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Mechanism and Biosynthetic Requirements for F Plasmid Replication in *Escherichia coli*†

Bruce C. Kline

ABSTRACT: Results of isopycnic centrifugation show that covalently closed, circular F DNA, a sex factor plasmid of *Escherichia coli*, replicates semiconservatively. In chloramphenicol-treated cells, both F and chromosome synthesis decrease continuously for 2 hr and then stop. Most cells initiate F replication once in the presence of this antibiotic resulting in an 85% increase in the amount of F DNA. If thymine-starved cells are treated with chloramphenicol and thymine, a biphasic increase in chromosomal DNA occurs which reflects the premature and subsequent stable initiations of chromosome replication. A biphasic, coordinate increase of F DNA also occurs in these cells. Premature initiation of F replication does not occur. The relative patterns of DNA increases in chloramphenicol-treated cells suggest F replication is coordinate with replication of a terminal chromosome region. In contrast, F does not duplicate synchronously with duplication of any chromosome locus when chromosome replication is synchronized by brief periods of sequential amino acid and thymine starvation. Several inhibitors of RNA polymerase decrease the amount of chloramphenicol-limited F replication by 30–60% indicating a requirement for untranslated RNA in F replication. Other observations suggest RNA polymerase is bound to F DNA but inacessible to inhibitors until the time of replication. A model of F replication that integrates the above findings is presented.

F is an extrachromosomal genetic element, a plasmid, that enables its bacterial host to participate in conjugation. This plasmid is a covalently closed, circular DNA molecule of molecular weight 64 × 10^6 daltons (Kline and Helinski, 1971; Clowes, 1972). Thus, it is about one-fiftieth the size of its host chromosome. Biochemical studies indicate there are one or two copies of F per replicating chromosome (Bazaral and Helinski, 1970; Frame and Bishop, 1971). Thus regulation of F and chromosomal replication is very similar in spite of their substantial size difference.

This similarity has generated much interest in F replication as a model system, yet little is known about the biochemistry

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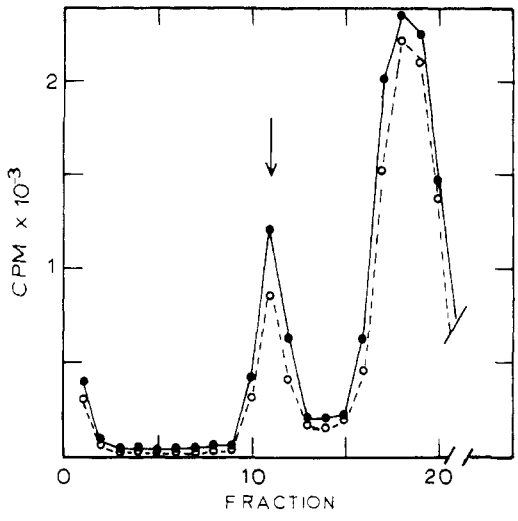

FIGURE 1: Sedimentation velocity analysis of isotopically labeled CCC F DNA. F DNA was labeled according to the protocol described under Materials and Methods and in Figure 7. Cleared lysate (0.3 ml) was layered onto a neutral sucrose density gradient and centrifuged as described under Materials and Methods. Sedimentation is from right to left. The DNA at the 80S position has been characterized as CCC F DNA (Kline and Helinski, 1971). The arrow indicates the position of the 80S CCC F DNA: (●) [^{14}C]DNA; (○) [^{3}H]DNA. [^{3}H]Thymine was incorporated over a 150-min period in the presence of both chloramphenicol (150 μg/ml) and rifampicin (50 μg/ml). Peak fractions were counted to at least 5% statistical accuracy. Two-tenths to 1.5% of the total ^{14}C cpm in the crude lysates was observed routinely as 80S DNA in the cleared lysates. The average recovery was 0.8%. The ^{3}H/^{14}C value in F DNA does not fluctuate when the recovery of F DNA varies fourfold as the result of different (± rifampicin) cell treatments prior to F purification (Kline, 1973, and unpublished data).

of this process. The mechanism of F replication is probably semiconservative (Matsubara, 1968) but this has not been shown conclusively. Other results indicate a role for protein (Bazaral and Helinski, 1970; Davis and Helmstetter, 1973), untranslated RNA (primer) (Kline, 1973), and, perhaps, chromosome (Zeuthen and Pato, 1971; Collins and Pritchard, 1973) synthesis in F replication. This paper shows that F DNA replicates semiconservatively and examines the roles of protein, RNA, and chromosome synthesis in this process.

Materials and Methods

Bacteria and Media. A K-12 strain of *E. coli*, strain CR34F, was employed in this work. Physiological properties of the bacteria and the origin of the F sex factor have been described previously (Kline and Helinski, 1971). The organism is auxotrophic for threonine, leucine, vitamin B_1, and a low concentration of thymine. The M9 media (Kline, 1972) used herein was supplemented with glucose (2 g/l.), Casamino acids (Difco lot 0231-01) (5 g/l.), and [2-^{14}C]thymine (0.45 μCi/μg; 1.1 μg/ml) or [*Me*-^{3}H]thymine (3–10 μCi/μg, 1.0 μg/ml). Bacteria were grown in media supplemented with bromouracil and thymine (20:1, w/w) to yield heavy DNA. The generation time of CR34F is 90–100 min in the presence of bromouracil and 50–55 min in its absence. All chemicals were of reagent grade quality. Rifampicin (lot 100990) was purchased from Calbiochem. It was manufactured by Lepetit, S.p.A., Italy. Streptovarcin and streptolydigin were generously supplied by Dr. John Whitfield, The Upjohn Company, Kalamazoo, Mich.

DNA Synthesis. The general technique (Kline, 1973) consists of prelabeling a logarithmic culture for three–four generations with [2-^{14}C]thymine, washing the cells at 0°, starving for 5 min to deplete the intracellular [2-^{14}C]thymine pool, and mixing the bacteria with [*Me*-^{3}H]thymine containing media with various combinations of antibiotics that inhibit protein and/or RNA synthesis. The final concentration of bacteria in [^{3}H]thymine media was about 10^8/ml (60–70 Klett units). At intervals after the label switch, aliquots of cells are removed, chilled, and lysed. In most cases where the exposure to chloramphenicol was longer than 120 min, 10 μg/ml of rifampicin was added during the last 5 min of incubation to improve the recovery of F DNA two- to fourfold (Kline, 1973). Bacteria cultured with rifampicin, actinomycin, or streptovarcin were not treated by freeze–thaw to prevent the (mechanical?) generation of chromosomal fragments similar in s^0 value to CCC F DNA.[1] The relative amount of F DNA synthesis was determined by examining the ^{3}H/^{14}C ratio in the CCC F DNA peak on a 20–31% sucrose gradient (Figure 1). This ratio was determined by dividing the sum of the ^{3}H counts in the 80S peak by the sum of the ^{14}C counts in the same peak. Both sums were corrected before division by subtracting base-line contamination (Figure 1). The relative amount of chromosome synthesis was determined by examining the ^{3}H/^{14}C ratio of crude lysate. Both F and chromosomal DNA samples were prepared in the same way (Kline, 1972) for liquid scintillation counting. Sucrose gradients contained 0.05 M $NaPO_4$ (pH 7.6), 0.005 M EDTA, and 0.5 M NaCl. Centrifugation was carried out for 90 min at 15° at 50,000 rpm in a Spinco SW 50.1 rotor.

The basic density-shift protocol employed in this work was to grow a culture of *Escherichia coli* CR34F overnight in medium containing a mixture of bromouracil (40 μg/ml) and thymine (2 μg/ml). The overnight culture was then subcultured into 20 ml of medium containing bromouracil and [2-^{14}C]-thymine (20:1, w/w), grown for three–four generations (final cell concentration, 2–4 × 10^8), harvested, washed by centrifugation, and resuspended in 20 ml of thymine-free medium. Finally, the culture was mixed with an equal volume of medium containing [*Me*-^{3}H]thymine (2 μg/ml) without bromouracil. Ten-milliliter aliquots were removed periodically and lysed, and F DNA was purified from cleared lysates (Kline and Helinski, 1971) as in Figure 1.

Isopycnic Centrifugation. Purified CCC F DNA or crude cell lysates (chromosomal DNA) were treated at 37° for 30 min with autodigested Pronase (1.5 mg/ml) and sarcosyl NL-30 (0.25%) and subsequently spun to equilibrium in self-generating CsCl isopycnic gradients, 1.735 g/cm^3. The gradients were made by dissolving 5.0 g of CsCl in 3.9 ml of the phosphate–EDTA buffer described above (NaCl omitted) and spinning at 40,000 rpm for 36–40 hr at 15° in a Spinco Ti 50 rotor. Gradient tubes were presoaked in 0.5 M phosphate buffer (pH 7.6) to ensure 90–100% recovery of centrifuged DNA. In some cases after Pronase treatment, F DNA was sheared on a Vortex machine for 5 min to break supercoiled forms, and the pH was adjusted to 12.4 with NaOH. This DNA was centrifuged to equilibrium in alkaline CsCl gradients, 1.77 g/cm^3, containing 0.2 M Na_3PO_4 (pH 12.4).

Graphing and Analysis of Isopycnic Data. Under the conditions of centrifugation slight overlaps occur with the density species on either side of the hybrid peak. The experimental design is such that heavy DNA is labeled only with ^{14}C and

[1] Abbreviation used is: CCC F DNA, covalently closed circular sex factor deoxyribonucleic acid.

light DNA only with ^3H. It is observed that in the hybrid peak a region of constant $^3H/^{14}C$ value exists. This is taken to be a region without significant contamination of either heavy or light density DNA. The shape of the hybrid peak is then calculated by correcting for the observed deviations from the constant $^3H/^{14}C$ value by simple arithmetic steps. The extra [^{14}C]- or [^3H]DNA after the corrections are assumed to be heavy and light DNA, respectively. Corrected density patterns for CCC F and chromosomal DNA are shown in Figure 2.

DNA replication for each time point after a density shift is quantitated as the fraction of substrate (heavy or hybrid density) DNA that has replicated. The fraction of heavy DNA that has replicated is numerically equal to the fraction of the total ^{14}C cpm appearing at the hybrid density. The fraction of hybrid density DNA that has replicated is numerically equivalent to the amount of light density DNA divided by the amount of hybrid density DNA. This number is calculated by dividing the ^3H cpm at the light density by 2(^3H cpm) at the hybrid density. Since hybrid density substrate is preserved in the act of duplicating, the amount of light DNA will eventually exceed the amount of hybrid DNA. Therefore, in the context of the fraction of hybrid substrate employed, the quantitation is only meaningful and valid to a value of 1.0. These two methods of representation are employed in preference to absolute counts per minute appearing in hybrid or light density positions because they normalize each gradient to internal standards and thereby correct for variations in sample size and recovery.

RNA Synthesis. Bacteria were handled according to the same protocol used in DNA synthesis experiments except cells were not prelabeled with [2-^{14}C]thymine. The RNA precursor was [2-^{14}C]uridine (1 μCi/μg, 5 μg/ml). The experimental design and protocol have been published (Lark, 1972). Bacteria were rendered permeable to all RNA polymerase inhibitors (except streptolydigin) by treatment with chloramphenicol (150 μg/ml) (Clewell *et al.*, 1972). EDTA (Leive, 1968) was used to render bacterial permeable to streptolydigin.

Results

Semiconservative Replication of F DNA. The mechanism of CCC F DNA replication has been investigated by use of the density shift technique described under Materials and Methods. The data in Figure 3A show the synthesis of hybrid and light density F and chromosomal DNA as a function of time. This synthesis is expressed as the fraction of heavy or hybrid template that has replicated. The data show that F replication is semiconservative. The premature appearances of light F and chromosomal DNA presented in Figure 3A are probably caused by bromouracil. An explanation of this point is given in the Discussion.

Sex Factor Replication in the Absence of Protein and RNA Synthesis. When protein synthesis is inhibited, cells can complete the current round of chromosome replication but cannot initiate a new round of replication (Maaløe and Hanawalt, 1961; Lark *et al.*, 1963). In contrast, F retains the potential to initiate a new round of replication for 2 hr in the absence of protein synthesis (Bazaral and Helinski, 1970; Kline, 1973). The rate of F synthesis in this circumstance was unknown. This point was examined by following the increases in the $^3H/^{14}C$ ratio in F DNA after shifting a [^{14}C]thymine-labeled culture to medium containing [^3H]thymine and chloramphenicol (150 μg/ml). Figure 4 shows that the rates of both F and chromosome synthesis decrease continuously from the time of antibiotic addition. The average maximal increases in the

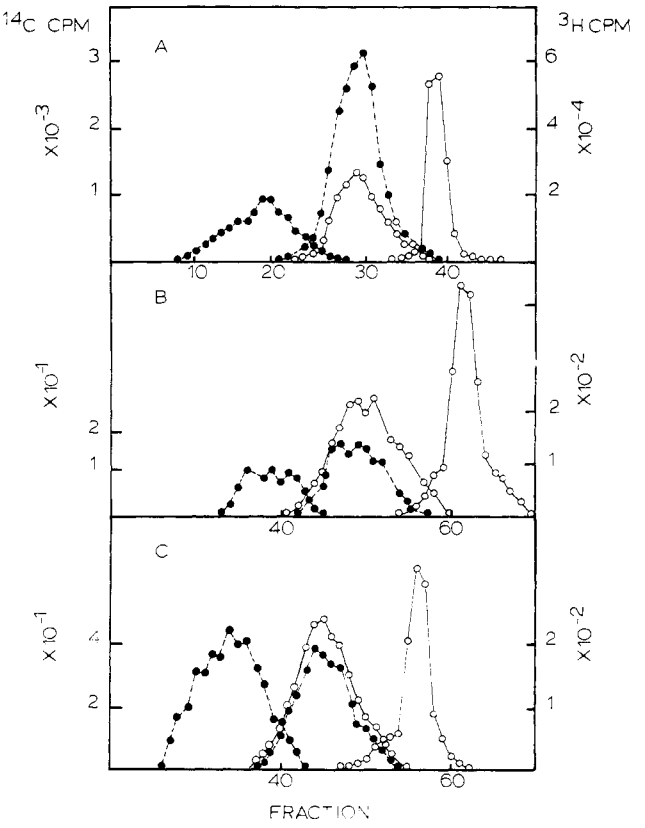

FIGURE 2: Density distribution of F and chromosome after isopycnic centrifugation. Experimental design, culture conditions, and analytical procedures are explained under Materials and Methods: bromouracil-[^{14}C]thymine-labeled DNA (●); [^3H]thymine-labeled DNA (○). The average density of the CsCl gradients is 1.735 g/cm^3. Density increases from right to left. (A) Density distribution of chromosomal DNA extracted from cells sampled 67 min after the density shift in the experiment described in Figure 3A. (B) Density distribution of CCC F DNA (>90% purity) extracted from cells sampled 120 min after a density shift into medium containing chloramphenicol. (C) Aliquots of same culture and media used in B except rifampicin (5 μg/ml) also was present in the light medium. The high purity for CCC F DNA in B and C was achieved after two preparative zonal centrifugation steps. In most density shift experiments aliquots of DNA were sheared and subsequently centrifuged in alkaline CsCl gradients. The ^{14}C- and ^3H-labeled DNA separated into distinct, symmetrical peaks indicating a lack of repair replication. In all density experiments, samples were counted until the standard deviations were $\leq$10% for ^{14}C and $\leq$5% for ^3H.

amounts of F and chromosomal DNA in the presence of chloramphenicol calculated from the results of 13 determinations are 85 ± 10 and 50 ± 5%, respectively.

Since low concentrations of [^{14}C]- and [^3H]thymine (1 μg/ml each) were used in the chloramphenicol experiments, an experiment of similar design was repeated using 4 μg/ml of thymine throughout. Results essentially identical with those with the low thymine concentrations were obtained. Also, low thymine concentrations do not induce observable aberrations in the coupling of F and chromosome replication since the $^3H/^{14}C$ ratios for both DNAs increase identically for one generation after a label switch to [^3H]thymine (unpublished data; see legend of Figure 7).

Measurement of DNA replication as a ratio of two labels has the advantage of correcting for variable recoveries of molecules and provides a comparison for the relative doubling rates of F and chromosome. Thus, when each species of DNA is doubling at the same rate, the ratio of the F and chromosome $^3H/^{14}C$ ratios (designated F/chromosome) is 1.0. This is

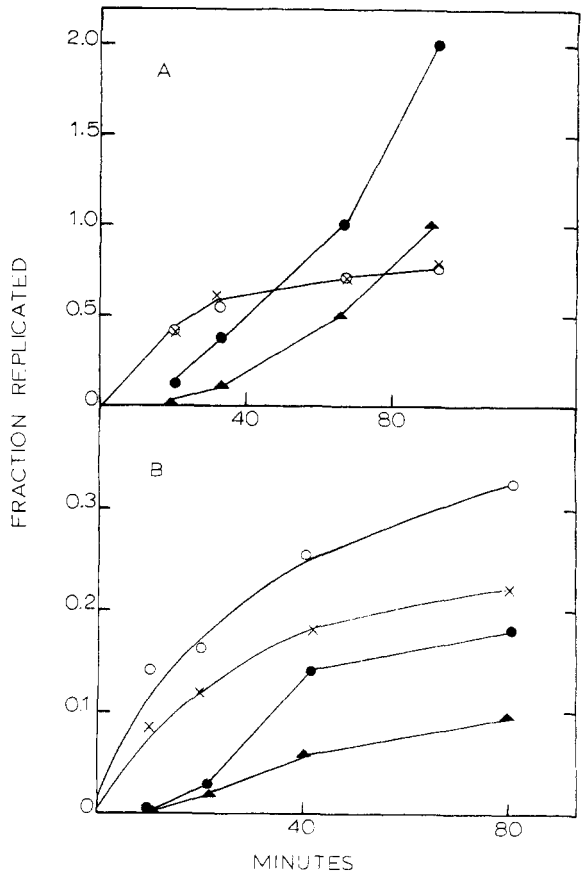

FIGURE 3: Mechanism of F DNA replication determined by density shift technique and isopycnic centrifugation. Experimental design and procedures are described under Materials and Methods. (A) Bromouracil-[^{14}C]thymine-labeled cells transferred into [^{3}H]-thymine medium lacking chloramphenicol. (B) Cells transferred into [^{3}H]thymine medium containing chloramphenicol. In both experiments the fractions of heavy (○ or ×) and hybrid (● or ▲) density DNA replicated (measured as relative amounts of hybrid and light DNA, respectively) are plotted: (○,●) CCC F DNA; (×,▲) chromosomal DNA. The abcissa represents minutes of DNA synthesis in [^{3}H]thymine media. Evidences of repair replication were not observed in an aliquot of F DNA (67 min, A) or in aliquots of chromosomal DNA (67 min, A; 80 min, B) when assayed by alkaline isopycnic centrifugation (unpublished data).

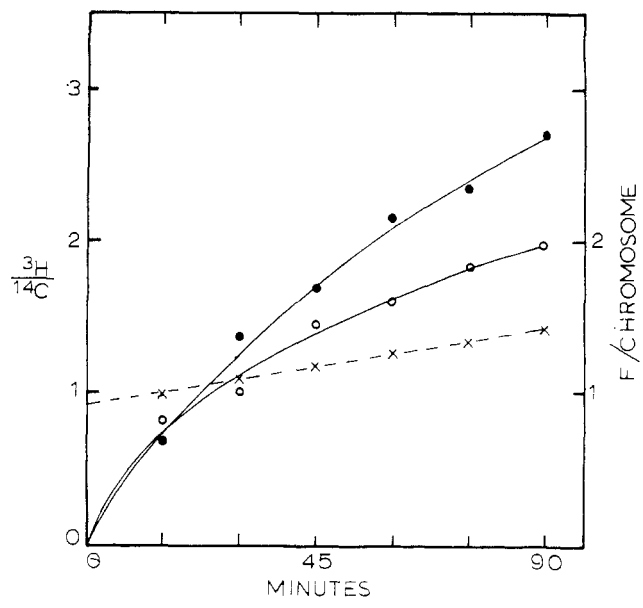

FIGURE 4: Synthesis of F and chromosome in the presence of chloramphenicol. Bacteria in the exponential phase of growth were treated with chloramphenicol and simultaneously [^{14}C]thymine was replaced by [^{3}H]thymine. Time of chloramphenicol addition is represented as zero time. Samples were withdrawn periodically and F (●) and chromosome (○) synthesis were examined by determining their respective $^{3}H/^{14}C$ values as explained under Materials and Methods. The standard error in the $^{3}H/^{14}C$ values is estimated at $\leq 10\%$ for F DNA and less for chromosome. The dimensionless quantity F/chromosome (×) is the amount of F synthesis divided by the amount of chromosome synthesis as explained under Results and in Table I.

seen, for example, when chloramphenicol is added to an exponentially dividing cell population (Figure 4).

The effects of chloramphenicol on F replication after a density shift are shown in Figures 2B, 2C, and 3B. Again, replication is observed to be semiconservative. Chloramphenicol (150 μg/ml) inhibits protein synthesis by 99% in 3 min at the cell concentration used in these experiments (B. Kline, unpublished data). While it might be argued that hybrid density F DNA synthesized in chloramphenicol medium represents replication "initiated" before an antibiotic addition, no such argument is possible for synthesis of light F DNA since it is the product of a second round of replication which occurs (Figure 3B) well after total inhibition of protein synthesis. Clearly, these data show F DNA replication is initiated in the absence of protein synthesis.

In some experiments cells were thymine starved for 45 min before chloramphenicol treatment. Kogoma and Lark (1970) have shown that DNA synthesis proceeds rapidly for 2 hr and then continues linearly at about one-half the initial rate for up to 18 hr if thymine is restored in the presence of chloramphenicol. The data of the Figure 5 insert depict this pattern of response as well as the profile of DNA synthesis in unstarved cells treated with chloramphenicol.

In two other experiments these procedures were repeated and the synthesis of F and chromosome followed from 0 to 2 or 2 to 5 hr (Figure 5). The data show: (1) the rates of F replication after thymine starvation and chloramphenicol treatment are biphasic instead of continuously decreasing as in Figure 4; (2) a greater amount of F is synthesized in thymine-starved cultures compared to the unstarved controls which were allowed to replicate F for an equivalent time (see legend to Figure 5); (3) F and chromosome double at about the same rates for 5 hr (for 8 hr in a different experiment); (4) premature initiation of F DNA replication does not occur since it takes about 1 hr (not 5 min) to double the amount of F DNA ($^{3}H/^{14}C$ ratio equivalent to 6) after the addition of thymine.

Next, F replication was examined in bacteria during synchronous chromosome replication. Synchronous chromosome replication occurs when thymine is fed to bacteria which were previously starved for required amino acids and subsequently exposed to amino acids in the absence of required thymine (Cerdá-Olmedo and Hanawalt, 1968). In such cells F replication occurs throughout the entire period of synchronous chromosome replication (Figure 6) indicating that replication of F is not synchronous with replication of a particular chromosome locus.

The apparent continual initiation of F DNA replication in the presence of a translational inhibitor makes it possible to study the requirement for untranslated RNA synthesis in F replication (Kline, 1973). The data in Figure 7 show the synthesis of F DNA in the presence of chloramphenicol and low (5 μg/ml) or high (50 μg/ml) concentrations of rifampicin, an inhibitor of RNA polymerase initiation. Several points

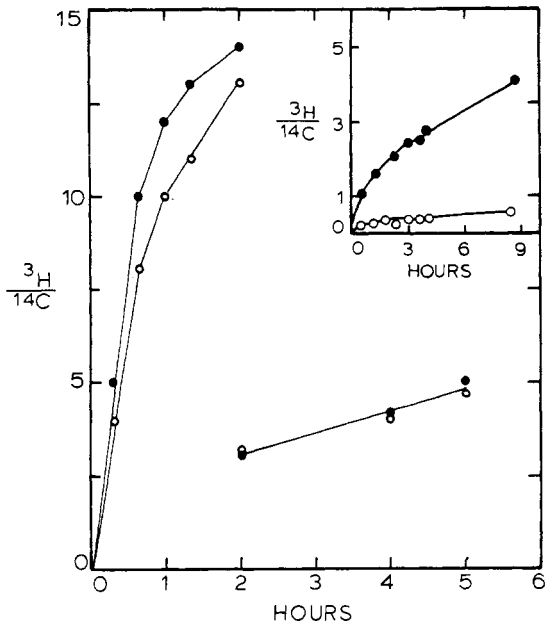

FIGURE 5: Synthesis of F and chromosome after sequential thymine starvation and chloramphenicol treatment. This figure contains the data of three separate experiments each done in the same way. In the first experiment (insert) ^{14}C-labeled bacteria were (●) or were not (○) thymine starved for 45 min at 37° and then treated with chloramphenicol coincident with an isotope switch to [^3H]thymine. The $^3H/^{14}C$ values in trichloroacetic acid insoluble material (chromosome) were determined from 0.2-ml aliquots of the appropriate cultures. In the second experiment, represented by the 0–2-hr time points, both chromosome (●) and CCC F (○) were examined. The $^3H/^{14}C$ ratios from control cells that were not thymine starved in the second experiment, but were chloramphenicol treated for 120 min, were 4.5 for chromosome and 6.0 for F. In the third experiment (2–5 hr) symbols are the same. In the chloramphenicol control culture for the third experiment, the $^3H/^{14}C$ values for chromosome and F were, respectively, 1.2 and 2.0 after 5 hr. In each experiment, control cultures were constructed from aliquots of the same media and cells used in the test cultures.

TABLE I: Inhibition of Chloramphenicol-Limited F DNA Replication[a] by Inhibitors of RNA Polymerase.

Antibiotic[b] (μg/ml)	$^3H/^{14}C$ F	$^3H/^{14}C$ Chromosome	F/Chromosome[d]
Rifampicin			
0	3.0	1.6	1.9
5	1.3	1.4	0.9
200	1.4	1.3	1.1
Actinomycin D[c]			
0	3.5	2.1	1.7
10	2.3	2.1	1.1
20	2.7	2.0	1.4
50	2.5	2.1	1.2
Streptovarcin			
0	2.9	2.1	1.4
5	1.8	2.1	0.9
50	1.2	2.1	0.6
250	1.2	2.0	0.6

[a] The experimental design and procedures are described under Materials and Methods and in Figure 4. [^3H]Thymine was incorporated for 150 min in the presence of antibiotics before $^3H/^{14}C$ ratios were determined. [b] Bacteria are rendered permeable to antibiotics as the result of exposure to chloramphenicol. [c] Actinomycin D caused a 70% decrease in RNA synthesis activity within 15 min at a concentration of 20 μg/ml when compared to RNA synthesis in a culture treated with only chloramphenicol (unpublished data). [d] F/chromosome is calculated by division of the ratio in column 2 by the ratio in column 3.

are observed in these data. (1) F replication does not continually decrease as in Figure 4 but shows several rates. (2) The fastest rate, which occurs within the first 5 min of antibiotic treatment, is greater than the chromosome duplication rate as indicated by an F/chromosome ratio ≥ 2.0 for the culture exposed to 5 μg/ml of rifampicin. The increase is not the result of a decrease in the rate of chromosome synthesis because rifampicin does not decrease the rate or final amount of chromosome synthesis (unpublished data and Table I). (3) F replication occurs for a significant time after DNA transcription has ceased. (4) Both high and low concentrations of rifampicin inhibit F replication to the same extent. (5) Rifampicin decreases the final amount of F synthesis by 50–60% when compared to the cells treated with chloramphenicol alone.

If RNA polymerase function is required for F DNA replication in the absence of protein synthesis, then antibiotics that inhibit this enzyme by other mechanisms should also inhibit F DNA replication. The data of Figures 8A and 8B show the kinetics of chloramphenicol-limited F replication in the presence of streptovarcin and streptolydigin. Streptovarcin has the same mechanism of action as rifampicin while streptolydigin is an inhibitor of RNA chain elongation (Wehrli and Staehelin, 1971). These data show that: (1) accelerated F synthesis typical of rifampicin-treated bacteria is absent; (2) both antibiotics immediately decrease the rate of F duplication relative to chromosome duplication (F/chromosome <1.0); these antibiotics do not reduce the amount of chromosome duplication (unpublished data and Table I); (3) F replication

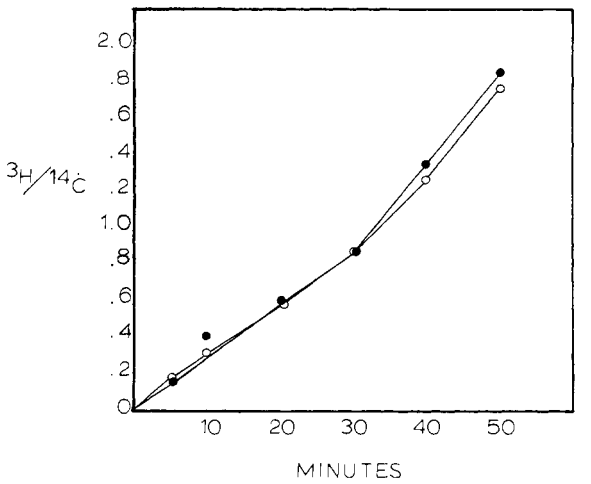

FIGURE 6: Replication of F and chromosome after sequential amino acid and thymine starvation of bacteria. Bacteria were cultured to exponential phase in [^{14}C]thymine–Casamino acid–M9 medium, and then transferred to [^{14}C]thymine–M9 medium, and incubated for 180 min at 37°. After this, bacteria were transferred to thymine-free, Casamino acids–M9 medium and incubated for 45 min at 37°. DNA synthesis was initiated by the addition of [^3H]thymine (2 μg/ml) and samples were taken at the times indicated on the abscissa. The $^3H/^{14}C$ ratios for F and chromosome are represented by ● and ○, respectively, and were determined as in Figure 1.

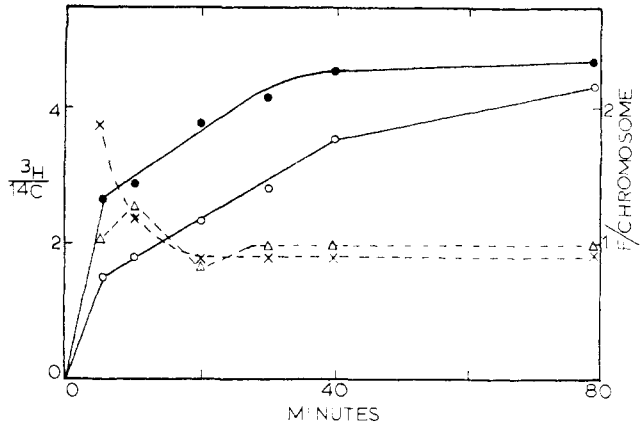

FIGURE 7: Affect of rifampicin on chloramphenicol-limited replication of F DNA. Aliquots of culture were treated with a mixture of chloramphenicol (150 μg/ml) and rifampicin (●, 5 μg/ml; or ○, 50 μg/ml) at the time of label switch to [^{3}H]thymine. The amounts of F DNA synthesis (^{3}H/^{14}C) were determined as in Figure 1 and are plotted as the solid lines. × and △ indicate the F/chromosome ratios for low and high rifampicin concentrations, respectively. The ratios were determined as in Figure 4. As one control, an aliquot of the above culture was treated with chloramphenicol alone for 120 min. The ^{3}H/^{14}C values for chromosome and F from this control culture were 5.50 and 9.0, respectively. In an untreated control, a one-generation culture, the ^{3}H/^{14}C values for chromosome and F were 10.4 and 10.2, respectively. RNA synthesis is 99% inhibited in 20 and 10 min at rifampicin concentrations of 5 and 50 μg/ml, respectively, as determined in separate experiments (Kline, 1973).

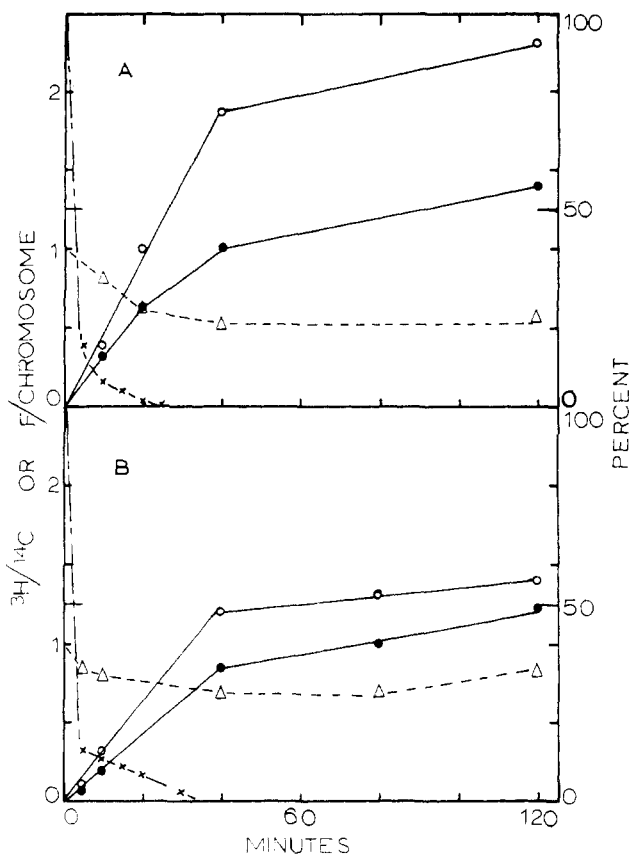

FIGURE 8: F, chromosome, and RNA synthesis in the presence of chloramphenicol and RNA polymerase inhibitors. The experiments and assays were performed as in Figure 7 except that rifampicin was replaced by (A) streptovarcin (50 μg/ml) or (B) streptolydigin (100 μg/ml). The symbols are the same in A and B. The ^{3}H/^{14}C values at the times of sampling are represented by ○ for chromosome and by ● for F DNA. The F/chromosome ratio is represented by △. In comparison to a culture treated with chloramphenicol alone, total inhibition in the amount of chloramphenicol-limited F replication by streptovarcin was 60% and by streptolydigin was 40%, but neither polymerase inhibitor significantly decreased the amount of residual chromosome replication (see also Table I). RNA synthesis was measured in separate experiments using conditions as identical as possible with the DNA synthesis experiments. The amount of RNA synthesis obtained in a 0–3-min pulse in a culture treated only with chloramphenicol was taken as 100%. The remaining RNA synthesis activity was measured in 3-min pulses of [^{14}C]uridine starting at times indicated by the position of ×. The 100% value for A was 35,000 cpm and for B was 50,000 cpm.

continues for at least 20–35 min in the absence of protein and detectable RNA synthesis. It is concluded from these results that the rifampicin-induced acceleration of F duplication is artifactual. The results of Table I show that nearly maximal inhibitions of F replication are caused by rifampicin and streptovarcin at the concentrations employed in Figures 7 and 8A. The data in Table I also show that actinomycin D inhibits chloramphenicol-limited F (but not chromosome) replication about 30%. In summary, the effects on F replication exerted by antibiotics which inhibit RNA polymerase by distinctly different mechanisms are similar and clearly indicate that F replication depends on the synthesis of RNA which is not translated.

The observation that replication of F DNA occurs after RNA synthesis has stopped (Figures 7, 8A, and 8B) suggests that the untranslated RNA required for F replication is reusable. A density shift experiment with chloramphenicol–rifampicin treated cells (Figures 2B and 2C) was performed to test the validity of this suggestion. In medium with these antibiotics, the second round of F replication should not occur until 20 min after antibiotic treatment (Figure 3B) at a time when RNA synthesis is 99% inhibited (Kline, 1973). The amount of F replication observed after 120 min in the chloramphenicol treated control (Figure 2B) shows that 70% of the heavy DNA is converted to hybrid density while 46% of the hybrid density DNA replicated to give light DNA. In the chloramphenicol–rifampicin treated cells only 40% of the heavy DNA was converted to hybrid density while 28% of hybrid DNA was replicated to give light DNA. If the amount of F replication in the absence of rifampicin is taken as unity, then rifampicin causes a 43% inhibition of the first round of replication and a 39% inhibition of the second round of replication. Thus, it appears that new RNA is synthesized for each new round of F replication, that is, the untranslated RNA is not reusable. In contrast, rifampicin had no inhibitory effect on the replication of parental (heavy) or daughter (hybrid) chromosomal DNA in the same experiment. In the presence or absence of rifampicin 56% of the heavy chromosomal DNA was converted to hybrid density and 10% of the hybrid density chromosomal DNA participated in the synthesis of light density DNA.

Discussion

The data presented show requirements for protein, RNA, and possibly chromosome synthesis in the semiconservative replication of CCC F DNA. The protein synthesis requirement is demonstrated by chloramphenicol inhibition of F replication, modification of this inhibition as the result of thymine starvation (protein enrichment), and by noting that, unlike F DNA, the amount of plasmid *col* E_1 DNA increases 150-fold during a 15-hr exposure to chloramphenicol (Clewell, 1972).

Results from the density shift experiments with chloramphenicol indicate that most cells initiate F replication once in the absence of protein synthesis (Figure 2B). This suggests F "initiator" protein (protein whose existence is inferred from the phenomenon of integrative suppression [Nishimura *et al.*, 1971]) is in gross excess or is reusable. Further, replication of F might stop in the presence of chloramphenicol because initiator proteins are consumed or degraded, because hypothetical membrane replication sites become totally occupied, or because chromosome replication has stopped.

Based on the timing of sex factor and chromosome duplication in the cell division cycle, Zeuthen and Pato (1971) suggested that sex factor replication is coupled to chromosome replication. Consistent with this, Collins and Pritchard (1973) have reported that F replication is coupled to replication of chromosome terminus. The similar duration and relative amount of F and chromosome replication in chloramphenicol-treated cells is consistent with the hypothesis of Collins and Pritchard. This interpretation also provides a simple explanation for the nearly coordinate replication of F and chromosome that occurs after sequential thymine starvation and chloramphenicol treatment (Figure 5). Thus, in Figure 5, the initial fast rate of chromosome synthesis reflects the activity of multiple replication forks induced by thymine starvation. The slower initial rate of F replication is expected if coupling occurs to a terminal chromosome region since the majority of chromosome replication precedes F replication.

The coupling concept also provides a basis for interpreting the premature appearances of light density DNA in the density shift experiments (Figures 3A and 3B). Given that cells replicating in bromouracil medium have multiforked chromosomes (Pierucci, 1969), it follows that as the second replication fork traverses the hybrid density chromosome terminus it will induce a second round of F DNA synthesis. Since F is much smaller than chromosome, light density F should be synthesized faster than light density chromosome. This is observed in Figure 3A.

Coincident initiation of sex factor and chromosome replication can occur (Cooper, 1972; Davis and Helmstetter, 1973) and indicates F replication does not necessarily depend on replication of the chromosome terminus. In fact, Davis and Helmstetter (1973) propose that sex factor replication is induced by attainment of a critical cell mass. It is shown in this paper, however, that exceeding the critical mass without concurrent chromosome synthesis during the mass increase does not induce premature F replication when chromosome synthesis resumes (Figure 5). Premature F replication is expected if growth beyond the critical mass is necessary and sufficient for F replication. Thus, some unknown event or factor in chromosome replication also appears to be required for F replication. The net effect of this event or factor is that it maintains the coordinate doubling of F and chromosome even under conditions of abnormal DNA replication (Figure 5).

During exponential cell growth, F DNA replicates synchronously with a given chromosome region (Cooper, 1972; Davis and Helmstetter, 1973). The particular region appears to be a function of the cell growth rate. The experiment reported in Figure 6 shows that this synchrony can be broken by aligning chromosome replication with sequential periods of amino acid and thymine starvation. Yet, breaking the synchrony does not break the coupling between F and chromosome replication since both molecules double at the same rate. The break in synchrony is best understood if some unknown cell element, whose quantity or effect is related to cell mass, is controlling F replication since the techniques used here to align chromosome replication should not generate cells of uniform mass.

In summary, some experimental results are best interpreted as replication of a terminal chromosome locus inducing F replication while others indicate attainment of a critical cell mass induces F replication. Quite possibly both mechanisms are operative and interact in a complex fashion such that alteration of the normal physiological state of the cell causes one of the mechanisms to predominate.

The function of untranslated RNA in F replication is unknown. Presumably, the RNA serves as a primer for the initiation of DNA synthesis as in M13 viral DNA synthesis (Wickner *et al.*, 1972). With this in mind, replication of F DNA in the absence of detectable RNA synthesis (Figures 7, 8A, and 8B) is paradoxical. The immediate inhibition of F replication by streptovarcin indicates primer RNA is synthesized just before replication; otherwise a lag in the inhibition of replication is expected. It seems unlikely that RNA primer is reused since rifampicin inhibits a second round of F replication as efficiently as the first round (Figures 2B and 2C). Sex factor replication is almost completely resistant to rifampicin in thymine-starved, chloramphenicol-treated cells (B. C. Kline, unpublished data). It is unlikely, however, that inhibitor-resistant replication of F results from thymine starvation because the culture conditions used in Figures 7, 8A, and 8B do not sustain F replication beyond 2 hr as is the case with deliberate thymine starvation (Figure 5).

The paradox is resolved if each F molecule is bound to RNA polymerase that is insensitive to or shielded from inhibitor until moments before replication. If the length of the primer is quite small, then the minute RNA synthesis required to start F replication would not be detectable. Similar insensitivity to or shielding from inhibitors of the RNA polymerase used in priming plasmid *col* E_1 replication has been observed (Clewell and Evenchik, 1973).

The following hypothesis integrates the role of protein, RNA, and chromosome synthesis in controlling F replication. Assume an F-specific initiator protein triggers F replication by activating an RNA polymerase molecule that is bound to F DNA throughout the cell division cycle. Normally, this polymerase–F DNA complex is "shielded" from initiator protein by an interaction with chromosome or some unknown cellular structure affected by chromosome replication. Once each chromosome replication cycle, the shield disappears and allows interaction of polymerase and initiator protein. Under normal physiological conditions, initiator protein functions once and is inactivated or released. Immediately after initiation, the shield reappears to prevent a second initiation of F replication. It should be recognized that the step in which the shield "disappears" might reflect biosynthesis of a molecular component required for F replication. Synthesis of this component, according to the hypothesis, would depend on replication of chromosome. Similarily, Goebel (1973) has proposed that chromosome replication may be required to induce synthesis of the *dna* C gene product which is required for initiation of R factor and *Hly* plasmid replication.

The finding that F replication is semiconservative is not unexpected. The *col* E_1 plasmid (Bazaral and Helinski, 1970) and some R factor plasmids (Kasamatsu and Rownd, 1970; Inselburg, 1971) have also been shown to replicate semiconservatively. Both *col* E_1 and R factor replication are under relaxed control. Thus, during one generation these plasmids may duplicate twice, once, or not at all. In contrast, the F replication system is under stringent control and normally replicates once in a generation. The results of this paper rep-

resent the first detailed demonstration of semiconservative replication in this latter class of plasmids. It should be recognized the results were obtainable because the methods used preserve the CCC form of the plasmid DNA.

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^{13}C Nuclear Magnetic Resonance Spectra and the Tautomeric Equilibria of Ketohexoses in Solution†

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ABSTRACT: The proportions of pyranose and furanose forms at equilibrium in aqueous solutions of L-sorbose, D-fructose, D-tagatose, and D-psicose have been determined by ^{13}C nuclear magnetic resonance spectroscopy. The assignments of the ^{13}C resonances were based on a study of configurationally related 1,5-anhydrohexitols and 1,4- and 2,5-anhydropolyols. With the exception of D-psicose, pyranose forms predominate, and the observed conformations and $\alpha:\beta$ ratios of these forms are in good agreement with values calculated from the interaction energies of nonbonded substituents. The $\alpha:\beta$ ratios of the furanose forms are determined principally by the geometry of hydroxymethyl and hydroxyl groups at C-2 and C-3. In every case, the C-2 hydroxymethyl group and the C-3 hydroxyl group are trans in the predominant furanose anomer.

Because of their biological importance, the composition and conformation of sugars in solution have been the subjects of intense investigation. Monosaccharides exist in several tautomeric forms in solution at equilibrium, and a knowledge of the relative abundance of these forms is necessary in order to understand their chemical and enzymatic reactivities. The tautomeric composition of aldoses has been determined principally by proton magnetic resonance spectroscopy (Lemieux and Stevens, 1966; Angyal, 1969). This method relies on the fact that the anomeric proton signals appear at lower field than the other proton signals and have chemical shifts and coupling constants characteristic of the configuration and conformation of the ring form. The tautomeric composition is determined by integration of the anomeric proton signals.

The equilibrium composition of ketohexoses has not been determined. There are eight isomeric ketohexoses comprising four enantiomeric D,L pairs, and a member of each of these pairs was examined in this investigation. These were L-sorbose (**1**), D-fructose (**2**), D-tagatose (**3**), and D-psicose (**4**).[1] The absence of an anomeric proton has made it impossible to observe and identify the tautomeric forms of ketoses by proton

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[1] The nomenclature of ketohexoses and other carbohydrates cited herein follows the IUPAC–IUB Rules for Carbohydrate Nomenclature [(1971), *Biochem. J. 125*, 673].