*THE NEGATIVE CONTROL MECHANISM FOR E. COLI DNA REPLICATION**

By Barbara Hatch Rosenberg, Liebe F. Cavalieri, and Grace Ungers

DIVISION OF GENETICS, SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH, NEW YORK

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Abstract.—Evidence is presented to show that the initiation of DNA replication in *E. coli* 555-7 requires synthesis of a protein whose production is correlated with total protein synthesis. Once replication is initiated, however, reinitiation will occur if *all* further protein synthesis is prevented; a small amount of protein synthesis is sufficient to prevent this unregulated reinitiation. This shows that the initiation of DNA replication is under negative control. A mechanism for the control of DNA replication is proposed; in this mechanism a replication repressor is synthesized periodically, while an antirepressor protein is synthesized continuously. Derepression of initiation results after sufficient accumulation of the antirepressor protein, and repression is re-established by repressor synthesis after the initiation of replication.

The rate of DNA replication and its correlation with cell growth are determined primarily by the rate at which new replication cycles are initiated.[1] Some kind of control mechanism must link initiation with other cell processes such as protein synthesis, and this control may be either positive[2] or negative in nature. Positive control implies the periodic availability of some factor (the initiator) that participates in the initiation or replication process. If the control is to be sufficiently tight, it can be shown that a number of initiator subunits must be synthesized during the course of each generation; that the active initiator must contain *all* the subunits made, and become active only upon addition of the last subunit; and that once a single initiation has taken place, the initiator must be incapable of acting again (at least until another initiator has been produced). These considerations seem to rule out an enzymatic function for an initiator. They also pose other problems.

For these reasons we have considered a negative control mechanism more likely. Moreover, negative control could perhaps account for the fact that many physiological perturbations that initiate new replication cycles[3] also derepress prophage.

The initiation of replication has a fixed locus[4, 5] at the origin of the genome, which therefore constitutes a unique site at which a replication repressor could act to prevent initiation. We have formulated a theory for the negative control of initiation, involving a replication repressor protein whose synthesis occurs periodically, just after the beginning of each replication cycle, and an antirepressor protein, capable of complexing the repressor, whose synthesis is continuous and correlated with the growth rate. The theory predicts (1) that new replication cycles will be initiated whenever the total protein of the cell doubles and (2) that, if all protein synthesis is prevented after initiation, no new repressor will be synthesized and further initiation will occur without the necessity for

bulk protein synthesis. The initiator theory, on the other hand, requires that each initiation be preceded by protein synthesis. The experiments reported here were designed to test these predictions. They fully support the repressor-antirepressor theory.

Materials and Methods.—E. coli 555-7, a 15T$^-$ derivative requiring thymine, methionine, arginine, and tryptophan, was originally obtained from D. Billen. It was grown with aeration in phosphate-glucose minimal medium supplemented with the required amino acids and 1 μg/ml thymine. The generation time was 50 min at 37°. Medium changes were accomplished by filtration on membrane filters and washing with mineral medium at 37°. The wash volumes were kept as small as possible in order to minimize the cell killing which results from excess washing; the affected cells do not synthesize DNA efficiently. When DNA synthesis was to be studied, the cells were prelabeled with C^{14}-thymine (specific activity 52 mc/mmole) for at least three generations. All studies were carried out with log-phase cells at a density of about 3×10^7/ml.

C^{14}-thymine incorporation was measured by precipitating culture samples in cold 10% trichloroacetic acid, washing on membrane filters, dissolving the filters in scintillation fluid, and counting in a Beckman scintillation counter. Culture turbidity was measured in a Beckman DU spectrophotometer. Culture viability was measured by plating suitable dilutions in nutrient agar pour plates.

In some experiments, thymine starvation was followed by a period of about 15 min in the absence of both required amino acids and thymine, in order to exhaust amino acid pools, before adding chloramphenicol and C^{14}-thymine. Subsequent experiments showed that this was unnecessary, and indeed the results were the same even if amino acids were present throughout the period of treatment with chloramphenicol. Apparently 300 μg/ml of chloramphenicol efficiently stops protein synthesis whether amino acids are present or not.

Results.—Dependence of the initiation of DNA replication on total protein synthesis: The rate of initiation of bacterial DNA replication cycles is correlated with the cell growth rate.[1] Protein synthesis must precede initiation.[6] These facts suggest that initiation of DNA replication is somehow related to total protein synthesis. When DNA synthesis is prevented for a time by thymine starvation, "premature" replication cycles are subsequently initiated, provided that amino acid starvation does not accompany thymine starvation.[3] As in normal growth, net protein synthesis apparently must occur during thymine starvation in order to initiate replication (which is manifested when thymine is restored to the medium).

Thymine starvation provides a convenient method for the study of initiation, since protein synthesis and DNA synthesis can be separated in time. When thymine is removed from a culture of *E. coli* 555-7, the culture turbidity rises at a gradually decreasing rate to a maximum of about 1.8 times the original in about two hours and then decreases very slowly. The maximum is a characteristic of the particular strain. As the turbidity increases, the cells elongate; turbidity is clearly proportional to the amount of protein present. Net protein synthesis can be stopped at any point by the removal of the required amino acids from the culture. If C^{14}-thymine is restored simultaneously with this removal, the amount of DNA synthesis that follows will depend upon how many cells of the random population are able to initiate a new replication cycle as well as to complete the cycle that was in progress when thymine was removed. Completion of the current cycles in a random population, with no new initiation, theoretically

increases the total DNA of the culture to 1.4 times the original amount. If *all* cells also initiate and complete a new cycle from every DNA origin in the cell, the total DNA will theoretically rise to 2.8 times the original; the factor doubles for each additional initiation. We assume that, in each individual cell, a doubling of the protein that it possessed at the time of the previous initiation results in initiation of a new replication cycle at the origin of every genome in the cell, whether or not it has been completed. Since we are dealing with a random culture, the amount of protein synthesis necessary for initiation varies from cell to cell. On our assumption, as the total protein in the culture doubles during thymine starvation, the number of cells initiating a new replication cycle will gradually increase from 0 to 100 per cent. The resulting theoretical curve, relating protein synthesis in a random culture during thymine starvation to the amount of DNA synthesis obtained subsequently in the absence of amino acids, is shown in Figure 1. The agreement of the experimental points with the theoretical curve shows that our assumption is correct: initiation of DNA replication depends on the doubling of the total protein of the cell.

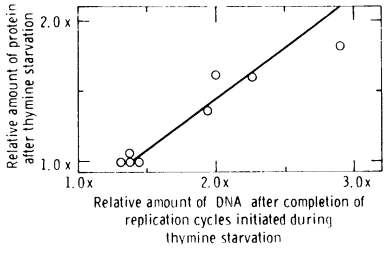

FIG. 1.—Relation of the amount of protein synthesized during thymine starvation to the subsequent initiation of DNA replication cycles in *E. coli* 555-7. Cultures were starved for thymine for various lengths of time. The increase in culture turbidity during starvation was taken as a measure of total protein synthesis. DNA synthesis was measured by C^{14}-thymine incorporation in the absence of amino acids to prevent further net protein synthesis (which would result in initiation of more DNA replication cycles) once thymine starvation was terminated. The points are experimental; the curve is theoretical, for a random culture, based on the assumption that replication is initiated at the origin of every genome, complete or incomplete, whenever the protein of the cell containing them doubles in amount. Protein and DNA are given in the figure as multiples of the initial amounts in the random culture at the start of thymine starvation. A protein value of 1.0× indicates no change in protein (i.e., a thymine starvation period of 0 min.).

Inhibition of the initiation of DNA replication by a specific protein: The absence of required amino acids prevents *net* protein synthesis, but may not eliminate protein turnover. We have found that RNA synthesis is not prevented in *E. coli* 555-7 by amino acid starvation,[7] so that synthesis of new proteins is possible. That such synthesis must occur,[8] in the experiments discussed above, is indicated by the fact that the results are considerably changed if 300 μg/ml of chloramphenicol are added when the cells are transferred from medium without thymine to that without amino acids. Chloramphenicol, at this level, very efficiently prevents protein synthesis, including that resulting from turnover. Figure 2 and Table 1 show that chloramphenicol *increases* the amount of DNA that can be synthesized after thymine starvation. The size of the increase depends on the amount of protein synthesized during thymine starvation, i.e., on the number of cells capable of initiating new replication cycles. It appears that once a new cycle has been initiated in a given cell, still *other* cycles can be initiated if chloramphenicol is present.[9] Synthesis of a small amount of protein must be

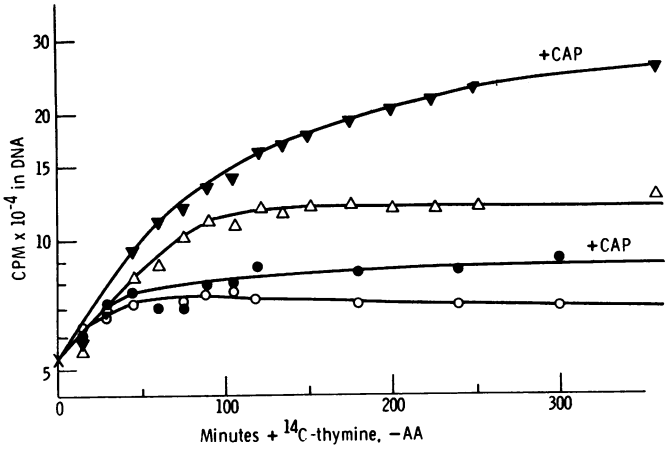

FIG. 2.—Effect of chloramphenicol on DNA synthesis after thymine starvation of *E. coli* 555-7. All curves have leveled off by 350 min except the top one, which ultimately reaches about 30 × 10^4 cpm. *Circles:* After 5 min of thymine starvation (during which the culture turbidity increased to 1.06 times the original, i.e., a 6% increase), required amino acids were removed and 11 min later C^{14}-thymine was added. *Triangles:* After 88 min of thymine starvation (culture turbidity had increased to 1.60×), required amino acids were removed and 16 min later C^{14}-thymine was added; just before thymine addition, 0.5 mg/ml norleucine and 10 μg/ml 5-methyl-tryptophan were added (subsequent experiments showed that these are irrelevant). *Closed symbols*: 300 μg/ml chloramphenicol added just before C^{14}-thymine. *AA*, amino acids. *CAP*, chloramphenicol.

required to *prevent* unregulated reinitiation. This protein synthesis is distinct from the bulk protein synthesis required to initiate in the first place.

Reinitiation of DNA replication in the presence of chloramphenicol does not go on forever, probably because the capacity for DNA synthesis—for continuation of cycles, not merely for initiation—is gradually lost in the absence of protein synthesis. Some normally noncontrolling factor in DNA synthesis, such as precursor supply, seems to become temporarily exhausted after a certain time in the absence of net protein synthesis.[10] To demonstrate this, a culture was starved for thymine for a long time, well beyond the cessation of protein synthesis, before the addition of chloramphenicol and thymine; thus, there was no protein synthesis not only during DNA synthesis but also for a time before it. As a result, the amount of DNA synthesis was curtailed. This is illustrated in Table 1 (compare the two entries from expt. 9). When the experiment was repeated with amino acid starvation instead of chloramphenicol, the expected amount (2.5×) of DNA synthesis was obtained. The synthetic capacity was apparently still sufficient for one new cycle, but was exhausted in the course of the second cycle that occurred when chloramphenicol was present.

All the experiments presented here have been concerned with DNA synthesis after thymine starvation. Because it is possible that thymineless death might disrupt the normal DNA replication controls of the cell, perhaps permitting abnormal modes of replication, we have utilized data mainly from experiments in which thymineless death was negligible. In Figure 1 this is true of all but the

TABLE 1. *DNA synthesis in the presence of chloramphenicol after thymine (T) starvation.*

Expt. No.	Culture turbidity (∝ total protein) after $-T$	Total time $-T$ (min)	Viability (%)*	Total DNA after Completion of Synthesis: Experimental	Total DNA after Completion of Synthesis: Predicted by Fig. 1
7	1.0 ×	0	100	1.7 ×	1.4 ×
7	1.6 ×	59	88	5.8 ×	2.2 ×
9	1.8 ×	111	8	5.2 ×	2.5 ×
9	1.8 ×	182	0.4	3.5 ×	2.5 ×
7	1.8 ×	175	0.2	3.5 ×	2.5 ×

Total protein constant after 112 min

After *E. coli* 555-7 was starved for thymine, chloramphenicol (300 μg/ml) and C^{14}-thymine (specific activity 52 mc/mmole) were added to the cultures. There was no further increase in turbidity. C^{14} incorporation was measured as an index of DNA synthesis. Samples were taken at intervals for 20 hr (however, incorporation was nearly maximal by 6 hr or sooner). Protein and DNA values (second and last columns) are given as multiples of the initial amounts at the start of thymine starvation. In the sample described in the last line, required amino acids were removed shortly before addition of chloramphenicol. The differences between the experimental and predicted values for DNA arise from the fact that chloramphenicol prevents protein synthesis more completely than does amino acid starvation, which was employed in Fig. 1.

* At the time of addition of chloramphenicol and thymine.

uppermost point. Table 1 shows that the chloramphenicol effect is independent of viability, *per se*. Thus, we are confident that we have been studying normal replication.

Theory for the Control of Replication.—The data presented here are consistent with a model in which the initiation of DNA replication is related to total protein synthesis through a protein which we shall call the antirepressor. The antirepressor is synthesized constitutively at a rate proportional to the over-all rate of protein synthesis (i.e., to the growth rate). The antirepressor protein has a very strong affinity for another protein, which we shall call the replication repressor; the replication repressor also has a strong, but lesser, affinity specifically for the origin of replication on the cellular genome. We propose that the replication repressor is synthesized only during a brief period at the start of each round of replication, in an amount more than sufficient to saturate all origins and thus to prevent immediate reinitiation. As antirepressor accumulates, the repressor is gradually bound in a repressor-antirepressor complex; when the amount of antirepressor becomes equal to the total amount of repressor, all the repressor is preferentially complexed by antirepressor, thus leaving all the DNA origins in the cell free to initiate replication again. We assume that the repressor-antirepressor complex either destroys itself or forms a stable component of the cell. The process of repressor synthesis and antirepressor accumulation then begins again.

The time required to produce enough antirepressor to complex all the repressor must be equal to the protein doubling time of the cell. Thus, replication will be initiated whenever protein has doubled, regardless of whether or not preceding DNA replication cycles have been completed. In this way the over-all rates of DNA synthesis and protein synthesis will remain the same, as they must. This is true even if the cell is temporarily starved for thymine.

If the binding constants of repressor to antirepressor and to the DNA origin, respectively, are of the order of 10^{13} and 10^{10}, the latter being the same as that

estimated for the binding of the lambda repressor to lambda DNA,[11] and there are ten or more repressor molecules per genome, it can be shown that the probability of initiation remains essentially zero until 90 per cent or more of the antirepressor made per generation has been produced. The probability then rises to 1 during synthesis of the remaining antirepressor. The interval between initiations will therefore differ from the protein doubling time by less than 10 per cent.

It is essential to the theory that a fixed amount of replication repressor is made immediately after replication starts, and at no other time. The continuous synthesis of antirepressor then gradually reduces the repressor concentration to zero, at which time new replication cycles are initiated, followed by resynthesis of repressor.

How can the synthesis of the replication repressor be restricted to this period? The repressor might repress not only DNA initiation, but also transcription of its own gene; this could be done simultaneously if the origin and operator were to overlap. Removal of the repressor would then permit both the initiation of replication and the transcription of the repressor gene, rapidly followed by repressor synthesis and renewed repression of both processes. However, this model implies that derepression would immediately be followed by transcription and repressor synthesis, producing renewed repression, even if DNA replication were prevented by thymine deprivation. This is contrary to the fact, since the capacity for DNA initiation can be maintained indefinitely during thymine starvation, even though RNA and protein synthesis are possible. Therefore, transcription of the repressor gene cannot take place until replication has actually started. One way of explaining this is as follows: The replication repressor gene is located near the origin of the genome and is controlled by another repressor, the transcription repressor, which is made constitutively. The replication repressor controls DNA initiation only. When all the replication repressor has been complexed by antirepressor, replication begins and progresses through the replication repressor gene, thereby displacing the transcription repressor for sufficient time to allow transcription to follow. Translation of the transcribed message produces more replication repressor, which binds to the origin and prevents reinitiation. Meanwhile, when the growing point has progressed further on, the transcription repressor returns to its former site, preventing transcription. Thus, replication repressor synthesis cannot occur again until sufficient antirepressor has accumulated to initiate replication and the new growing point has progressed through the replication repressor gene. Other, more complex hypotheses can be proposed to explain the observations, but we consider these less likely.

General Implications of the Theory.—The theory outlined above implies certain suggestions and predictions, some of which are listed here.

(1) DNA replication probably displaces repressors controlling RNA synthesis, such as the transcription repressor proposed above, as the growing point moves through their sites. A local change in DNA configuration could be responsible. Displacement may last long enough for transcription of repressed genes to occur. This could account in part for the basal level of repressed enzymes and for the

position effect in "escape synthesis" of repressed enzymes by replicating transducing phages.[12]

(2) There is probably a maximum frequency of initiation, caused perhaps by an altered DNA configuration for some distance behind the growing point. This would allow time for the replication repressor to be synthesized before reinitiation becomes possible.

(3) Although the replication repressor gene is probably transcribed only once per generation, while the antirepressor gene is continuously available for transcription, the two protein products must be made in the same amount per generation. This implies not only that the number of translations per messenger differs for the two genes,[13] but also that, if the amount of repressor is small, like the amount of lambda repressor,[14] the amounts of each protein must be strictly (rather than statistically) determined in order to synchronize derepression with the protein doubling time. Otherwise, the average deviation of the generation time would be too great. It has already been shown[15] that transcription of an operon can occur at a fixed frequency; it may also be true that the number of translations per messenger RNA molecule of a given type has a fixed value.

(4) We suggest that the active antirepressor protein may be a membrane component, composed of several identical subunits; and that the antirepressor cistron is located near the origin of the genome. Thus, the antirepressor gene dosage would always remain the same as that of the replication repressor and would not further complicate the production of the two proteins in equal amounts. If the active antirepressor were to have multiple subunits, this would maximize the number of antirepressor polypeptides, making it easier to keep the rate of antirepressor synthesis coordinated at all times with the rate of total protein synthesis. There are several reasons why membrane attachment seems desirable—among them, the need for equal segregation of antirepressor present at the time of cell division. It is not necessary for the antirepressor to be diffusible (even if complementation should prove to be possible), since the replication repressor is diffusible.

(5) The theory predicts three possible types of conditional DNA initiation mutants in *E. coli:* those defective for antirepressor, or for transcription repressor, or for some factor(s) involved in initiation but present in excess under permissive conditions (if any such factors should exist).

(6) The F factor and other independently replicating entities must themselves produce replication repressors and antirepressors (unless their DNA replicates constitutively, as in the case of virulent phages). There is no proof as yet that these control proteins are completely specific; it would not be surprising if some cross-reaction were possible.

The temperature-sensitive F′ mutants of Jacob, Brenner, and Cuzin[2] may include initiation mutants, but there is no evidence that their defects are not in factors required for the continuation rather than the initiation of DNA synthesis. Complementation of an initiation mutant by a wild-type F′ factor could be readily explained for any of the mutant types mentioned in item (5) above without implicating a diffusible initiator.

(7) Temperate phages have no need for an antirepressor of their own, since

they replicate under host control in the prophage state and constitutively in the vegetative state. However, prophage induction occurs under conditions in which the host makes excess antirepressor (i.e., conditions causing the cessation of DNA but not protein synthesis). It seems likely that phage induction is caused by the complexing of phage repressor with the antirepressor of the host or of an independent episome.

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[6] Maaløe, O., and P. Hanawalt, *J. Mol. Biol.*, **3**, 144 (1961).

[7] Rosenberg, B., and D. Packer, in preparation.

[8] In studying thymineless death, we have found that protein must be synthesized during the lag period if death is to follow. In *E. coli* 555-7, thymineless death can occur in the absence of required amino acids, implying that sufficient protein can be made under those conditions.[7]

[9] A high level of chloramphenicol prevents protein synthesis and permits reinitiation of replication in *E. coli* 555-7. In *E. coli* K12, chloramphenicol is not necessary; reinitiation occurs to some extent merely in the absence of required amino acids (Rosenberg, B., and G. Ungers, unpublished data). This is because amino acid starvation stops protein synthesis more completely in K12 than in 555-7; for example, without amino acids K12 cannot make the protein required for thymineless death.[8]

[10] Even current cycles of DNA synthesis cannot be completed in *E. coli* 555-7, if there has not been net protein synthesis for some time (Rosenberg, B., unpublished data).

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