Control of Initiation of DNA Synthesis in *Escherichia coli* B/r

C. B. Ward and D. A. Glaser

VIRUS LABORATORY, UNIVERSITY OF CALIFORNIA, BERKELEY, CALIFORNIA 94720

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Abstract. The pattern of new growing forks in the *Escherichia coli* genome after a period of inhibition of DNA synthesis has been examined. Synchronous cultures of *E. coli* B/r were treated with nalidixic acid to stop DNA synthesis for various lengths of time. After removal of the nalidixic acid the location of active growing forks was determined by measuring the reversion rate of a histidine requirement induced by the "growing-point mutagen," nitrosoguanidine.

It was found that the ability to initiate a new round of DNA synthesis upon release from the nalidixic acid block was attained at the cell age at which a new round of replication would have been initiated had DNA synthesis not been blocked. However, if the nalidixic acid was removed before the normal initiation age, the initiation of the next round of synthesis was delayed past the normal time, which indicates that initiation of new rounds is not completely independent of DNA synthesis.

The *E. coli* chromosome is a single circular molecule of double-stranded DNA which, at sufficiently slow growth rates, is replicated by a single replicating fork. Maaløe and Kjeldgaard have suggested that the control of the rate of DNA synthesis is accomplished by controlling the frequency with which new growing forks are initiated, rather than by controlling the speed with which the growing forks move. Several experiments have yielded data supporting this model.

Helmstetter and Cooper have proposed a simple model to account for the regulation of initiation. The model assumes that some cellular component other than DNA accumulates over the cell cycle until its concentration reaches a critical level which triggers initiation of a new round of DNA synthesis. The model predicts that the capacity to initiate a new round of DNA synthesis is independent of DNA synthesis, so that if DNA synthesis is blocked artificially for a sufficiently long period a new round of DNA synthesis should be initiated immediately upon release of the DNA block. The capacity to initiate a new round of synthesis upon release should be gained at the cell age at which a new round would have been initiated had DNA synthesis not been blocked.

We have shown that a new round of DNA synthesis is initiated after treatment with nalidixic acid (a specific inhibitor of DNA synthesis). Similarly, Pritchard and Lark have shown that a new round is begun in thymineless mutants of *E. coli* after a period of thymine starvation. Recently, Stein and Hanawalt have examined DNA synthesis in a temperature-sensitive, DNA-replication deficient mutant of *E. coli* after a period at the restrictive temperature. These authors found that new rounds were initiated following the period...
of DNA synthesis inhibition, and that the ability to initiate a new round upon release is independent of the presence or locations of existing replicating forks. Although these results support models in which the control of initiation is independent of DNA synthesis, they do not prove them.

Consider a synchronized population of bacteria which would normally initiate a new round of synthesis at cell age A and in which DNA synthesis has been specifically blocked. The model described above predicts that initiation of a new round of synthesis will occur immediately upon release if and only if the cells are older than cell age A when the DNA synthesis block is removed. If the cell age at the time of release from the DNA inhibitor is less than cell age A, the model predicts that the next initiation should occur at cell age A.

Previous investigators tested only the first of these predictions. The work presented in this paper has been done in order to examine the above predictions in more detail and to ascertain the pattern of initiation of new growing points on the genome after nalidixic acid (NAL) treatment.

The basic experimental technique employed in this work is based on the finding of Cerda-Olmeda et al.\textsuperscript{16} that the chemical mutagen \textit{N}-methyl-\textit{N}'-nitro-\textit{N}-nitrosoguanidine (NG) makes mutations preferentially in those genes being replicated at the time of treatment. Thus if the variation in the NG-induced mutation rate in a specific gene is measured as a function of time in a synchronous population of bacteria, a large increase in this mutation rate will occur at the time the gene in question is replicated. Hence the time at which a given gene is replicated may be determined by observing the NG-induced mutation rate in that gene.

**Materials and Methods.** \textbf{Bacterial strain:} This work was done with DG336, a histidine-requiring derivative of \textit{E. coli} B/r strain HB60 (from Herbert Boyer).

\textbf{Media:} Minimal salts medium is as previously described.\textsuperscript{11} Minimal salts medium was supplemented with \textit{DL}-histidine (50 \textmu g/ml) and glucose (0.2%).

\textbf{Cell synchrony:} The membrane collection technique of Helmstetter and Cummings\textsuperscript{17} was used to synchronize the cells. Our modifications of this procedure have been previously described.\textsuperscript{18}

\textbf{NG mutagenesis:} The procedure for NG mutagenesis is as previously described.\textsuperscript{18}

\textbf{Density gradient centrifugation of DNA samples:} 6-ml culture samples were rapidly pipetted onto 3 g of a frozen solution which was 0.01 M in Tris and in EDTA. The samples were then thawed and centrifuged to pellet the bacteria. The pellets were then resuspended in 0.1 ml of a 0.01 M Tris–0.01 M EDTA solution. The cells were lysed by the addition of 0.025 ml of a 15\% sodium dodecylsulfate solution followed by incubation at 47\^\circ C for 10 min. After the lysate was digested with pronase, 0.05 ml samples were mixed with CsCl (dissolved in 0.01 M Tris, pH 8.5) to give a final density of about 1.75. The CsCl samples were centrifuged at 33,000 rpm for 48 hr in a SW39 rotor in a Spinco L2 centrifuge. The tubes were punctured and drops were collected directly into bottles of scintillation fluid (Aquasol, New England Nuclear Corp.).

\textbf{Results.} \textbf{Cell age at which the ability to reinitiate immediately upon release from NAL is attained:} We have shown\textsuperscript{18} that strain DG336 grown on glucose minimal media normally initiates a new round of replication at a cell age of 25 min. Synchronized populations of bacteria were treated with NAL for various times. After removal of the NAL block, aliquots were taken from the culture at regular intervals, and the NG-induced reversion rate of the histidine marker was measured to determine if a new round of replication was initiated immediately upon removal of the NAL.
Consider a synchronous population of bacteria 5 min after cell division (cell age 5 min). If DNA synthesis is blocked by treatment with NAL for 20 min or longer, a new round of replication should be initiated immediately upon removal of the NAL. Suppose a new round is initiated upon removal of the NAL; then since the time required to replicate the portion of the genome between the origin of replication and the histidine operon is $35 \pm 5$ min, a large increase in the NG-induced reversion rate of the his marker should be observed about 35 min after the removal of the NAL. If the round of replication that was in progress at the time DNA synthesis was blocked continues its replication of the genome upon removal of the NAL, a peak in the NG revertibility of the his marker should be observed 10 min after the removal of the NAL, since the old growing forks would have replicated the histidine operon at a cell age of 15 min had DNA synthesis not been blocked.

The results of such a set of experiments are shown in Fig. 1. For NAL

![Graphs showing NG-induced mutation rate in 5-min old cells after treatment with NAL. A synchronous population of E. coli B/r was allowed to grow to a cell age of 5 min and then NAL (10 μg/ml) was added to the culture for the length of time shown in each graph. The NAL was then removed by filtration and washings with minimal salts medium. After the removal of the NAL, aliquots were removed at regular intervals, treated with NG, and plated on selective media to determine the reversion rate of the histidine requirement.](image-url)

**Fig. 1.** NG-induced mutation rate in 5-min old cells after treatment with NAL. A synchronous population of E. coli B/r was allowed to grow to a cell age of 5 min and then NAL (10 μg/ml) was added to the culture for the length of time shown in each graph. The NAL was then removed by filtration and washings with minimal salts medium. After the removal of the NAL, aliquots were removed at regular intervals, treated with NG, and plated on selective media to determine the reversion rate of the histidine requirement.
treatment times of 20 and 30 min, a peak in the NG-induced revertibility of the
his marker is observed 30 min after the removal of the NAL, indicating that a
new round of replication was begun upon release from the NAL. For a treat-
ment time of 10 min no such reinitiation is observed. These results are in good
agreement with the predictions of the model.

The data also indicate that the old growing forks do not continue replicating
the genome when premature reinitiation occurs, as evidenced by the very small
peak at 10 min after the 30-min NAL treatment. This result is probably not
due to NAL damage of the old forks, since these forks continue normal synthesis
after a 10-min treatment, in which case premature reinitiation does not occur.
The fact that both peaks are present after the 20 min treatment is consistent
with the imperfect synchrony since cells which are slightly younger than 20 min
would not be expected to reinitiate prematurely and hence would give an NG
revertibility pattern like that observed with the 10-min treatment, while cells
which are older than 20 min would be expected to give a pattern like that ob-
served with the 30-min treatment.

In order to verify that the ability to reinitiate prematurely upon removal of the
NAL depends only on the cell age at the time of removal of the NAL and not on
the length of the NAL treatment, the above experiments were repeated starting
with a synchronized population of bacteria of cell age 15 min. If the control of
initiation is independent of DNA synthesis, one expects to obtain premature
initiation for NAL treatments of 10 min or longer. As in the previous case, if a
new round is initiated immediately upon release from the NAL, there will be a
large increase in the NG-induced reversion rate of the his marker 35 min after the
removal of the NAL, since it takes 35 min for the new growing forks to replicate
the portion of the genome from the origin to his. However, since the old growing
forks are in the process of replicating his at a cell age of 15 min, if the old growing
forks continue upon removal of the NAL, they will give a peak in the NG re-
vertibility of his immediately upon removal of the NAL rather than 10-min
later, as in the previous case.

As can be seen from Fig. 2, a new round is initiated immediately upon
removal of the NAL for treatment times of 10, 20, and 30 min, as evidenced by
the large increase in the NG revertibility of the his marker about 35 min after the
removal of the NAL. Furthermore, as in the previous experiments, the old
growing forks do not appear to continue when a new round is prematurely ini-
tiated upon removal of the NAL. Again, the presence of the peak for the old
growing points in the 10 min NAL pulse experiment is a reflection of the imper-
fect synchrony.

From the NG experiments alone, one cannot rule out the possibility that the
old growing points continue after premature reinitiation, but with a reduced
sensitivity to NG mutagenesis. In order to rule out this possibility, a synchro-
nized culture of a thymine-requiring derivative of DG336 was given a 5-min
pulse of [3H]thymine and then grown for 35 min in unlabeled medium, so that
the labeled segment of the genome was just about to be replicated. After a 45-
min treatment with NAL, the culture was transferred to fresh growth media in
which 5-bromouracil replaced thymine. Aliquots of this culture were taken at
regular intervals and analyzed on CsCl gradients to determine when the $^3$H-labeled material was replicated. If the old growing points do not continue, the labeled material should not be replicated until the newly initiated growing points have replicated the portion of the genome from the origin to the labeled material; however, if the old growing points continue, the labeled material should be replicated immediately after the removal of the NAL. The results of these experiments indicated that the labeled material was not replicated until about 70 min after the removal of the NAL, whereas in the control culture which was treated exactly like the experimental culture except that the 45-min incubation in NAL was omitted, the labeled material was replicated at about 20 min. It should be noted that the rate of DNA synthesis in the bromouracil-containing media is about one-fifth the rate in thymine-containing media. If this difference in rate is taken into account, the experiment is in good agreement with the results of the NG experiments, which indicated that the old growing points did not continue synthesis after premature initiation occurred.

Initiation of new rounds after short periods of DNA inhibition: A synchro-
nized culture of newly divided cells (cell age zero) was treated with NAL for 15 min. If control of initiation is independent of DNA synthesis, the next round should be initiated at a cell age of 25 min, that is, 10 min after the removal of the NAL. After this round is initiated there should be two sets of growing forks on the genome; the old forks which have been delayed 15 min by the NAL treatment, and the newly initiated forks. Since the time normally required for a single growing fork to replicate the genome is about 41 min, one would expect to observe the his marker to be replicated first by the old forks and then about 26 min later by the newly initiated forks. As can be seen from Fig. 3 the his marker is not replicated by the new growing forks until about 50 min after it is replicated by the old growing forks. Thus the initiation of the new rounds is delayed past the normal time in the cell cycle, possibly to allow the old round of replication to be completed.

Pattern of DNA replication after premature initiation: The fact that the old growing fork does not continue to synthesize DNA after premature initiation leads one to ask how the new forks get by the old nonfunctioning fork. One possibility which cannot be ruled out by the NG experiments is that the old fork starts synthesizing DNA again when the newly initiated forks catch up with it. If this were the case one would expect to observe an increase in the rate of

![Fig. 3. NG-induced mutation rate in newly divided cells after a 15-min exposure to NAL. A synchronous population of E. coli B/r at cell age zero was treated with NAL (10 μg/ml) for 15 min. After removal of the NAL by filtration, samples were taken in the manner described in Fig. 1.](image-url)
DNA synthesis at the time the old forks recommenced synthesizing, about one-half a generation after the removal of the NAL. Measurements of the rate of DNA synthesis (Fig. 4) show that the rate of synthesis remains constant for approximately one and a half generations after the removal of the NAL. The fact that the rate of synthesis after removal of the NAL is twice that observed before the addition of the NAL indicates that two new forks are initiated.

Fig. 4. Rate of DNA synthesis before and after treatment with NAL. A synchronized culture of E. coli B/r was allowed to grow to cell age 5 min and then NAL (10 μg/ml) was added. After 30 min the NAL was removed by filtration and washings with minimal salts medium. During this procedure, samples were taken at regular intervals, and [3H]thymidine was added, and the samples were incubated for 2 min at 37°C, after which growth was stopped by the addition of cold trichloracetic acid to a final concentration of 5%.

Conclusion. Our data indicate that the capacity to initiate a new round of DNA synthesis upon release from a period of DNA inhibition is attained at the cell age at which the cells would have normally initiated a new round of synthesis had DNA synthesis not been blocked. However, the control of initiation cannot be completely independent of DNA synthesis, since a delay in the time of initiation can be produced by blocking DNA synthesis and then releasing the block before the cells have reached the point in the life cycle at which a new round would have normally been initiated. Thus there must be at least one more control condition which must be satisfied in addition to the accumulation of some cellular component.

For example, one could imagine that there is a separate system in the cell which regulates the maximum number of growing forks that can operate in a particular growth media. This would be the case if the number of attachment sites on the cell membrane was limiting. The maximum number of forks which operate normally in cells grown on glucose minimal medium is two. Suppose that cells grown in glucose minimal medium cannot support more than two active growing forks at any one time; then when two new forks are initiated after a sufficiently long period of DNA inhibition one might suppose that one of the three forks on the genome (the two new forks and one old fork) might be inactivated, and as we have seen, this is the case. Furthermore, our data indicate that if the DNA inhibition is released before the point in the cell cycle at which the capacity to prematurely initiate is attained, the initiation of the next round
of synthesis is delayed by a time consistent with that needed to complete the round of synthesis in progress. This is consistent with the idea that the cell cannot support more than two growing forks in this growth medium and hence must delay the next initiation until the current one is completed.

Two different DNA blocking techniques in addition to NAL have been used to study the effects of selective inhibition of DNA synthesis. Pritchard and Lark\textsuperscript{14} have shown that a period of thymine starvation results in premature reinitiation in \textit{E. coli} 15T\textsuperscript{-}. Yoshikawa and Haas\textsuperscript{19} have shown that in \textit{B. subtilis}, thymine starvation results in premature initiation and that the capacity to initiate prematurely is gained at the cell age at which the cells would normally have initiated a new round had DNA synthesis not been blocked. Stein and Hanawalt\textsuperscript{16} have shown that a period of DNA inhibition imposed by placing a DNA temperature-sensitive mutant at the nonpermissive temperature also results in reinitiation of DNA synthesis immediately upon return to the permissive temperature. The fact that all these techniques for blocking DNA synthesis selectively result in premature initiation strongly argues that this effect results from the selective inhibition of DNA synthesis rather than from the specific treatment used to block DNA synthesis.

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Abbreviations: NAL, nalidixic acid; NG, N-methyl-N'-nitro-N-nitrosoguanidine.