Replication of the Bacterial Chromosome: Location of New Initiation Sites After Irradiation

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New loci of replication along the bacterial chromosome are observed after irradiation of Escherichia coli. It was conjectured that, after X-irradiation, the new initiation site was random with respect to the fixed-origin, whereas, after ultraviolet light exposure, it was selective and appeared to be from the fixed-origin. Evidence presented here shows that, after X-irradiation of E. coli, the new initiation site(s) for the onset of deoxyribonucleic acid replication is induced at chromosomal regions not restricted to the fixed-origin. After ultraviolet light exposure, the new initiation site is preferentially from the fixed-origin. In these studies amino acid starvation was used to synchronize chromosome replication and to allow for differential radioisotopic labeling of the chromosomal origin and terminus. To facilitate interpretation, growing cells actively replicating their chromosome were compared with cells lacking growth points at the time of irradiation. The role of these new replication sites in the observed kinetics of deoxyribonucleic acid replication following X-ray or ultraviolet light exposure is discussed.

An alteration in the expected sequence of chromosome replication in Escherichia coli occurs after exposure to ultraviolet light (UV; reference 13) or X-rays (7). The reorientation of the replication sequence is not an artifact of the methodology (14) or a consequence of repair synthesis (8), but results from a semiconservative synthesis starting at a region differing from that occupied by the growth point at the time of irradiation.

The present studies were designed to determine whether the new initiation sites were at the fixed-origin of cycles of replication or elsewhere on the chromosome. Advantage was taken of the amino acid deprivation method which allows synchronization of replication starting from the vegetative origin (fixed-origin) of the bacterial chromosome (1, 9, 21, 23). The sequence of replication of radioisotopically marked origin and terminus was then followed by density-transfer.

The resulting data show that, in the immediate round of replication following X-irradiation, the new initiation sites are non-origin in location, whereas, after UV they are predominantly from the origin.

MATERIALS AND METHODS

Bacterial growth. The multiauxotrophic E. coli 15T^- (555-7) requiring thymine, arginine, methionine, and tryptophan was used. The cells were grown with forced aeration at 37 C in a minimal salts medium (3) supplemented with 20 μg of arginine, 30 μg of methionine, 14 μg of tryptophan, and 20 μg of thymine per ml.

Harvesting of cells. Cells were harvested and washed on membrane filters (Millipore Filter Corp., Bedford, Mass.) at ambient temperature to reduce trauma. Care was exercised to avoid drying the cells on the filter. The washing consisted of adding an equal volume of minimal salts solution followed by filtration. The sequence was repeated three times. The total time required for washing was less than 5 min. There was no observable deleterious effect on cell growth or macromolecular synthesis resulting from this step.

Radioisotopes. The radioisotopes were procured from Schwarz Bio Research Inc., Orangeburg, N.Y. For 3H-thymidine labeling, the cells were exposed to 1 or 2 μc of 3H-thymidine (11.3 c/micromole) plus 2 μg of thymine per ml. For 14C-thymidine labeling, 0.1 or 0.25 μc of 14C-thymidine (51 mc/micromole) and 2 μg of thymine per ml were used.

Cell lysis preparation and density gradient equilibrium sedimentation were as previously described (13).

Irradiation of bacteria. All irradiations were carried out at room temperature in minimal salts. The details for X-ray and UV exposure were as described earlier (7).

Determination of per cent of radioisotopically labeled deoxyribonucleic acid (DNA) replicated. Details of the procedure for determining per cent of radioisotopically labeled DNA, and the rationale of the method, were
as described previously (13). Briefly, replication of labeled DNA was determined by incubation of the cells in a 5-bromouracil medium. Portions (5 ml) were removed at intervals, harvested, washed, and subjected to the cell lysis procedure. The cell lysate was then subjected to density-gradient centrifugation in CsCl. The percentage of radioactivity in DNA of hybrid density was obtained from the radioactive profile of the resulting gradient (that radioactivity found between 1.730 and 1.775 g/ml) and was taken to represent that portion of the labeled DNA which had been replicated.

RESULTS

Origin and terminus identification by radioisotopic labeling. Selective labeling of the origin and terminal regions (hereafter referred to as "origin" and "terminus," respectively) of the bacterial chromosome was accomplished by employing a procedure described by Bird, Ranger, and Lark (Cold Spring Harbor Symp. Quant. Biol., in press). An earlier, similar, but less fruitful approach was described by Hewitt and Billen (13). The method is based on the alignment of chromosom es by starvation for essential amino acids (21, 22).

E. coli in exponential growth (10^6 to 4 × 10^8 colony-forming cells per ml) were incubated at 37 °C for 90 min in medium lacking tryptophan and arginine, to allow completion of previously initiated cycles of replication (alignment). The required amino acid methionine was present during alignment to allow normal methylation of the DNA formed during this period (5, 18). Arginine and tryptophan were then added and ³H-thymidine was added 2 min later. Incubation was continued for 15 to 20 min to label the origin. The cells were harvested, washed, and subjected to a second period of amino acid starvation. All initiated cycles of chromosome replication should continue toward the terminus. After 40 to 45 min, ¹⁴C-thymidine was added and the culture was incubated for an additional 15 to 20 min to accomplish terminus labeling.

Nonaligned cells. The cells were diluted two to three times in a complete medium and incubated for 95 or 120 min to effect "randomization" of the replication cycles (nonaligned cells). A portion of nonaligned cells were next shifted into a 5-bromouracil medium to determine their pattern of origin and terminus replication. Nonaligned cells should show little selectivity between origin and terminus replication upon incubation with 5-bromouracil. The results (Fig. 1, control) show that the expectation was realized since both ³H- and ¹⁴C-labeled DNA were replicated simultaneously.

Aligned cells. A second portion of cells was again aligned by amino acid starvation (aligned cells) prior to incubation in the 5-bromouracil medium. In the aligned cells, ³H-labeled DNA (origin) should appear prior to ¹⁴C-DNA (terminus) in the hybrid density band. A typical response is shown in Fig. 2. Following a brief delay, ³H-DNA and then ¹⁴C-DNA were replicated. Thus, the origin and terminus can be labeled by this procedure and realigned a second time by amino acid starvation. In four experiments testing origin and terminus replication in aligned cells, the onset of terminus replication after initiation of origin replication varied between 20 to 40 min.

The results are independent of the nature of the radioisotope since the results were similar when ¹⁴C-thymidine instead of ³H-thymidine was used for origin label.

Initiation sites after X-irradiation: nonaligned cells. If, after X-ray exposure, DNA synthesis is initiated from the chromosomal fixed-origin, then ³H-DNA (origin) should be selectively replicated in nonaligned cells. The original growth point may or may not be active.

When growing cells containing chromosomes labeled at origin and terminus are studied after exposure to 5,500 r of X-rays (30% survival), the results were as shown in Fig. 1 (irradiated). It is apparent that both the origin and terminus have an equal probability of being replicated during the initial postradiation cycle of DNA.

![Graph](http://jb.asm.org/Downloaded from http://jb.asm.org/ On October 28, 2017 by guest)
replication was in decrease whereas that X-irradiation, replication was indicate replication premature (Fig. 4).

parison of the almost equal frequency Both origin and terminus are radioresistant first.

if DNA 8,000 r, and (10,000 r, 20% survivors) were incubated with 1H-thymidine for 5 min, shifted to a medium containing thymine synthesis and that there is no selective initiation from the origin.

Initiation sites after X-irradiation: aligned cells. A growing population of E. coli contains chromosomes most of which possess at least one active growth point (12, 20). This could complicate identification of the location of the new "site" induced by irradiation. Accordingly, a population of origin and terminus-marked cells were aligned just prior to X-irradiation. Such cells lacked an active "growth point," since DNA synthesis had ceased after 90 min of amino acid starvation (13, 21).

As expected in the nonirradiated aligned population, the origin was replicated first (Fig. 3a). If DNA synthesis was also initiated at the origin after irradiation, then the origin label (1H-DNA) would appear in the hybrid density DNA first. However, the aligned cells, given 8,000 r of X-rays [53% survivors; aligned cells are radioresistant (4)], showed an altered pattern. Both origin and terminus were replicated with almost equal frequency (Fig. 3b, X-ray). A comparison of the kinetics of origin and terminus replication indicate that the timing of origin replication was initially close to normal after X-irradiation, whereas that of the terminus was premature (Fig. 4). At a higher X-ray dose (10,000 r, and 20% survivors), a noticeable decrease in the initial rate of origin and terminus replication was observed.

Pattern of labeling after X-irradiation: “chase experiment.” Cells in exponential growth were exposed to 5,500 r of X-rays (32% survivors). Cells were incubated with 1H-thymidine for 5 min, shifted to a medium containing thymine

Fig. 2. Origin and terminus replication pattern in aligned cells. A portion of nonaligned cells (from the same culture as in Fig. 1) containing origin and terminus label were aligned by starvation for amino acids. Pattern for replication of radioactive label in such cells is shown. Symbols as for Fig. 1; △, 1H or origin label; ○, 14C or terminus label.

Fig. 3. Origin and terminus replication pattern of aligned cells after X-ray or UV exposure. The experiments and control were from the same culture. The protocol for origin and terminus was as described in the Results. (a) Control; (b) exposed to 8,000 r of X-rays; (c) exposed to 600 ergs/mm² of UV at a rate of 7.4 ergs per mm² per sec. Per cent of total radioactivity in DNA appearing at the hybrid density is shown. Symbols are as for Fig. 1; △, 1H or origin label; ○, 14C or terminus label.
(chase) for various times, then exposed to $^{14}$C-thymidine for 5 min. The cells were then aligned by amino acid starvation for 90 min. Replication of the DNA was followed in a fully supplemented 5-bromouracil medium. If the new replication site is from the fixed-origin after irradiation, then $^3$H-label should be restricted to the origin. Subsequent alignment should lead to early selective replication of the $^3$H-DNA in the 5-bromouracil medium. The results (Fig. 5) clearly showed that, regardless of the chase time between labels, there was no selectivity for the $^3$H-DNA over that of the $^{14}$C-DNA in the irradiated, aligned population. After X-irradiation, the fixed-origin identified by amino acid starvation differs from the replication sites active after X-ray exposure. Thus, there is no selective initiation from the fixed-origin after X-irradiation, a conclusion in agreement with that obtained in the previous experiments.

Location of onset of replication following UV-exposure. Exposure of bacteria to UV temporarily inhibits DNA synthesis (16) and is in contrast to ionizing radiation, which does not have as gross an effect on the DNA replication rate (2). When semiconservative DNA synthesis resumes after UV exposure, a new initiation site is evident (13). Hewitt and Billen concluded "that chromosome regions at or nearest the origin have the highest probability of being replicated following the restoration of DNA synthetic capacity." In these earlier experiments, active growth points were present at the time of irradiation, and, while their activity is observed to be inhibited, some residual activity is evident (25; D. Billen, Proc. Intern. Atomic Energy Agency, in press). To provide a more definitive answer to the location of the new site after UV exposure, labeled cells were aligned just prior to irradiation, allowing completion of the replication cycle as before. The replication pattern of aligned cells after exposure to 600 ergs/mm² of UV (24% survival) is shown in Fig. 3c. While there is a longer delay before DNA synthesis resumes in the irradiated cells as compared to the control (Fig. 4), a portion of the chromosomes that undergo replication initiate synthesis preferentially from the fixed-origin.
DISCUSSION

The evidence presented in this report permits the conclusion that, after X-ray exposure of bacterial cells, new initiation sites for the onset of DNA replication are induced at chromosomal regions not restricted to the fixed-origin. After UV irradiation, the new initiation site is preferentially the fixed-origin. The implications of these observations justify further comment.

Replication pattern after X-irradiation. The new site(s) may be located at one or more of a number of specific regions scattered along the chromosome (alternate-site model, Fig. 6b). This new site is not restricted to the fixed-origin (Fig. 6a), since the replication sequence expected from a fixed-origin site was not observed. Whether these new sites are associated with cellular components as observed for the active replicative forms of phage \( \Phi X174 \) during infection (17), or reflect an unregulated and therefore abnormal initiation of replication is currently unknown. An additional model for nonsequential replication of origin and terminus is illustrated in Fig. 6c. Replication could commence from the origin but with divergent growth points such that both origin and terminus are being replicated at the same time. Huberman and Riggs (15) observed what they interpreted to be adjacent diverging growth points in the DNA of mammalian cells. However, the "chase experiment" (Fig. 5) argues against this model. If divergent growth points are active after irradiation, then the \(^4\text{H}-\)thymidine label immediately after exposure should have labeled both origin and terminus, while the \(^1\text{C}-\)thymidine label would be elsewhere on the chromosome, depending on the length of chase between labels. Following alignment of these cells, \(^4\text{H}-\)label should be replicated first and then \(^1\text{C} \)in sequence. However, both \(^4\text{H} \)- and \(^1\text{C} \)-DNA replication occur at the same time and not in the sequence predicted by this model. Thus, of the several models presented, the alternate-site model best fits the observations.

The biological significance of the new initiation site(s) is currently unknown. In the experiment from which the data of Fig. 3 was obtained, 53% of the X-irradiated cells were able to form macroscopically visible colonies. During the starvation period just prior to irradiation, conclusion of a cycle of replication and division of those cells containing four completed chromosomes should result in a population of cells with no greater than two completed chromosomes (12, 20, 21). Therefore, on the premise that at least one functional chromosome per bacterium is required for colony formation, as many as

![Diagram showing replication patterns](image)

Fig. 6. Schematic presentation of chromosome replication models useful in evaluating the origin and terminus replication patterns. The "fixed-origin" refers to the normal vegetative origin for chromosome replication and the "alternate-origin" refers to any site differing from the fixed-origin location.
25% of the chromosomal population should have survived. Because there is only a slight selection of the fixed-origin during replication after irradiation (Fig. 3b, X-ray), it would seem that some of the cells capable of colony formation contain chromosomes in which the alternate initiation site was active at least for the initial postirradiation replication cycle. The subsequent replicative fate of the DNA synthesized during the initial cycle following irradiation is currently being investigated.

It is apparent that amino acid starvation subsequent to X-irradiation recalls a site (probably the fixed-origin) that is other than the radiation-induced site (chase experiment, Fig. 5). Perhaps the activity at the new site is limited to the initial postirradiation cycle of replication.

Pattern of replication following UV exposure. The data confirm an earlier observation (13) which indicated a preference in the location of the new site of DNA replication when semiconservative synthesis resumed after UV irradiation. This new location is seen to be preferentially but not entirely from the fixed-origin. UV produced a result resembling the effect of thymine starvation in that a new cycle of replication is initiated from the origin before the original cycle is completed (24). However, unlike thymine starvation, the DNA synthesis rate is not accelerated when DNA replication resumes after UV exposure (10, 16) but returns to, or is less than, the original rate. If, after UV exposure of exponentially growing cells, the fixed-origin is the starting point for a new cycle of replication, then it is unlikely that more than one new growing point is activated or that the original growth point present at the time of irradiation resumes its normal activity.

To account for the pattern of DNA replication observed after UV exposure, the following is offered. Photoproduts alter the DNA structure. The replication point active at the time of irradiation may be completely blocked, as in E. coli B- (26), or markedly delayed by dimers or other photoproducts, as in cells of intermediate sensitivity (25), or, as in resistant cells, capable of excision and repair synthesis (Rupp, Intern. Congr. Photobiol., 5th, p. 143, 1968). Howard-Flanders et al. (Cold Spring Harbor Symp. Quant. Biol., in press) estimate that a segment of DNA containing 15 to 20 dimers requires about 10 min for replication. Thus, with increasing dose, less and less of the total DNA would be replicated by the original replication point, as the number of unrepaired photo lesions accumulate either because the excision and repair system is lacking or because of saturation of the repair system (Billen, Proc. Intern. Atomic Energy Agency, in press).

In resistant cells [such as E. coli 15T- (555-7) or E. coli B/r of hcr phenotype] receiving 200 ergs, the chromosome would contain approximately 700 to 1,000 dimers. DNA replication is initially depressed but, after a dose-dependent lag, eventually recovers. During the lag, both protein and ribonucleic acid synthesis continue at or close to the normal rate as during a thymineless incubation period. When gross DNA synthesis resumes at about 20 to 30 min, it preferentially starts from the fixed-origin on one of the two daughter segments as is observed after thymineless growth. At this time many, but perhaps not all, of the photosolles have been repaired in the chromosomes.

Since initiation of DNA replication is primarily from the normal fixed-origin after UV irradiation, there need not be a pathological consequence other than that which may result from a premature initiation of a replication cycle causing an abnormal gene dosage distribution within the cells. The ultimate fate of the original growth point, when present at the time of irradiation, is not known. Too little is known about this component to warrant speculation.

The original replication point apparently is never restored to full activity since the DNA replication rate after the lag never exceeds that of the prereplication incubation rate. In considering the final rate of DNA replication, the limiting factor may be a combination of the availability of initiator protein (19) present at the time of irradiation and unrepaired lesions or gaps.

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LITERATURE CITED


