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Initiation of Deoxyribonucleic Acid Replication in *Escherichia coli* B/r: Chronology of Events and Transcriptional Control of Initiation

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Three processes necessary for the initiation of deoxyribonucleic acid replication have been separated in time in synchronously growing *Escherichia coli* B/r. One can be inhibited with 2 μg of chloramphenicol per ml and occurs about 20 min prior to initiation. A second step, occurring 5 to 10 min prior to initiation, is sensitive to 4 to 30 μg of chloramphenicol per ml. A third process occurs at the time of initiation and can be inhibited with rifampin. The experiments suggest that transcription itself, or the synthesis of a relatively small ribonucleic acid, is required for initiation of replication.

The rate of deoxyribonucleic acid (DNA) replication in bacteria is regulated by mechanisms which control the frequency with which the synthesis of DNA molecules is initiated. Once initiated, the replication point travels with constant velocity (6, 14). Since cell division is dependent on a previous round of DNA replication (2, 8), the frequency of initiation also determines the division rate. The regulation of a periodic event, such as initiation of replication, in the life cycle of a cell and its implications on some of the most essential cell functions were of interest to many investigators (see A. Klein and F. Bonhoeffer, Ann. Rev. Biochem., *in press*).

Initiation of replication is dependent on protein synthesis (12, 13). Experiments in which initiation was inhibited by amino acid starvation, chloramphenicol, or phenethyl alcohol demonstrated that more than one process is involved in the control of initiation (9, 10). Recently some of these processes have been separated in time on the basis of their differential sensitivity to chloramphenicol (11, 19). There is, however, some disagreement about the number of processes which can be observed and their exact timing.

Experiments with *Escherichia coli* B/r presented in this paper show that three processes can be separated in time. Two of the processes provide proteins required for initiation. Transcription, the third process, is required at the time of initiation of replication.

MATERIALS AND METHODS

E. coli B/r-301 (F^-, *leu*$^-$, *pro*$^-$, *lac*$^-$, *gal*$^-$, *trp*$^-$, *his*$^-$, *arg*$^-$, *thy*$^-$, *str*^{r}, *met*$^-$, *dra*$^-$, or *drm*$^-$, *hsp*^{K12}) is a derivative of strain HB50 from H. Boyer. The spontaneous rifampin-resistant mutant was isolated from a colony grown on nutrient agar (Difco) containing 100 μg of rifampin/ml.

Glucose-minimal medium (5) contained 2 g of NH_4Cl, 6 g of $Na_2HPO_4 \cdot 2\ H_2O$, 3 g of KH_2PO_4, 3 g of NaCl, 0.175 g of $MgSO_4 \cdot 7\ H_2O$, and 2 g of glucose in 1 liter of demineralized water. Amino acids and thymine were added at 20 μg each/ml.

A modification of the membrane selection technique of Helmstetter and Cummings (7) was used for synchronization. Freshly divided cells were collected in ice for 60 to 90 min and concentrated about 10 times by filtration through 0.22-μm membrane filters (Millipore Corp.), prerinsed with hot distilled water. Cells were resuspended in prewarmed medium. This modification of the original technique was without influence on the degree of synchrony. Synchronous growth of the cultures was followed with a Coulter counter combined with a Nuclear Data multichannel analyzer.

Rate of DNA synthesis was measured with 4-min pulses of ^{3}H-thymidine, 2.5 μCi/ml, 20 to 30 Ci/mmole. Incorporation was stopped with trichloroacetic acid, 5% final concentration. The precipitate was collected on 0.45-μm membrane filters (Millipore Corp.), presoaked with 5% trichloroacetic acid containing 0.5 mg of thymidine/ml, washed thoroughly with 5% trichloroacetic with thymidine, and counted in a Beckman scintillation counter. When rifampin was present, filters were rinsed with ethanol after the trichloroacetic acid washings.

E. coli B/r is much more sensitive to chloram-

phenicol than *E. coli* 15 or *E. coli* K12 (19). The minimum concentration required for a complete block of initiation was 1.5 μg/ml as opposed to 25 μg/ml in *E. coli* 15. The strain is relatively insensitive to rifampin, 70 μg/ml being the minimum inhibiting concentration for initiation. This is due to a permeability barrier since mutants can be obtained which at the same time become more sensitive to rifampin and actinomycin (17).

RESULTS

E. coli B/r-301 growing in a glucose-minimal medium with a 45-min generation time was synchronized as described above. The rate of DNA replication increased by a factor of 2 at an average cell age of 17 to 22 and 57 to 62 min as taken from the inflection points of the control curves in Fig. 1–3. This is consistent with the time of initiation predicted in the Helmstetter model (6), giving a time of about 40 min to replicate a chromosome. The dependence of rate of uptake on cell age was identical whether ^{3}H-thymidine or ^{3}H-thymine

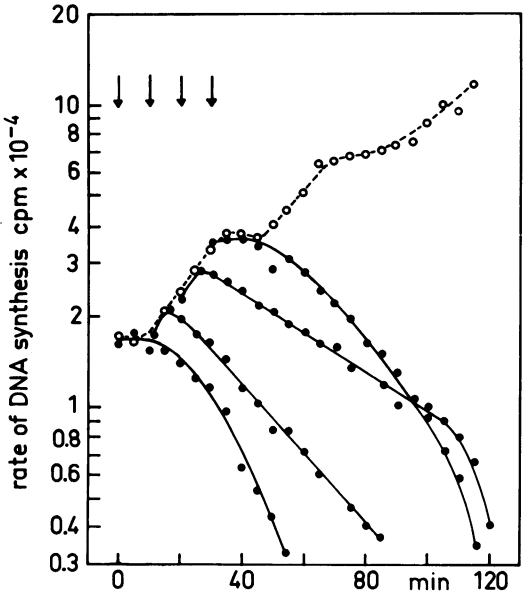

FIG. 1. *Rate of DNA replication in synchronized E. coli B/r-301 after the addition of 10 μg of chloramphenicol/ml. E. coli B/r-301 growing in a glucose-minimal medium with 45-min generation time was synchronized as described in Materials and Methods. Chloramphenicol (10 μg/ml) was added at different times during the life cycle (arrows), and the rate of DNA replication was measured in these samples by the uptake of ^{3}H-thymidine during a 4-min pulse (●). Control without chloramphenicol (○). Cell number at the beginning of the experiment was 4.5 × 10⁷/ml.*

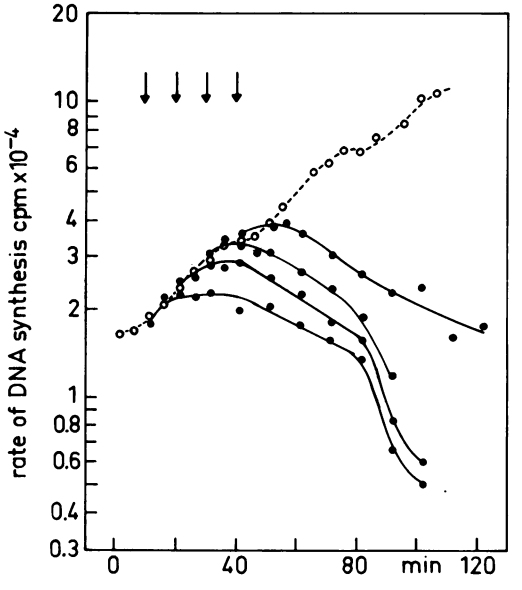

FIG. 2. *Rate of DNA replication in synchronized E. coli B/r-301 after the addition of 2 μg of chloramphenicol/ml. To E. coli B/r-301, grown and synchronized as in Fig. 1, 2 μg of chloramphenicol/ml was added at different times during the life cycle (arrows) and the rate of DNA replication was measured (●). Control without chloramphenicol (○). Cell number at the beginning of the experiment was 4.3 × 10⁷/ml.*

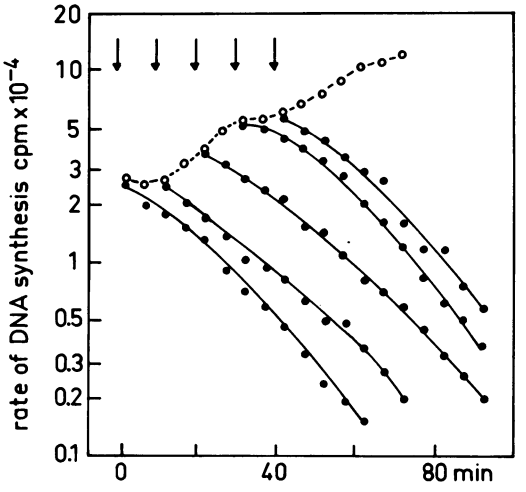

FIG. 3. *Rate of DNA replication in synchronized E. coli B/r-301 after the addition of 100 μg of rifampin/ml. E. coli B/r-301 was grown and synchronized as in Fig. 1. Rifampin (100 μg/ml) was added at different times during the life cycle (arrows). Rate of DNA replication in the presence of rifampin (●). Control without rifampin (○). Cell number at the beginning of the experiment was 5.2 × 10⁷/ml.*

was used in the pulse except that incorporation was higher with ^{3}H-thymidine.

At various times in the growth cycle, different concentrations of chloramphenicol or rifampin were added and the rate of replication was determined in these samples. The question will be whether and how long continued initiation occurs after addition of the inhibitors, as measured by an increase in the uptake of ^{3}H-thymidine.

Effect of high chloramphenicol concentrations in synchronized cultures. Chloramphenicol (10 μg/ml) was added to a synchronized culture at different times during the life cycle. The results presented in Fig. 1 show that initiation continues for about 5 min when chloramphenicol is added, provided the cells are potentially able to initiate (i.e., at cell age 10 min and 20 min, curves 2 and 3, respectively).

The end of a round of replication occurs at the same time as initiation under the conditions used. As soon as the formation of new growing points stops, we expect a decrease in the rate of replication, which should be about symmetrical to the increase in rate in the control culture. This is due to those cells which were not able to initiate after the addition of the drug but reach the end of their replication cycle.

Addition of chloramphenicol during the time at which initiation occurs in the population reflects the effects of the drug on both the number of growing points and the rate of DNA synthesis per growing point. If chloramphenicol is added at the beginning of the plateau, i.e., at cell age zero min and 30 min, the effect of chloramphenicol on the replication rate per growing point can be studied. As shown in Fig. 1, curves 1 and 4, the rate of replication stays essentially constant for the first 10 min and then drops, again due to the normal cessation of replication rounds. There is, however, a reduction in the rate of replication per growing point during prolonged treatment with chloramphenicol (*see below*).

In individual experiments the time interval during which continued initiation occurred after addition of 10 μg of chloramphenicol per ml was between 5 and 10 min. After the addition of 4 μg and 30 μg of chloramphenicol per ml, initiation continued for the same time interval.

Effect of low chloramphenicol concentrations in synchronized cultures. If chloramphenicol at 2 μg/ml is added during the initiation period in a synchronized culture, initiation continues for about the same time period as the initiation period in the control culture, i.e. for a maximum of about 20 min if the drug is added at cell age 10 min (Fig. 2). Chloramphenicol at 2 μg/ml thus allows continued initiation for about 20 min.

Afterward, a decrease in the rate of replication occurs, which is probably a composite of some cells reaching the end of the replication cycle and a reduction in the rate of synthesis per growing point.

At 85 to 90 min, an additional decrease in the rate of replication in the cultures treated with chloramphenicol before 40 min can be observed, because all cells complete the replication cycles in progress and then stop synthesizing DNA. This completion occurs about 30 min later than the end of a round of replication in the control culture, being coincident with the initiation step at 60 min under the growth conditions used.

Effect of rifampin on initiation of replication in synchronized cultures. A striking difference from the experiments with chloramphenicol can be observed when rifampin at 100 μg/ml is added at various times in the life cycle (Fig. 3). At all cell ages there is an immediate reduction in the rate of DNA replication. No initial increase in the rate can be observed.

Since rifampin has a more pronounced effect on the rate of chain elongation than chloramphenicol (*see below*), samples to which rifampin was added at a time when no initiation occurred (cell age 0 min and 30 min, curves 1 and 4 in Fig. 3) showed an immediate reduction in the rate of DNA synthesis.

The rate of replication in the samples to which rifampin was added during the initiation period (cell age 10 and 20 min, curves 2 and 3 in Fig. 3) show that, in addition to the effect on chain elongation, rifampin blocks initiation without any detectable delay.

To insure that rifampin inhibition of initiation of replication is due to the effect of the drug on ribonucleic acid (RNA) polymerase, and not to a direct action on the DNA initiation complex, a rifampin-resistant mutant was isolated. In this rifampin-resistant mutant RNA polymerase was affected because the enzyme was rifampin resistant when tested in cell extracts. In this mutant rifampin was without effect on DNA replication and initiation. The rates of DNA synthesis in the presence of rifampin were identical to the controls without rifampin.

Effect of chloramphenicol and rifampin in exponential cultures. The differential effects of different concentrations of chloramphenicol and of rifampin can also be observed in exponential cultures.

To an exponential culture of *E. coli* B/r-301 growing in glucose-minimal medium with a 45-min generation time at 5×10^7 cells/ml, different concentrations of chloramphenicol or 100 μg of rifampin/ml were added. The rate of DNA replication, as measured by a 4-min pulse of ^{3}H-thymidine, was determined at different times after addition of the inhibitors (Fig. 4).

Again it is obvious that the effects of different concentrations of chloramphenicol can be classified into two groups. Low concentrations (1.5 to 2.0 μg/ml) allow continued initiation, i.e., an initial increase in the rate of DNA replication, for about 20 min; high concentrations (4 to 30 μg/ml) of chloramphenicol allow continued initiation for about 10 min. This initial increase in rate is followed by a more or less exponential decrease in the rate of thymidine uptake. Rifampin (100 μg/ml) does not allow any initiation to occur after the addition of the drug.

The delayed effect of chloramphenicol is not due to a delayed action on protein synthesis. With 30 μg of chloramphenicol per ml protein synthesis stops immediately. Chloramphenicol at 2 μg/ml gives an immediate reduction of the rate of protein synthesis to about 10% of that observed in the control. On the other hand, with rifampin (100 μg/ml) it takes about 12 min until protein synthesis stops completely (data not shown).

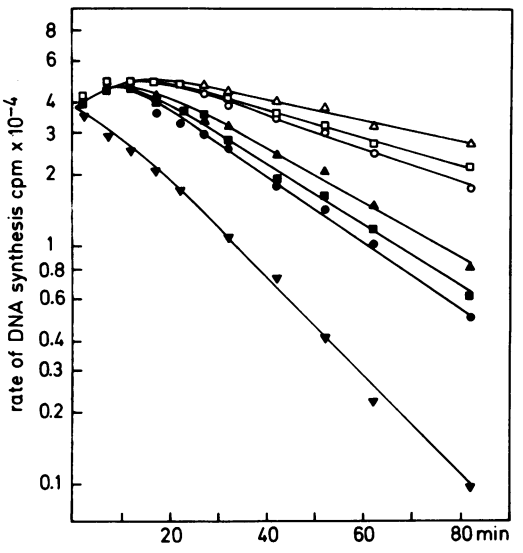

FIG. 4. *Rate of DNA replication in exponentially growing E. coli B/r-301 after the addition of chloramphenicol or rifampin. E. coli B/r-301 was growing exponentially in glucose-minimal medium at* 5×10^7 *cells/ml. Chloramphenicol or rifampin was added at time zero and the rate of DNA replication was determined as in Fig. 1. Inhibitor added: low concentrations of chloramphenicol: 1.5 μg/ml (△), 1.8 μg/ml (□), 2.0 μg/ml (○). "High" concentrations of chloramphenicol: 4 μg/ml (▲), 10 μg/ml (■), 30 μg/ml (●). Rifampin, 100 μg/ml (▼).*

DISCUSSION

In any model for DNA initiation we must postulate regulatory components and components which serve as material during the act of initiation. The time at which some of these components are synthesized can be determined with the help of antibiotics of known specificity.

A step which is sensitive to 2 μg of chloramphenicol/ml occurs about 20 min prior to initiation, indicated by a corresponding delay of chloramphenicol action. Chloramphenicol at concentrations of 4 to 30 μg/ml inhibits a process which occurs 5 to 10 min prior to initiation. These results agree with results obtained by Lark and Renger (11) in *E. coli* 15 T$^-$, using a completely different technique. The timing of the chloramphenicol-sensitive step agrees with results of Ward and Glaser (19) obtained with *E. coli* B/r; however, these authors found the step resistant to low concentrations of chloramphenicol to occur at the time of initiation. This discrepancy may be due to strain differences or to the fact that the technique used by Ward and Glaser is very sensitive to the observed reduction of replication velocity.

At least for the process sensitive to low concentrations of chloramphenicol, it is unlikely that it reflects a regulatory component. If the rate of protein synthesis is increased by the addition of Casamino Acids after the time of synthesis of the chloramphenicol-sensitive product, initiation occurs earlier than in the control (Messer, *unpublished data*). This indicates that with the synthesis of the chloramphenicol-sensitive protein the time of initiation is not strictly determined.

Besides its effect on initiation, inhibition of protein synthesis reduces the rate of chain elongation. An estimate for this reduction of replication velocity can be obtained from the final slopes of the curves in Fig. 4. For 2 μg of chloramphenicol per ml, a reduction in the rate of DNA synthesis by about 15% per 10 min is observed, about 20% for 10 μg of chloramphenicol per ml, and about 40% for rifampin. This is an upper estimate since it includes both the reduction of the rate of chain elongation per growing point and cells in which growing points reach the end of the chromosome.

The main effect of this reduction in replica-

tion velocity is that the end of a round of replication is shifted to later times. If the drugs would inhibit only the initiation process, in an exponential culture the rate of DNA replication would have dropped to zero after about 40 min with rifampin and 50 or 60 min, respectively, with chloramphenicol. As seen in Fig. 4, there is substantial synthesis after these times because more cells still synthesize DNA, although at a reduced rate. When 2 μg of chloramphenicol per ml was present throughout the cycle, the end of a round of replication occurred 30 min later than in the control (Fig. 2).

The interpretation of the timing of the different processes is not affected by this reduction of replication velocity since the changes in rate due to the formation of new growing points are much greater. The timing of the process inhibited by low concentrations of chloramphenicol, however, is not precise since the maximal rates of replication are decreased due to the prolonged action of the drug.

The use of chloramphenicol in the study of initiation of DNA replication has been questioned by Cooper and Wuesthoff (3). These authors argue that intermediate concentrations of chloramphenicol allow the residual synthesis of intermediate amounts of DNA. Recently, it has been demonstrated by Lark (*personal communication*) that initiation can occur with equal efficiency in the presence of low concentrations of chloramphenicol, once the chloramphenicol-sensitive protein has been synthesized. However, these replication cycles are not completed in the presence of the inhibitor, and the amount of replication decreases with increasing drug concentrations. These observations agree perfectly with our experiments showing that chloramphenicol has an effect on chain elongation. At least for chloramphenicol concentrations between 4 and 30 μg/ml, we could demonstrate that the use of chloramphenicol is justified since an identical time lag between the addition of the drug and its effect on initiation was observed with 4, 10, and 30 μg/ml.

The most striking result obtained is that rifampin inhibits initiation without any detectable delay at a time when inhibition of translation is no longer effective. Rifampin is known to block the initiation of transcription; polymerase molecules already in the process of transcription will continue to synthesize upon addition of rifampin (see Mosteller and Yanofsky [15] for a survey of the relevant literature). The synthetic capacity of RNA polymerase is about 40 nucleotides per second (16). We estimate that the limit of detection of a delay in rifampin action with our technique would be about 30 sec. Thus, if the requirement for transcription in the initiation of DNA replication reflects the synthesis of a RNA molecule, this must be less than about 1,000 nucleotides long.

The synthesis of a very small RNA used as a primer for DNA synthesis and covalently linked to DNA has been postulated for phage M13 initiation by Brutlag et al. (1). The conversion of M13 single strand to its replicative form in vitro requires both the action of RNA polymerase and ribonucleoside triphosphates (20). On the other hand, the replication of ϕX174 is not affected by rifampin (18, 20). Another possibility could be that the act of transcription itself is required, the local unwinding at a specific site during the initiation of transcription presenting the substrate for the initiation of DNA replication.

Lark (J. Mol. Biol., *in press*) measured the ability to replicate labeled chromosome origins of *E. coli* 15 T^- following exposure at different times to rifampin. In his system initiation could no longer be inhibited by rifampin 5 min prior to initiation. The same time interval between addition of the drug and the effect on initiation was observed when streptolydigin was used as an inhibitor of RNA chain elongation. This again suggests, consistent with our results, that transcription itself or the synthesis of a relatively small RNA is required for initiation of replication.

Two processes, distinguishable in time of occurrence and sensitivity to chloramphenicol, which reflect the synthesis of a protein (or proteins) precede initiation. At the time of initiation transcription is necessary for initiation to occur. There is a striking similarity to the processes observed in the initiation of lambda replication. The products of genes O and P are required for initiation, but, in addition, transcription has to occur at the origin of replication (4).

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