Replication of the bacterial chromosome

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In this paper I shall confine myself to only one aspect of chromosome replication in bacteria: its control and co-ordination with growth and cell division.

The nature of the problem to be considered is made clear by two features of chromosome replication in *Escherichia coli*. First, under conditions of rapid growth, involving generation times of up to about one hour, DNA synthesis is essentially continuous; there is no detectable resting period corresponding to the G period typically found in higher organisms. Secondly, in glucose minimal media, as the data of Cairns (1963) and others have shown, a single replication point, or growth point, traverses the length of the chromosome during each cycle of replication. It follows that, although the rate of replication in *E. coli* might be determined by the supply of DNA precursors, the maintenance of the proper sequence of events cannot be controlled in this way since in a system in which DNA synthesis is continuous these precursors must be present at all times. Under the conditions mentioned above in *E. coli*, for example, the cell must have some means of ensuring that a new cycle of replication is not initiated until the previous one is complete. Consequently the important point of control of replication in bacteria must be over the initiation of replication rather than replication itself.

Although the need for control of initiation of replication is clear under conditions of continuous DNA synthesis as in bacteria, the same logic indicates that control of initiation is equally necessary in systems in which there is an interval between successive cycles of replication. The S period—period of DNA synthesis—in cell division in higher organisms occupies a measurable length of time. If replication in these systems were controlled primarily through changes in the level of DNA precursors, it is difficult to understand how the cell could avoid a situation in which repeated acts of replication would be initiated successively once the level of precursors had reached that necessary for replication to commence. It seems likely, therefore, that the striking changes in the level of DNA precursors which have been observed in plant and animal cells before the onset of DNA synthesis (reviewed by Lark 1963) reflect a system of control which exists primarily for reasons of cell economy and is not directly related to control of DNA synthesis itself.

**Control of initiation**

The first clear demonstration that initiation of replication and replication itself were separate processes was the demonstration by Lark, Repko & Hoffman (1963), following the pioneer work of Maalée and others (Maalée & Hanawalt 1961; Maalée 1961), that amino acid starvation of an amino acid auxotroph inhibits initiation of replication but does not prevent the completion of a cycle of replication in course at the time the required amino acid is removed. We have recently
shown (Pritchard & Lark 1964) that control over initiation can be modified in another way. Thymine starvation of a thymineless auxotroph stops DNA synthesis but does not stop protein and RNA synthesis—growth becomes 'unbalanced'. If thymine is restored, 30 to 60 min later, DNA synthesis is resumed but it now occurs not only at the pre-existing growth point but also commences at the normal site of initiation (which we have termed the chromosome origin).

**Figure 38.** Double-labeling technique to determine sequence of replication along a bacterial chromosome. Diagram to right of curve illustrates technique. Serrated line indicates chromosome segment labelled with $[^3]H$ thymine. Solid curve indicates segment labelled with BU. Points on curve represent samples taken at different times after transfer of culture to BU medium. Dashed curve indicates theoretical curve assuming sequential replication of chromosome and no heterogeneity in rate of replication. Modified from Lark, Repko & Hoffman (1963).

The principal method we used to demonstrate this effect is illustrated in figures 38 and 39. A culture growing exponentially in minimal medium is pulse labelled with $[^3]H$ thymine. After the pulse the density label 5-bromo-uracil (BU) is substituted for thymine in the growth medium and samples are taken at intervals for analysis in a density gradient. In each sample the amount of radioactive label ($^3H$) in light (T-T) DNA and hybrid (BU-T) DNA is measured, and the percentage of the radioactivity in hybrid DNA plotted as a function of the total percentage of the DNA which is of this type. If DNA synthesis proceeds sequentially along the chromosome, no $^3H$ activity should appear in hybrid DNA until the growth point responsible for insertion of the $[^3]H$ thymine has traversed the remainder of the chromosome and recycled back to the same point. This is essentially the result obtained (figure 38). It indicates that the technique is a valid one for determining the sequence of replication and provides a control for the experiments illustrated in figure 39 which demonstrate the premature initiation induced by thymine starvation. In this experiment we first deprived the culture of three required amino acids until DNA synthesis had ceased. The culture should now be effectively synchronized since all growth points should proceed to the chromosome terminus and no re-initiation should be possible. When the required amino acids are restored
[³H] thymine is substituted for thymine and growth in this medium is permitted until about 10% of the DNA has replicated. Cold thymine is then substituted for [³H] thymine and growth permitted until about three cell doublings have occurred.

The culture is now again starved of its required amino acids until DNA synthesis has stopped. The amino acids are again restored but BU is now substituted for thymine and samples assayed as before. As can be seen (figure 39, curve 1) the radioactive label now appears preferentially in DNA made immediately after

**Figure 39.** Initiation of replication by thymine starvation. The experimental procedures (b) are indicated in diagram below graph (a). The first line indicates expected distribution of ³H label incorporated after amino acid starvation (−AA + T). Procedures 1 to 3 show subsequent treatment of culture as described in text. * indicates site of initiation induced by thymine starvation. Note that initiation has been indicated on one partially completed replica only since this and other experiments indicate that after thymine starvation only 50% of the BU label is incorporated at the chromosome origin.
emino-acid addition. This shows that the point on the chromosome at which DNA synthesis ceased is the same after successive periods of amino acid starvation, and provides our second control. Our third control (figure 39, curve 2) shows what happens when BU is substituted for thymine without a prior second amino acid starvation. The label now appears in BU-T DNA at random, indicating that the synchronization induced by the first period of starvation has been lost. Finally we tested what happened if BU was added after a period of thymine starvation (figure 39, curve 3). Radioactivity again appears early, indicating that replication is now occurring preferentially at the chromosome origin. Thus thymine starvation de-randomizes the incorporation of 3H in BU-T DNA in the same way that amino acid starvation does.

Our conclusions from this experiment were confirmed by a number of other methods, but the interpretation of this induction of replication by thymine starvation has not yet been satisfactorily explained. There seem to be two quite different possibilities. First, initiation may be a consequence of specific inhibition of DNA synthesis. Alternatively, it may be a direct or indirect result of thymine deprivation itself.

There is independent evidence which at first sight seems to favour the former alternative. The extensive work of Maaløe and his collaborators, already referred to, on the pattern of re-initiation of DNA synthesis after amino acid starvation, and also on the way in which the rate of DNA synthesis responds to changes in growth rate, suggests that the signal for initiation of replication may be the attainment of a critical cell mass or some other growth parameter. Since mass increase continues during thymine starvation it might be supposed that this critical mass is attained in the majority of cells during this treatment so that, on re-addition of thymine, DNA synthesis resumes not only from the pre-existing growth point but also from the chromosome origin. Further support for this possibility comes from recent work of Oishi, Yoshikawa & Sueoka (1964). They have obtained evidence which suggests that under conditions of very rapid growth in Bacillus subtilis (giving a generation time of about 20 min) each chromosome has two growing points, although in synthetic media permitting generation times of 40 min or more there is probably only a single growth point as there is in E. coli under similar conditions.

In order to understand the possible analogy between the existence of two growing points in rapidly growing cells and in cells that have been deprived of thymine we must consider what happens under conditions which severely reduce the growth rate (in synthetic media with a poorly utilized carbon source, such as succinate, for example). It has been reported (see Maaløe 1961) that under these conditions a 'resting period' with no DNA synthesis now appears in the cell cycle. The popular idea which would connect all these observations together is that the rate at which a polymerase molecule travels along a DNA duplex is relatively independent of changes in growth rate induced by different nutritional conditions. This seems a reasonable assumption, provided the supply of precursors is never rate limiting, since the rate of replication might then be a function solely of the properties of the polymerase. It follows that if the generation time were longer than the time taken
to replicate a whole chromosome, a resting period would occur in the cell cycle. If the generation time were less than the replication time, on the other hand, DNA synthesis could only keep pace with the growth rate by the insertion of additional replication points such that their rate of arrival at the chromosome terminus matched the rate of cell division. Such an equilibrium would be ensured if the signal for both cell division and initiation of replication were correlated in some way.

Elegant as it is, current evidence suggests that the induction of replication at the chromosome origin by thymine starvation cannot be interpreted on the basis of this model. It leads to the prediction that specific inhibition of DNA synthesis by any means should have the same consequence: initiation of replication at the chromosome origin. We had already noted (Pritchard & Lark 1964) that inhibition of DNA synthesis by deoxyribooside starvation and by addition of deoxyadenosine in other species of bacteria did not appear to lead to initiation. Lark & Lark (1964) have now shown that cytosine arabinoside, which also specifically inhibits DNA synthesis, does not lead to re-initiation of DNA synthesis in *E. coli*. An important aspect of this last work is that simultaneous addition of cytosine arabinoside and removal of thymine again leads to re-initiation, as does thymine starvation alone.

On the basis of these observations we believe that induction of replication by thymine starvation is probably a direct or indirect result of thymine starvation and is independent of its effect on chromosome replication. A possible clue to the nature of its effect is provided by another consequence of thymine starvation: induction of prophages such as $\lambda$ (Korn & Weissbach 1962).

**Induction of prophage**

It is tempting to assume that the induction of chromosome replication and induction of prophage replication by thymine starvation are related, and indeed it was this possibility which led us to the hypothesis that thymine starvation would induce replication at the chromosome origin.

We can consider at least four possible mechanisms for induction of prophage: (i) thymine starvation causes damage to some specific site on the prophage or on the bacterial chromosome necessary for maintenance of the prophage state; (ii) induction is the result of specific inhibition of DNA synthesis (i.e. unbalanced growth); (iii) it is due to accumulation of a DNA precursor or other material which interferes with repression of the prophage; (iv) it is due to depletion of some material necessary for repression of prophage.

Hypothesis (i) seems unlikely since damage to the chromosome would presumably be confined to the neighbourhood of the growing point. In only a small fraction of the population would a growing point be located in a specific region of the chromosome yet nearly the whole population is induced during starvation. Moreover, we have found that in *E. coli* K 12 strains removal of both a required amino acid and thymine inhibits thymineless induction although attempted DNA synthesis is occurring under these conditions. There is also no evidence for any DNA breakdown or measurable change in the physical properties of DNA during or following
In E. coli (Smith 1964). A report of a drastic reduction in viscosity of DNA after thymine starvation, indicative of single strand breaks (Mennigmann & Zybalski 1962), has not been confirmed (see Maaløe 1963; Smith 1964).

Hypothesis (ii) seems to be ruled out by two quite different observations. First, we have found that under conditions of limiting thymine concentration, permitting about 20% of the normal rate of DNA synthesis and leading to typical

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unbalanced growth, induction of \( \lambda \), and also thymineless death, are almost abolished (figure 40). In connexion with this result it should also be noted that mutants which are ‘leaky’ and give between 10 and 20% of the normal rate of DNA synthesis in the absence of thymine, nevertheless undergo typical induction when deprived of thymine. Secondly, it has been shown that the kinetics of induction of phage P1 in E. coli B are identical under conditions of thymine deprivation and when BU is substituted for thymine in the growth medium, although in the presence of BU the rate of DNA synthesis is only slightly reduced (Seno & Melechen 1964).
Thus there seems to be no direct connexion between induction and unbalanced growth.

Hypothesis (iii), which may be regarded as a particular case of (ii), has the attraction that it might explain why the majority of inducing agents are also inhibitors of DNA synthesis. Evidence apparently in favour of it is based largely on the behaviour of a mutant of E. coli which, when lysogenic for \( \lambda \), is induced at high temperatures (Goldthwaite & Jacob 1964). At intermediate temperatures the degree of induction depends on the environmental conditions. Addition of adenine, adenosine, or deoxyadenosine, but not the other naturally occurring purines and pyrimidines or their ribosides or deoxyribosides, enhances the degree of induction. Since it has also been shown that in E. coli \( 15T^- \) thymine deprivation leads to an accumulation of deoxyadenylic acid (dATP) (Munch-Petersen & Neuhard 1964) and to excretion of D-ribose into the medium (Smith 1964), it might be assumed that thymineless induction was due to interference with repression of \( \lambda \) by dATP or a derivative. We have found, however, that excretion of D-ribose occurs only in certain thymineless mutants of E. coli K12 and that all of these are double mutants, one mutation preventing synthesis of thymine and the other leading to accumulation of D-ribose when DNA synthesis is inhibited. The two mutations are not linked and the fact that many strains in current use are double mutants is probably due to the fact that the second mutation permits them to utilize thymine at low external concentrations and hence can confer on them a selective advantage.

Thymineless mutants that do not excrete detectable amounts of D-ribose are nevertheless induced by thymine starvation as effectively as those that do. This suggests that accumulation of deoxyribotides is not the cause of induction although it does not rule out the possibility of accumulation of some unknown inducing substance. There is further evidence which is unfavourable to this hypothesis however. It seems likely that the sensitive target in the case of u.v. induction is the same as that involved in thymineless induction. The two treatments act additively in causing cell death (Gallant & Susskind 1961) and we know that conditions necessary for thymineless death and induction are in most cases identical (see, for example, figure 40). It has been shown that induction occurs in an un-irradiated F\(^-\)\( \lambda^+ \) culture if it is mated with an F\(^+\) \( \lambda^- \) or Col I\(^+\) \( \lambda^- \) donor which has been irradiated with u.v. light (Borek & Ryan 1960). An extensive re-analysis of this phenomenon of cross-induction (see Monk & Devoret 1964) shows that the inducing substance transferred during mating is either a cytoplasmic component formed in the male as a consequence of u.v. irradiation or is the episome itself with which a u.v. product has become associated either by synthesis or attachment. If this product accumulated as a result of inhibition of DNA synthesis by u.v. then cross-induction should occur also when thymine-starved F\(^+\) cells are used as donors. This is apparently not found (R. Devoret, personal communication). It seems clear that these results rule out (iii) as a unitary hypothesis to account for both u.v. and thymineless induction.

Hypothesis (iv), on the other hand, will account for all the features of induction I have described provided one makes the admittedly unorthodox assumption that the inhibitory substance, which we assume to be depleted by thymine starvation
and damaged by u.v. irradiation, is a labile molecule which is either DNA or sufficiently similar to DNA to act as a substrate for the repair enzymes known to exist in E. coli (see Boyce & Howard-Flanders 1964). The latter assumption is necessary by virtue of the fact that the sensitivity to induction by u.v. is different in hcr+ and hcr− lysogenic strains, in which the repair enzyme is present or absent (Monk & Devoret 1964).

Some preliminary experiments designed to test this last hypothesis were carried out some while ago, but although the results obtained were compatible with it they were not unequivocal. The technique we used is based on the observation (Stahl et al. 1961) that the sensitivity of BU-DNA to long wavelength u.v. (the source was a fluorescent tube) is much greater than that of normal DNA. This difference in

Table 5. Decay of sensitivity of a BU-grown culture to long-wavelength u.v. after transfer to thymine

<table>
<thead>
<tr>
<th>time (min)</th>
<th>viable count/ml.</th>
<th>before exposure</th>
<th>after exposure</th>
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<tr>
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In experiment 1 an exponentially growing culture in minimal medium (50 μg/ml thymine) was washed by filtration and transferred to BU medium (50 μg/ml BU and 5 μg/ml thymine) and incubated for 2 h. It was again washed and transferred back to thymine minimal medium. At times indicated samples were removed, diluted × 100 in buffer, assayed for viable titre and induced cells (after treatment with λ antiserum), exposed to fluorescent light (12.5 cm from two 20 W Osram ‘warm white’ tubes) for 30 min and again assayed for induced cells.

In experiment 2 the washed culture was transferred to BU medium (50 μg BU only) and incubated for 40 min only and then treated as in experiment 1.

Sensitivity is probably due at least in part to a difference in the absorption spectrum of BU and thymine (Boyce & Setlow 1963). Lysogenic BU-grown cells are also induced by long-wavelength u.v. light and this allows one to determine how the sensitivity to long-wavelength u.v. changes when a culture which has grown in BU is transferred back to medium containing thymine. If the target were BU incorporated into the chromosome in place of thymine, and if light sensitivity was independent of the distribution of this BU, then subsequent growth in thymine should not lead to a reduction in light sensitivity (measured as total number of induced cells per unit volume of culture). In fact we observed a rapid decay of light sensitivity (table 5), the half life being 10 to 15 min, as would be expected for a labile BU-containing target in which BU was rapidly replaced by thymine. The decay could also be explained by (a) production of BU-T DNA from BU-BU DNA or alternatively by (b) production of cells with one nucleus unlabelled and one labelled

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as a result of growth in thymine. These interpretations seem unlikely, however, since cultures grown in BU for less than one generation time and cultures grown for several generations in the presence of BU show a similar rate of decay in sensitivity after transfer to media containing thymine (table 5).

Further analysis of this behaviour will be necessary in order to define more closely the nature of the inducing action of u.v. and thymine deprivation but our working hypothesis is that there is either a labile DNA molecule or a labile segment of the chromosome which is involved in control of both chromosome replication and induction of prophage.

REFERENCES (Pritchard)


