 h. This was because the viscous fraction of CR34 could not be obtained at 18 h under the condition used. This inability of collecting DNA from CR34 was observed only when glycerol was used as the sole carbon source and CM was added in the medium. Although we isolated the DNA fraction at 6 h from CR34 and at 18 h from Hfr(R6K) no. 1, this is not unreasonable because the generation time of CR34 and the Hfr at 40 C was about 2 to 3 and 8 to 9 h, respectively, in the absence of CM. The incubation time of 6 h for CR34 will be sufficiently comparable to that of 18 h for

FIG. 5. *Density profiles of DNA collected from cells grown in ^{15}N-labeling medium in the presence of 100 μg of CM per ml at 30 or 40 C. DNA from P. mirabilis grown in a light medium without CM was used as an internal standard (1.700). (A) DNA from Hfr(R6K) no. 1 at 40 C for 18 h; (B) DNA from Hfr(R6K) no. 1 at 30 C for 18 h; (C) DNA from CR34 at 40 C for 6 h.*

Hfr(R6K) no. 1 on the basis of the number of generations.

Two possibilities as to the replication of an integrated plasmid at a permissive temperature was suggested by Nishimura et al. (9). One was that the replication system of the host chromosome repressed that of integrated plasmid and the other was that both host chromosome and integrated plasmid could replicate simultaneously without ill effect. Our result that heavy-heavy DNA was not obtained from Hfr(R6K) no. 1 at a permissive temperature may support the former possibility.

We are anxious about the quantitative discrepancy of the results of the [^{3}H]thymine uptake and the density labeling experiment. As long as we could obtain so much heavy-heavy fraction in the density labeling experiment for the Hfr after the temperature shift-up and the addition of CM, we should have expected more increase of [^{3}H]thymine incorporation. However, both of these experiments were completely reproducible and degradation of DNA into an acid-soluble fraction was not observed (data not shown). This may be related to the observation that the light-light DNA was always more than the hybrid in Hfr(R6K) no. 1.

A possibility that the heavy-heavy DNA obtained from the Hfr strain at a nonpermissive temperature was due to autonomous R6K segregated from integrated state might be ruled out by the finding that the heavy-heavy fraction, when the labeling was made at a permissive temperature, was either undetectable or at most far less than that obtained at a nonpermissive temperature.

The second possible complication is that the heavy-heavy DNA fraction might have been derived from repeated replication of the integrated R6K region alone. This seems to be unreasonable as long as the plasmid has been covalently bound to the chromosome as indicated by the result at the permissive temperature.

A third alternative pertains to the possible existence of replication multiforks at the time of temperature shift-up and the addition of CM. But this is unlikely first because the sole carbon source was glycerol and secondly because of the result obtained at a permissive temperature. Since we divided the same culture for the permissive and the nonpermissive experiments at the time of the addition of CM, we would have obtained a heavy-heavy fraction from the culture grown at a permissive temperature, if multiple replication forks had been present.

In conclusion, we believe that the chromosome replication of this Hfr strain is under the control of the integrated plasmid, R6K, at a nonpermissive temperature.

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