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Replication Time of the Chromosome in Thymineless Mutants of *Escherichia coli*

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Reducing the concentration of thymine in the growth medium of *thy*$^-$ mutants of *Escherichia coli* progressively increases the replication time of their chromosomes, without affecting significantly the growth rate of the cultures, down to a concentration that is strain-specific. The replication time of the chromosome of *thy*$^-$ mutants of two unrelated strains of *E. coli* was estimated to be longer than that in *E. coli* B/r *thy*$^+$ even in the presence of saturating concentrations of thymine. The replication time could be further reduced by adding deoxyguanosine as well as thymine to the growth medium.

1. Introduction

The growth rate of a culture of *Escherichia coli* and the replication time of the chromosome can be varied independently of each other. For example, Helmstetter, Cooper, Pierucci & Revelas (1968) have shown that in strain B/r the replication time (C) of the chromosome is constant in cells growing with mean generation times between 22 and 65 minutes. Conversely, we have shown (Pritchard & Zaritsky, 1970) that in the *thy*$^-$ strain 15T$^-$ (555-7) the time taken to complete a round of chromosome replication can be increased by a factor of at least 2·5 by reducing the concentration of the thymine in the growth medium without significantly affecting the mean generation time of the culture.

The discovery that the replication velocity (reciprocal of the replication time) is affected by the concentration of thymine in the growth medium, and that a substantial change in velocity occurs without any detectable effect on growth rate, introduces a potentially serious source of error in the interpretation of data involving measurements of rates of DNA synthesis and determination of the macromolecular composition of cells in *thy*$^-$ mutants, since these parameters are sensitive to changes in the replication time of the chromosome as well as to changes in growth rate (see Pritchard & Zaritsky, 1970). In the case of strain 555-7 this problem is probably not a serious one since only a small increase in the replication velocity occurs on increasing the thymine concentration above 1·0 μg/ml. and, in most published work with this strain, concentrations greater than this have been used. This strain is exceptional, however, in being able to grow normally on very low concentrations of thymine. Concentrations greater than 0·2 μg/ml. will support normal growth (Maaløe & Rasmussen, 1963; Lark, Repko & Hoffman, 1963; Pritchard & Zaritsky, 1970), and the major change in replication

velocity occurs over the range 0·25 to 1·0 μg/ml. Most strains of *E. coli* with a "low" thymine requirement need about ten times this concentration for normal growth (unpublished data).

The main purpose of the experiments reported in this paper was to determine whether an effect of thymine concentration on replication velocity also occurs in other thymineless mutants of *E. coli*. We chose the strain CR34 since it has been widely used in studies of DNA synthesis (e.g. Caro & Berg, 1968; Wolf, Newman & Glaser, 1968) and requires at least 2·5 μg thymine/ml. for normal growth in glucose minimal medium. By comparison with 555-7 it might be anticipated that over the concentration range 2·5 to 10·0 μg thymine/ml., used by most authors in experiments with this strain, there would be significant changes in the chromosome replication velocity. Between thymine concentrations of 2·5 μg/ml. and 16 μg/ml. the replication time was found to change from 120 minutes to 66 minutes in CR34.

In this paper we have also investigated the effect of addition of deoxyguanosine to the growth medium on replication velocity in both CR34 and 555-7. In both strains, addition of this compound as a source of deoxyribose-1-phosphate resulted in an increased replication velocity.

In the following paper (Beacham, Beacham, Zaritsky & Pritchard, 1971) the replication velocity is compared with the intracellular concentration of thymidine triphosphate in both CR34 and 555-7.

2. Materials and Methods

(a) *Bacterial strains*

P162 (CR34), *E. coli* K12 *thy*$^-$ *dra*$^-$ *thr*$^-$ *leu*$^-$ *thi*$^-$. P162-8, *drm*$^-$ derivative of P162 (Beacham *et al.*, 1971). P178 (555-7), *E. coli* 15T$^-$ *thy*$^-$ *drm*$^-$ *arg*$^-$ *trp*$^-$ *met*$^-$. Symbols are those used by Taylor (1970).

(b) *Cultural conditions and experimental procedure*

M9 glucose medium was used in all experiments. Cultural conditions, changes of medium, sampling technique, measurement of absorbance and measurement of isotope incorporation have been described in detail previously (Pritchard & Zaritsky, 1970). Specific activities of [^{14}C]thymine given in the text are approximate only and comparisons of absolute amounts of incorporation are consequently valid only within a given experiment. Particular care was taken to ensure that cultures were in a steady state of balanced, exponential growth by keeping optical density (at 450 nm) below 0·4.

Strain P162 grows normally on thymine concentrations down to 2·5 μg/ml. Our criteria (Pritchard & Zaritsky, 1970) for normal growth are that (a) mass increases exponentially and indefinitely at a constant rate which is not changed significantly by increasing the thymine concentration in the growth medium, (b) the DNA : mass ratio remains constant, and (c) the ratio of particles (measured on an electronic particle counter) to colony-forming units remains constant and is not significantly different from unity.

(c) *Estimates of replication time* (C)

These were obtained (a) from measurements of the increment in DNA (ΔG) after a shift down to medium lacking required amino acids, and (b) from the DNA: mass ratio ($\overline{G}:\overline{M}$) of cultures grown on various concentrations of thymine. The principles underlying each method have been discussed in detail previously (Pritchard & Zaritsky, 1970).

The *thy*$^-$ mutation present in P162 and P162-8 was shown to be non-leaky using the technique described by Pritchard & Zaritsky (1970). The details of the method are given in the following paper (Beacham *et al.*, 1971).

3. Results

(a) *Effect of thymine concentration on replication time* (C) *in CR34*

The increment in DNA (ΔG) in a culture following transfer of an amino acid auxotroph in a steady state of exponential growth to medium lacking amino acids provides an estimate of the replication time of the chromosome provided the doubling time (τ) of the cultures before the shift down is known. The assumptions underlying this method are (a) that replication forks traverse the chromosome at uniform velocity during steady-state growth conditions, (b) that new rounds of replication cannot commence after transfer, and (c) that existing forks progress to the chromosome terminus (Maaløe & Hanawalt, 1961; Lark *et al.*, 1963; Caro & Berg, 1968; Wolf *et al.*, 1968).

TABLE 1

ΔG as a function of thymine concentration before amino acid deprivation

Thymine concn (μg/ml.)	τ (min)	ΔG (%)	C (min)
8·0	54	57·6	77
2·83	60–61	78·5	114

Cultures of P162 were grown in the presence of the indicated concentrations of [^{14}C]thymine (0·05 μCi/μg) for at least 6 generations. When each culture reached $A_{450} = 0{\cdot}4$ it was filtered, washed with approximately the same volume of warm medium without amino acids or thymine and resuspended in a small volume of the same medium. This procedure usually took less than 3 min. At time zero, portions were withdrawn into the same medium supplemented with [^{14}C]-thymine (0·05 μCi/μg) and the amount of ^{14}C-incorporation into acid-insoluble material determined as described before (Pritchard & Zaritsky, 1970). Each value of ΔG is the average of three determinations obtained from post-shift cultures containing different thymine concentrations. The highest and lowest values did not differ by more than 5% from the average. Values of τ were determined by measuring optical density and DNA doubling times before the shift-down. Values of C were calculated from the equation

$$\Delta G = \frac{(2^{C/\tau}.C/\tau.\ln 2}{2^{C/\tau} - 1} - 1)\,100$$

(Sueoka & Yoshikawa, 1965).

Values for ΔG obtained with P162 grown in M9 medium containing 2·83 and 8·0 μg thymine/ml. are given in Table 1 together with the estimates of C calculated from them. As we have previously found with strain P178 (Pritchard & Zaritsky, 1970), ΔG is increased when the thymine concentration in the growth medium is reduced.

An independent method of obtaining relative replication times of the chromosome in cultures in steady states of exponential growth in medium containing different concentrations of thymine is to measure the DNA : mass ratio in such cultures. The main assumption underlying this method is that initiation of cycles of chromosome replication occur at a defined mass : chromosome-origin ratio (Donachie, 1968; Cooper & Helmstetter, 1968; Pritchard, 1968; Pritchard, Barth & Collins, 1969; Pritchard & Zaritsky, 1970). Provided the DNA : mass ratio is known for one value of C, the replication time can be calculated for any other DNA : mass ratio obtained.

Values for $\bar{G}/\bar{M}$ were therefore obtained for cultures growing on several concentrations of thymine (Table 2) and C was calculated from these, assuming a value of 77 minutes at 8·0 μg thymine/ml. (Table 1). Using this reference value, experiments 1 and 2 in Table 2 supply four additional estimates of C. One of these values (101 minutes for 4·0 μg thymine/ml.) was used in the same way as a reference value in experiment 3 (Table 2) to obtain a fifth estimate for C. These five values, as well as the two obtained

TABLE 2

$\bar{G}/\bar{M}$ *as a function of thymine concentration*

Thymine concn (μg/ml.)	τ (min)	$\bar{G}/\bar{M}$ (10^{-4} × cts/min/A_{450})	$R \times \bar{G}/\bar{M}$	C (min)
Experiment 1				
2·0	56	0·596	0·593	164
4·0	48	0·734	0·730	101
8·0	49·5	0·853	(0·848)	(77)
16·0	50	0·916	0·911	65–66
Experiment 2				
8·0	46·5	0·830	(0·825)	(77)
2·5	55·5	0·722	0·718	120
Experiment 3				
4·0	49	0·750	(0·738)	(101)
5·0	49	0·795	0·782	90–91

Parallel cultures of P162 were grown in medium containing the indicated concentrations of thymine. Specific activities within experiments were identical (ca. 0·05 μCi/μg) but varied a little from one experiment to another. $\bar{G}/\bar{M}$ was determined by measuring simultaneously the absorbance and ^{14}C-incorporation into acid-insoluble material as described previously (Pritchard & Zaritsky, 1970). Each value is an average of at least 5 samples taken in successive generations. To obtain C from experimental measurements of τ and $\bar{G}/\bar{M}$, we first found $\bar{G}/\bar{M}$ for an assumed value of C (obtained from ΔG). For this value of C we thus had the observed DNA : mass ratio—$(\bar{G}/\bar{M})_{obs}$—and the value —$(\bar{G}/\bar{M})_{calc}$—calculated from the equation $\bar{G}/\bar{M} = \tau/kC \ln 2\,(1\text{-}2^{-C/\tau})$ (Pritchard & Zaritsky, 1970). From these two values we obtained a normalization factor $R = (\bar{G}/\bar{M})_{calc}/(\bar{G}/\bar{M})_{obs}$ with which we multiplied other experimentally determined values of $\bar{G}/\bar{M}$. Values of C were calculated from the corresponding values of $R \times \bar{G}/\bar{M}$. The values in brackets served as reference values from which the other values of C were calculated.

from ΔG experiments (as described), are plotted in Figure 1 against the reciprocal of the thymine concentration. As previously found in similar experiment with P178 (Pritchard & Zaritsky, 1970), they fall on a straight line apart from one obtained for a culture growing on 2·0 μg thymine/ml., which is below the minimum concentration required for normal growth by our criteria.

(b) *Effect of deoxyguanosine on replication velocity*

The value of C extrapolates to 57 minutes for saturating concentrations of thymine (Fig. 1). An identical extrapolate was obtained previously for strain P178 (Pritchard & Zaritsky, 1970). This value is nearly 30% greater than that obtained in a *thy*$^+$ strain of *E. coli* (B/r) by Cooper & Helmstetter (1968), who used a different method to estimate the value of C.

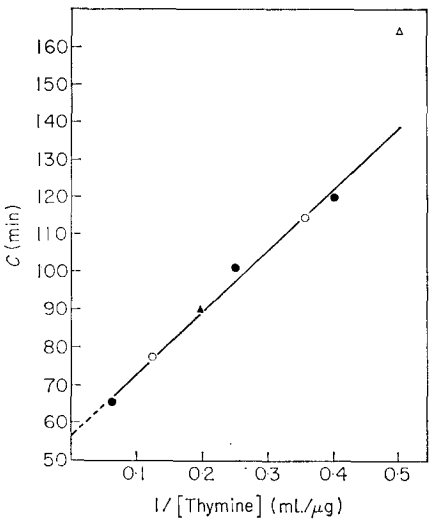

FIG. 1. The replication time of the chromosome of P162 as a function of the reciprocal of the thymine concentration. The source of the points on the graph is as follows: (○) from ΔG (Table 1); (●), (△) and (▲) from $\bar{G}/\bar{M}$ in experiments 1, 2 and 3, respectively (Table 2).

The most likely interpretations of this difference seemed to be either that there is a systematic error in our estimates of C or that the difference between these strains is real. If real, it might reflect either a difference in replication time between B/r on the one hand and strains K12 and 15 on the other, or might be due to the fact that both strains we have studied carry *thy*$^-$ mutations and have low intracellular thymidine triphosphate (dTTP) concentrations even at saturating thymine concentrations (Beacham *et al.*, 1971). In an attempt to test this last possibility, we obtained new estimates of C (Tables 3 and 4) for both P162-8 and P178 in cultures supplemented

TABLE 3

ΔG in cultures grown in the presence of deoxyguanosine before amino-acid deprivation

Strain	Thymine concn (μg/ml.)	τ (min)	ΔG (%)	C (min)
P178	5·0	47·5	38	48
P162-8	8·0	52·4	39	53

Cultures of each strain were treated as described in the legend to Table 1, with the addition of 200 μg deoxyguanosine/ml. to the growth medium in all stages of the experiment. Values of τ, ΔG and hence C, were determined as described under Table 1.

TABLE 4

$\overline{G}/\overline{M}$ in cultures grown in the presence of deoxyguanosine

Thymine concn (μg/ml.)	Deoxy-guanosine (200 μg/ml.)	τ (min)	$\bar{G}/\bar{M}$ (10^{-4} × cts/min/A_{450})	$R \times \bar{G}/\bar{M}$	C (min)
Experiment 1; P178					
5·0	−	37	1·765	(0·833)	(60)
5·0	+	41·5	2·00	0·945	49
Experiment 2; P162-8					
8·0	−	48	1·01	0·837	(77)
8·0	+	49	1·17	0·970	54
Experiment 3; P162-8					
8·0	−	46·5	0·830	(0·825)	(77)
1·25	+	49	0·967	0·961	55

Cultures of each strain were sampled as described in legend to Table 2. Values of τ, $\bar{G}/\bar{M}$, $R \times \bar{G}/\bar{M}$ and C were determined as described under Table 2. The reference value $C = 60$ min for P178 growing on 5·0 μg thymine/ml. is taken from Pritchard & Zaritsky (1970). Specific activities of [^{14}C]thymine were 0·05 μCi/μg (experiment 1) and 0·025 μCi/μg (experiments 2 and 3).

with deoxyguanosine, on the assumption that this deoxynucleoside would raise the dTTP concentration by providing an additional source of deoxyribose-1-phosphate. (It was necessary to use a *drm*$^-$ derivative of P162 for this experiment, since growth of P162, which is *dra*$^-$, is inhibited by deoxyguanosine, see Beacham, Eisenstark, Barth & Pritchard (1968).)

It was found (Tables 3 and 4) that addition of deoxyguanosine reduces ΔG and increases $\bar{G}/\bar{M}$ in both strains. This result suggests that the replication velocity of P178 growing in medium containing 5 μg thymine/ml. and of P162-8 growing in medium containing 8 μg/ml. is not the maximum attainable in these strains. We therefore conclude that even at high concentrations of thymine the replication velocity is being limited by the intracellular concentrations of the deoxynucleotide precursors and is lower than would be found in *thy*$^+$ derivatives of these strains. When C is calculated from ΔG in cultures of P178 supplemented with deoxyguanosine, a value of 48 minutes is obtained. This is quite close to the estimate of 46 minutes which was obtained by Cooper & Helmstetter in *E. coli* B/r after correcting their estimate for the difference in growth rate between batch cultures and in cells adhering to a membrane filter (Helmstetter & Cooper, 1968). In the case of P162-8, on the other hand, our estimates of C in cultures containing deoxyguanosine remain higher than 46 minutes.

4. Discussion

We have now provided evidence that in two *thy*$^-$ strains with very different thymine requirements, the replication time of the chromosome is altered by changing the thymine concentration in the growth medium without significantly altering the growth rate. These results support the hypothesis that chromosome replication is a process whose velocity is independent of the growth rate of the cell (Cooper & Helmstetter, 1968; Pritchard & Zaritsky, 1970).

In both strains, close quantitative agreement in our estimates of the *rate of change* in C as a function of thymine concentration was obtained from measurements of ΔG and $\overline{G}/\overline{M}$. In P178, also, this agreement held when another independent method of estimating C was used (Pritchard & Zaritsky, 1970). Although this suggests that there is no serious error in our estimates of the way in which C changes as a function of the thymine concentration in the growth medium, we can be less confident of the validity of the absolute value of C under any particular cultural condition, since this is determined ultimately from ΔG and therefore depends on the validity of the assumptions that when amino acids are removed from the growth medium all existing replication forks reach the chromosome terminus and that no further initiation occurs. Nevertheless the data presented in this paper suggest that estimates of C from ΔG in P178 are close to the true value, since the minimum value obtained when the growth medium was supplemented with deoxyguanosine as well as thymine, is similar to that found in *E. coli* B/r by Helmststter & Cooper (1968) using a more direct method.

On the other hand, there is a large discrepancy between our estimate of C in P178 and that of Bird & Lark (1968). Using 2 μg thymine/ml. they obtained a value of 40 minutes for C compared with our estimate of 65 minutes at this thymine concentration (Pritchard & Zaritsky, 1970). Bird & Lark used amino-acid starvation to label chromosome origins with [^{3}H]thymine and termini with [^{14}C]thymine. Amino-acid starved cultures labelled in this way were allowed to recommence DNA synthesis by returning them to full growth medium, pulse-labelled at various times with 5-bromouracil, and the fraction of ^{3}H and ^{14}C in hybrid DNA determined. The times at which the greatest fraction of ^{3}H and ^{14}C appeared in DNA of hybrid density were taken to measure the times of replication of origins and termini respectively, and the time between these maxima was 40 minutes. We believe that this method probably underestimates C substantially. Thus cultures were labelled with [^{3}H]thymine for 25 minutes after release from amino-acid starvation. Even if we were to assume that label is incorporated over only half this period it will be distributed over a substantial fraction of the chromosome length and the maximum of ^{3}H in hybrid DNA will occur later than the time of replication of the beginning of the chromosome. Any departure from perfect synchrony of DNA replication will increase this discrepancy. Similarly the time of replication of chromosome ends will be different from the time when the fraction of ^{14}C in hybrid DNA is at a maximum.

As anticipated from the minimum concentration of thymine required for normal growth of P162, significant changes in replication velocity are found in cultures maintained on concentrations in the range 2·5 to 10·0 μg/ml. typically used in studies with this and other *thy*$^-$ strains of *E. coli* K12. In Figure 2, we have redrawn part of our data (Pritchard & Zaritsky, 1970) giving C as a function of the reciprocal of the concentration of thymine in the growth medium for strain P178 in order to compare the slope obtained with that for P162. The very striking difference in slope is probably due to differences in the way in which the intracellular deoxynucleotide concentrations respond to changes in the external thymine concentration (Beacham *et al.*, 1971). It will be noticed that the degree of scatter in the estimates of C from measurement of $\overline{G}/\overline{M}$ and ΔG in P178 is much greater than that in P162 (Fig. 1). This may be due to the difficulty of maintaining a constant concentration of thymine in the growth medium in P178, since the concentration range over which there are substantial changes of C is such that a significant proportion of the added thymine will be metabolized during the experiment.

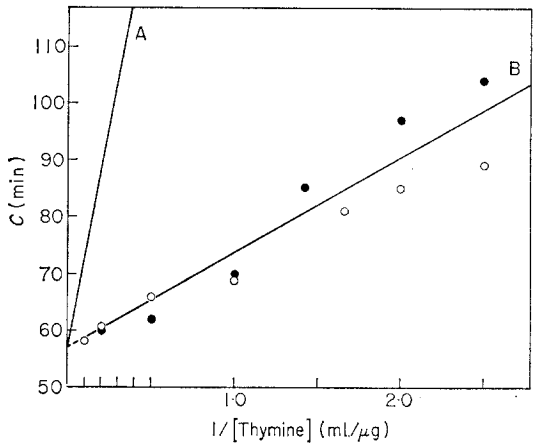

FIG. 2. C as a function of the reciprocal of thymine concentration in P162 compared with that in P178. Part of the line drawn in Fig. 1 is replotted (curve A) omitting the experimental points. The experimental points for curve (B) are taken from data previously published (Pritchard & Zaritsky, 1970). (●) Estimates of C from ΔG; (○) estimates from $\bar{G}/\bar{M}$.

A number of puzzling observations in the literature are resolved when the relatively long and variable replication time of thymineless strains is taken into account. Thus, as originally pointed out by Maaløe & Hanawalt (1961), although the increment in DNA predicted to occur in a culture deprived of a required amino acid is 39% if no further initiation occurs and all replication forks progress to the chromosome terminus, this increment is not usually found in practice in cultures of *thy*$^-$ mutants growing in synthetic media with glucose as carbon source with doubling times of 45 minutes or less (e.g. Billen & Hewitt, 1966). An increment of 39% is only predicted, however, for cultures in which $C = \tau$ (i.e. when the average number of forks per chromosome is one), and the frequently made assumption that this is so in such cultures is shown to be unjustified by our data. In strains P178 and P162-8 we did obtain an increment of 39% but only when the culture was grown on a mixture of thymine and deoxyguanosine (Table 3). It is also noteworthy that in related strains an increment of about 40% was also found under conditions in which $\tau = 60$ minutes (Donachie, 1969; Boyle, Goss & Cook, 1967).

Another puzzling phenomenon has been the degree of stimulation of DNA synthesis induced by a period of thymine starvation in cultures of strain 555-7, growing on synthetic medium with glucose as carbon source. This stimulation is due to initiation of new rounds of replication during the starvation period (Pritchard & Lark, 1964; Wolf *et al.*, 1968; Caro & Berg, 1969) and is understandable in terms of the hypothesis that initiation occurs at a defined mass : chromosome-origin ratio and is independent of replication itself, since during the period of thymine starvation the culture mass continues to increase. After a period of starvation sufficient to give one doubling in mass, the predicted increase in rate of DNA synthesis in a culture in which C was equal to τ (as has hitherto been tacitly or explicitly assumed for 555-7 growing on glucose synthetic medium) is threefold if reinitiation occurs at all chromosome origins, but the observed increase in rate is invariably closer to twofold (Pritchard & Lark, 1964; Donachie, Hobbs & Masters, 1968; Pritchard *et al.*, 1969). The observed increase in rate

is in good agreement with that predicted, however, for cultures in which $C = 60$ minutes. After a culture grown in the presence of thymine and deoxyguanosine (to achieve a situation in which $C = \tau$) has been starved of thymine for a period sufficient to give one doubling in mass, the rate of DNA synthesis is increased threefold as expected (Zaritsky, 1971). Similarly, when a related strain was grown under conditions in which $\tau = 60$ minutes, a threefold rate-stimulation was also found (Donachie, 1969). We conclude that the initial observation of Pritchard & Lark (1964) which led them to suggest that reinitiation of replication after a period of thymine starvation might take place on only one of the two arms of a replicating chromosome, is consistent with reinitiation on both arms.

Finally, we would like to note one interesting implication of the variable replication velocity of thymineless strains. A culture of P178 growing in medium containing both deoxyguanosine and thymine has a chromosome replication time of about 48 minutes and a doubling time of about 45 minutes. There will consequently be on average about one replication fork per chromosome and two copies of genes near the chromosome origin for every one near the chromosome terminus. When a culture of the same strain is grown in the presence of 0·25 μg thymine/ml. without deoxyguanosine the generation time is around 40 minutes but the replication time is increased to about 120 minutes (Pritchard & Zaritsky, 1970). In these circumstances, the average number of forks per chromosome will be about seven and the relative dosage for genes near the origin will rise to eight. The fact that such a large change in relative gene dosage can be accommodated without reduction in the growth rate suggests that the cell is well buffered against changes in gene dosage, presumably as a result of its capacity to regulate the output of gene products. Below a quite sharply defined thymine concentration, there is a reduction in growth rate. It may be that at this point the buffering capacity against changes in relative gene dosage is exhausted. Below this critical concentration the relative number of colony forming units to particles also falls. In both strains this condition occurs when the estimated value of C reaches about 130 minutes. The cause of loss of viability is not clear: possibly there is a maximum number of replication forks per chromosome, or a minimum DNA/mass ratio (this decreases as C increases), or a maximum DNA/cell ratio (this increases as C increases) compatible with normal growth and cell division. Alternatively, there may be an impairment in replication or damage to DNA at the replication fork.

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