# ROLE OF DNA TRANSCRIPTION IN THE INITIATION OF ESCHERICHIA COLI SEX FACTOR (F) DNA REPLICATION

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SUMMARY

The bacterium Escherichia coli, which behaves genetically as a male (or donor), harbors an extrachromosomal DNA molecule, the F sex factor. The in vivo ability of F DNA to replicate in the presence of nucleic acid transcriptional and translational inhibitors was examined. Sex factor DNA can initiate and replicate for 1.5 hours in the presence of the messenger RNA translation inhibitor, chloramphenicol, that is, in the absence of protein synthesis. The amount of replication, determined for the covalently closed form of F DNA under this condition, is about a two fold increase. When chloramphenicol and the DNA transcriptional inhibitor, rifampicin, are both present, the extent of F DNA replication is about one-half that observed in the presence of chloramphenicol. In contrast, chromosomal DNA replicates to the same extent in the presence of chloramphenicol alone or combined with rifampicin. It appears that initiation of F DNA replication has a requirement for the synthesis of an RNA molecule that is not translated into protein and that is stable for at least 15 minutes.

The extrachromosomal genetic element F was the first episome discovered that conferred conjugal ability upon its bacterial host, Escherichia coli. The autonomous F molecule that resides in the cell cytoplasm is a covalently closed circular (CCC) DNA of approximately 62 x $10^6$ daltons molecular weight (1). A number of investigations (2,3) have indicated there are only one or two copies of F DNA per chromosomal equivalent even though there is probably sufficient time for many rounds of F replication per round of chromosomal replication. Thus, F DNA replication appears to be under what might be termed rigid control.

The nature of this control and the preliminary biosynthetic events necessary for F DNA replication are poorly understood. It is known that protein synthesis is required for continued exponential replication of F DNA and that limited initiation and replication of F DNA can occur during amino acid starvation of the host (4). In this paper it is shown that during inhibition

of protein synthesis the limited replication of F DNA is itself partially inhibited if DNA transcription into RNA is blocked.

Methods: An $F^+$, thymine auxotroph of E. coli K-12, strain CR34, was employed in this study. The sex factor harbored by this strain was originally carried by E. coli W1485 $F^+$ (1). Media, growth procedures, isotopic labelling and CCC F DNA isolation procedures have been published previously (1, 5).

The rates of nucleic acid synthesis were determined according to the following protocol: A small amount (10-30 ml) of M9-casamino acid broth (5) was inoculated with a 15 hour stationary culture and allowed to incorporate [$^{14}$C]-thymine (0.45 μc/μg; 1.1 μg/ml) for 3 to 4 generations at 37°C (final cell concentration [5-8] x $10^8$/ml). The bacteria were then centrifuged, washed once with and resuspended in an equal volume of thymine-free media. All handling was at 0°C to prevent thymine starvation. Aliquots of washed cells were then mixed with equal volumes of M9-casamino acids medium supplemented with [$^3$H]-thymine (5-10 μc/μg, 2 μg/ml) and incubated at 37°C for specified times. By this technique the [$^3$H]-thymine specific activity of each sample was the same. Cultures were treated with chloramphenicol (CM) (150 μg/ml) or rifampicin (RIF) (Lepitit, Calbiochem.) at final concentrations of 5 or 50 μg/ml. Samples of treated cultures were harvested at desired times in iced test tubes and lysed according to the technique developed by Kline and Helinski (1). The crude lysates were then analysed directly by dye-CsCl isopycnic centrifugation (4) or centrifuged at 40,000 x g for 5 to 10 minutes to remove chromosomal DNA. The resultant supernatants (cleared lysates) were then layered onto neutral sucrose density gradients as described in (1) or onto alkaline sucrose density gradients containing 0.2M NaOH, 0.05M $Na_3PO_4 \cdot 12H_2O$, 0.005M NaEDTA and 1.0M NaCl. Recovery of DNA was greater than 90 percent of the amount applied to the gradients. Gradients were fractionated onto paper strips and the strips washed according to previously described procedures (6). RNA synthesis was determined with a 2 min. pulse of [$^3$H]-uridine (1 μc/μg, 5 μg/ml) given at different times after exposure to anti-

biotic(s). The experimental design and techniques have been published (7).

Results and Discussion: The experiments that follow were performed according to the basic protocol described in the Methods section. The initial experiments were performed with low concentrations of RIF (5 μg/ml) since the possibility is reduced that a contaminant of the antibiotic causes inhibition of F replication. Even though E. coli has a permeability barrier to low concentrations of RIF, this procedure was chosen since Clewell et al. (9) have shown that certain plasmid DNA replication can be sensitive to RIF concentrations as low as 0.25 μg/ml when CM is present.

Figure 1 shows the results of a typical experiment designed to show that an RNA synthesis requirement exists for F DNA replication even though M-RNA translation is inhibited by a high concentration of CM. The numerical analysis of the CCC F DNA peaks is given for this and other experiments in Table I. The data show that regardless of the yield or method of CCC F DNA isolation, cells treated with a mixture of CM and RIF exhibit approximately 40 percent inhibition of F replication compared to those cells treated with CM. This consistency in the amount of RIF inhibition, despite the fact that the yield of F DNA varies several fold, suggests that a random sample of the F-population is obtained. As a control, F DNA replication in a spontaneous RIF resistant mutant is not sensitive to RIF (Table I).

The data of Table I shows a consistently higher yield of [$^{14}$C]-CCC F DNA from cells treated with RIF. At first glance, one might ascribe the inhibitory effects of RIF to a preferential increase in the recovery of [$^{14}$C]-CCC F DNA, thereby lowering the [$^{3}$H]/[$^{14}$C] value. This seems unlikely because as Figure 1 shows the amount of [$^{3}$H]-CCC F DNA is also higher in the RIF treated culture. Moreover, increased recovery occurs whether or not the treated culture if Rif$^{s}$ or Rif$^{r}$; yet, in Rif$^{r}$ bacteria F replication is not inhibited by RIF, that is, the F DNA [$^{3}$H]/[$^{14}$C] value is not lowered.

It was of basic interest to examine the duration of F replication in the presence of CM or CM and RIF. The data in Figure 2 show that F DNA synthesis

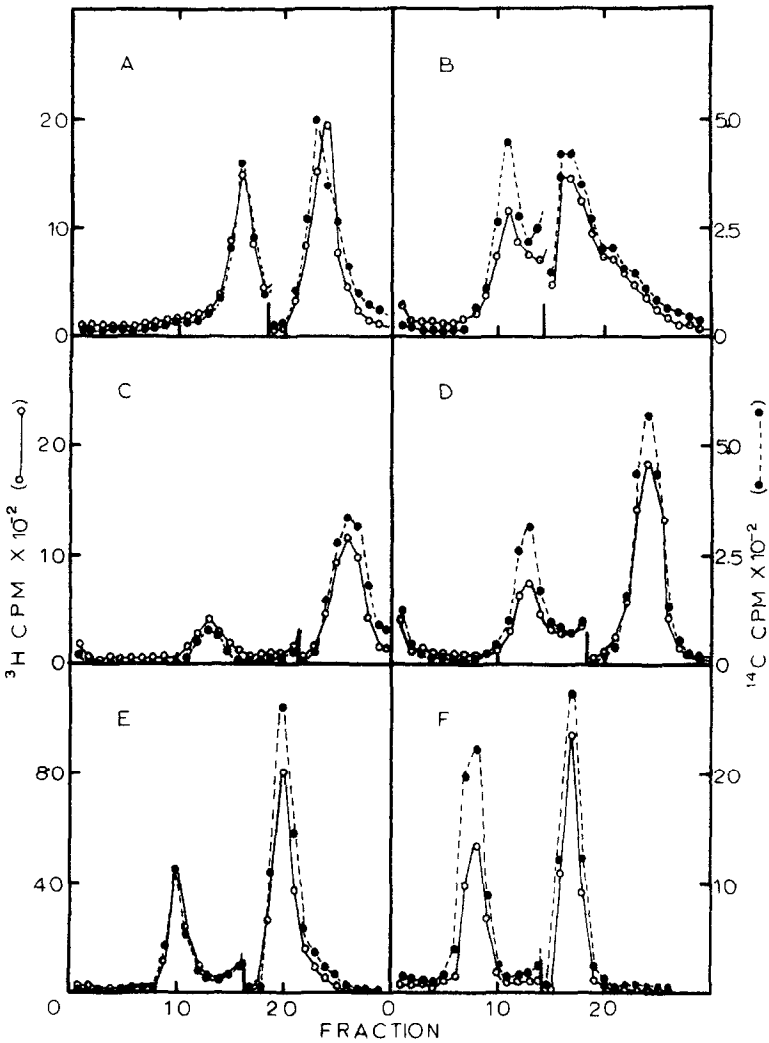


Figure 1. Inhibition by rifampicin of chloramphenicol restricted F DNA replication. Cleared lysates made from the treated cultures were analysed on neutral sucrose (Fig. A and B), alkaline sucrose (Fig. C and D) and dye-CsCl isopycnic (Fig. E and F) density gradients, respectively. Figures A, C, and E represent the culture treated with Cm. Figures B, D, and F represent the culture treated with a mixture of CM and RIF (5 µg/ml). Centrifugation conditions have been published (1, 4). To facilitate better separation of CCC F DNA, the results given in Fig. B were obtained from a 10' longer spinning time than Fig. A. Sedimentation is from right to left in Fig. A-D; density increases from right to left in Fig. E and F. In all gradients the CCC F DNA peak is closest to fraction 10. To accommodate the presentation of grossly different sized peaks, the chromosomal peaks represented to the right of the bar (|) in Fig. A-E have been reduced by a factor of 10 and in Fig. F by a factor of 30.

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continues approximately for 80 minutes to 120 minutes in the presence of CM; however, in the presence of CM and RIF, F replication is less than 40 minutes. In other experiments (data not shown) the duration of F replication was fixed at 35 to 40 minutes in the presence of CM and RIF.

TABLE I

INHIBITION OF F AND CHROMOSOMAL DNA REPLICATION

| Phenotype[1]: | Treatment[2] | Technique[3] | $^3H/^{14}C$ F | $^3H/^{14}C$ Chromosome | Recovery of F DNA[4] (% Total DNA) |
|---|---|---|---|---|---|
| Experiment 1 | | | | | |
| $Rif^s$ | None | I | 2.6 | 3.0 | 0.9 |
| | CM | I | 2.5 | 1.6 | 0.9 |
| | CM + RIF | I | 1.3(48)[5] | 1.5 | 2.2 |
| Experiment 2[6] | | | | | |
| | CM | I | 3.9 | 3.0 | 0.2 |
| | CM + RIF | I | 2.2(44) | 2.9 | 0.8 |
| $Rif^s$ | CM | NS | 3.6 | 2.9 | 0.3 |
| | CM + RIF | NS | 2.3(36) | 3.0 | 0.5 |
| | CM | AS | 3.7 | 3.0 | 0.1 |
| | CM + RIF | AS | 2.3(38) | 3.0 | 0.3 |
| Experiment 3 | | | | | |
| $Rif^r$ | CM | NS | 2.6 | 2.6 | 0.2 |
| | CM + RIF | NS | 2.7(0) | 2.6 | 0.8 |

[1] $Rif^s$ and $Rif^r$ respectively, refer to sensitivity and resistance of strain CR34F to growth in the presence of 100 µg/ml of RIF.

[2] Experimental design is given in the Methods section. Treatment was for 120 min. in all cases except, the None, which is a one generation (48 min.) control.

[3] Separation of CCC F DNA from other DNA was achieved by dye-CsCl isopycnic centrifugation (I) or by neutral (NS) or alkaline (AS) sucrose density gradient centrifugation.

[4] The recovery of CCC F DNA is calculated from the $[^{14}C]$-CPM of F DNA found in an aliquot of cleared lysate divided by the $[^{14}C]$-CPM found in an equal volume of crude lysate. For experiment 1 only, the recovery is based on the $[^{14}C]$-CPM in the CCC peak divided by the total CPM in the gradients since crude lysates were analysed.

[5] The number in brackets refers to the percent inhibition of the $[^3H]/[^{14}C]$ thymine value in the CM + RIF treatment when the value for CM treatment is taken as 100 percent.

[6] The data of Experiment 2 correspond to the data in Figure 1.

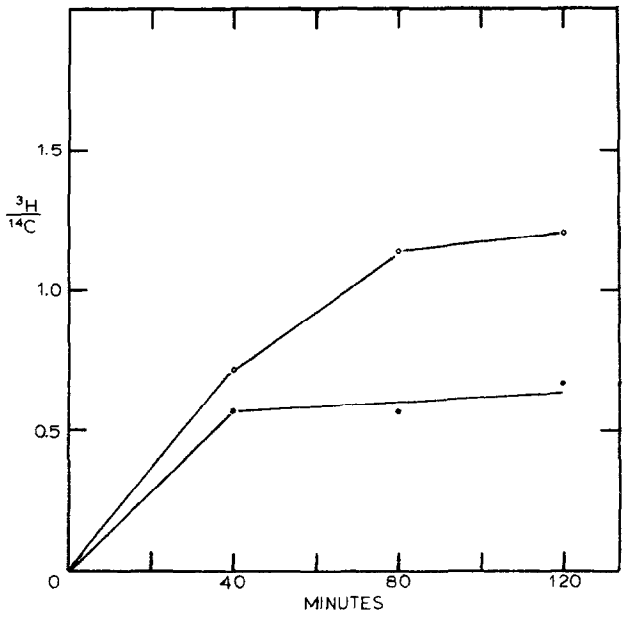


Figure 2. Kinetics of F DNA replication in antibiotic treated cultures. A culture was treated according to the protocol given in the Methods section for the indicated times. The $^3H/^{14}C$ values were determined in the CCC F DNA peaks obtained by centrifugation of cleared lysates on neutral sucrose density gradients. (O) Culture treated with CM; (●) culture treated with mixture of CM and RIF (5 μg/ml).

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The data of Figure 3 show that the partial inhibition of F replication observed in Figure 2 is not caused by an insufficient concentration of RIF. In the experiment shown in Figure 3 the RIF concentration was increased ten fold with only a slight drop in the final amount of replicated F DNA. Also given for comparison in Figure 3 are the data from a separate experiment designed to show the RNA synthesis activity during the various antibiotic treatments. Clearly, F replication continues after significant RNA synthesis has stopped.

The results of this work have confirmed and more rigorously substantiated the earlier observation of Bazaral and Helinski (4) that protein synthesis is required for continued exponential F DNA duplication but not limited initiation of F DNA duplication. In both of these works, initiation is inferred from the assumption that replication of F DNA requires only one or two minutes for

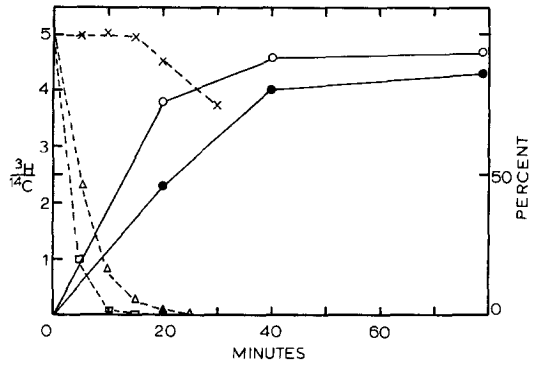


Figure 3. Sex factor replication and RNA synthesis at high and low concentrations of rifampicin. Sex factor replication was determined as in Figure 2.: Replication in presence of CM with 5 μg RIF/ml (O——O) or CM with 50 μg RIF/ml (●——●). The amount of sex factor replication is indicated by the $^3H/^{14}C$ values; the value for CM treatment at 80 min. is 7.5. The amount of RNA synthesis in a 2 minute pulse is related to the amount of synthesis in untreated cells in 2 minutes (76,000 CPM) and is expressed as percent of control. The results are plotted at the times the pulses were started. Treatments: CM (X--X), CM with 5 μg RIF/ml (Δ--Δ) and CM with 50 μg RIF/ml (□--□).

completion. Additionally, this work shows that about one doubling of the CCC F DNA population occurs during protein synthesis inhibition (Experiment 1, Table I) in a time period equal to 2.5 bacterial generations. Since this doubling is a statistical value, it does not show if some F molecules have replicated more than once or not at all.

The results of this work also have suggested that F replication has a requirement for RNA synthesis in the absence of gross RNA translation. Yet F replication persists for at least 15 minutes after the cessation of significant RNA synthesis. The obvious interpretation is that a stable RNA is involved in F replication. Nonetheless, the possibility should not be overlooked that an unstable RNA is synthesized just before use by a modified rifampicin-resistant RNA polymerase (11). If the latter possibility is true, then it is not clear why F replication is not completely resistant to RIF as is apparently the case for ϕ174 DNA replication (11). In any event, the finding of stable RNA involved in DNA synthesis would not be unique since

Lark (7) has made a similar observation in the initiation of E. coli 15T⁻ chromosomal synthesis.

It has been hypothesized for other replicon systems that a primer function exists for the RNA involved in initiation of DNA synthesis (8, 9). In this hypothesis the RNA provides a 3'-OH group which acts as an acceptor for the incorporation of the first 5'-deoxyribonucleotide in the process of DNA duplication. A similar function may exist for the RNA involved in F DNA synthesis.

Bazzicalupo and Tocchini-Valentini (10) have shown that low amounts of RIF (6 μg/ml) very efficiently cure logarithmically growing E. coli of F' lac DNA without affecting the growth rate of the bacteria. The results of their experiments could not be used to distinguish between curing as the result of a failure to initiate the synthesis of an RNA molecule required for translation into protein or curing as a failure to synthesize an RNA molecule hypothetically required as a primer for initiation of F DNA replication. While the data of this paper do not resolve these possibilities, they do suggest that there is a definite role for the RNA synthesis event per se in the synthesis of CCC F DNA.

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