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Initiation and Termination of Chromosome Replication in *Escherichia coli* Subjected to Amino Acid Starvation

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Initiation and termination of chromosome replication in an *Escherichia coli* auxotroph subjected to amino acid starvation were examined by following the incorporation of [^{3}H]thymidine into the *Eco*RI restriction fragments of the chromosome. The pattern of incorporation observed upon restoration of the amino acid showed that starvation blocks the process of initiation prior to deoxyribonucleic acid synthesis within any significant portion of the *Eco*RI fragment which contains the origin of replication, *oriC*. In this experiment, no incorporation of [^{3}H]thymidine into *Eco*RI fragments from the terminus of replication was observed, nor was it found when a *dnaC* initiation mutant was used to prevent incorporation at the origin which might have obscured labeling of terminus fragments. Thus amino acid starvation does not appear to block replication forks shortly before termination of replication. Attempted synchronization of replication initiation by including a period of thymine starvation subsequent to the amino acid starvation led to simultaneous incorporation of [^{3}H]-thymidine into all *Eco*RI fragments within the 240-kilobase region that surrounds *oriC*. It is shown that the thymine starvation step allowed initiation and a variable, but limited, amount of replication to occur.

In *Escherichia coli*, initiation and termination of rounds of chromosome replication are linked to cell growth and division. Initiation occurs at a fixed site (*oriC*) near 83 min on the genetic map (9, 13, 15, 23, 24) when a given amount of total protein per copy of *oriC* DNA is attained by the cell (6). From the origin, replication forks proceed in both directions around the large circular chromosome, meeting within the terminus region diametrically opposite the origin (2, 9, 20, 21). Termination is a prerequisite for cell division and appears to provide a signal that determines its timing (5, 7, 18).

Starvation of an auxotroph for a required amino acid stops the net accumulation of protein and thus prevents initiation of new rounds of chromosome replication (11, 14) although preexisting replication forks can continue to move around the chromosome to the terminus. The sensitive step in the initiation process is likely to be an early one, but, conceivably, initiation may be blocked even after a segment of DNA has been synthesized at the origin and before the normal DNA elongation process commences.

Amino acid starvation has also been reported to prevent termination of replication by causing the preexisting replication forks to halt with about 0.5% of the chromosome in the terminus region remaining unreplicated (17). However, similar experiments by Loehr and Hanawalt (12) do not support the existence of a unique terminal segment which remains unreplicated in amino acid-starved cells. In both cases, the evidence is indirect, as it is based upon [^{3}H]thymidine incorporation into total cellular DNA. To clarify the effects of amino acid starvation on DNA replication, we reexamined both initiation and termination by following the incorporation of [^{3}H]thymidine into *Eco*RI restriction fragments of the *E. coli* chromosomes. Amino acid starvation is directly shown to block initiation prior to DNA synthesis within any significant portion of the *oriC*-containing *Eco*RI fragment. No evidence for an unreplicated terminal segment was found.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain DG75 (F$^-$ *leu-6 thyA47 dra-3* and *rpsL153* [*str*]) and its temperature-sensitive derivative PC2 (*dnaC2*) (4) were provided by P. Carl.

Medium. Bacteria were grown in a Tris-hydrochloride-buffered (pH 7.5) minimal medium containing, per liter: NaCl, 0.54 g; KCl, 0.3 g; NH_4Cl, 1.1 g; $CaCl_2 \cdot 2H_2O$, 15 mg; $MgCl_2 \cdot 6H_2O$, 203 mg; $FeCl_3 \cdot 6H_2O$, 0.2 mg; KH_2PO_4, 87 mg; Na_2SO_4, 22.7 mg; and Tris, 12.1 g. The complete medium additionally contained 0.2% glucose, 80 μg of leucine, and 40 μg of thymine per ml.

Media changes were accomplished by rapid filtration of cultures through HAWP filters (Millipore Corp.). After thorough rinsing with fresh, prewarmed medium, cells were resuspended by immersing and swirling the filter in the new medium.

Radioactive labeling and isolation of DNA. Ex-

ponential cultures at 1×10^8 to 2×10^8 cells per ml were subjected to starvation for leucine and thymidine or to temperature shifts as described. DNA was labeled by adding 40 μl of [^{3}H]thymidine (0.5 mCi/ml and 60 Ci/mmol, Schwarz/Mann) per ml of culture. Labeling was terminated by pouring a 10-ml sample over ice in a centrifuge tube that contained enough nonradioactive thymidine and KCN to make their final concentration 100 μg/ml and 10 mM, respectively.

Total cellular DNA was purified as follows. Pelleted cells were resuspended in 0.4 ml of 20% sucrose–100 mM NaCl–10 mM Tris-hydrochloride (pH 8.2), treated for 20 min on ice with 0.1 ml of a 4-mg/ml solution of lysozyme in 120 mM Tris-hydrochloride (pH 8.2)–50 mM EDTA, and lysed with Sarkosyl (0.4 ml of a 2.5% solution). The lysate was heated to 65°C for 5 min, cooled, diluted with water to 7.7 ml, and digested for 4 h at 37°C with 0.1 ml of a 1-mg/ml solution of pronase (Calbiochem). Solid KI was added to bring the volume of the lysate to 10 ml, and a density gradient was formed by centrifugation in a Beckman 50 Ti rotor at 37,000 rpm for 40 h at 20°C. The DNA, which banded in the center of the gradient, was collected and dialyzed against 20 mM Tris-hydrochloride (pH 7.8)–0.2 mM EDTA.

Restriction endonuclease digestion. Restriction endonuclease *Eco*RI was purchased from Miles Laboratories, Inc. The reaction mixture contained 20 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$–10 mM NaCl–7 mM 2-mercaptoethanol–0.2 mM EDTA–0.1% gelatin. Sufficient enzyme was added to give complete digestion within a 4-h incubation period at 37°C.

Gel electrophoresis and fluorography. *Eco*RI digests were fractionated by electrophoresis on 18-cm-long slab gels of 0.6% agarose (Seakem) in an apparatus constructed as described by Sugden et al. (22). The gel itself contained 20% glycerol–80 mM Tris-maleate (pH 7.8). Running buffer was 40 mM Tris-maleate (pH 7.8). Up to 300 μl of the digest, adjusted to contain 10% sucrose, was placed in each well, and 30 V was applied for 16 h at room temperature.

The [^{3}H]labeled DNA was detected by fluorography, using a modification of the method described by Bonner and Laskey (3) for polyacrylamide gels. The agarose gels were first dehydrated by soaking in ethanol and were then immersed in acetone containing 10% (wt/vol) 2,5-diphenyloxazole (PPO). After equilibration, the gels were placed between a sheet of Whatman 3 MM filter paper and a sheet of thin Mylar plastic and were dried under reduced pressure under a heat lamp. These gels were exposed to Kodak RP Royal X-Omat film at −70°C for 2 days to 2 weeks, depending upon the ^{3}H input.

*Eco*RI fragments of phage λ were run as molecular-weight standards, assuming a size of 49 kilobases (kb) for the λ chromosome. This is 2.5 kb more than the value we used in an earlier report (16) and is mainly responsible for the revised molecular weights reported here for *Eco*RI fragments from the *E. coli oriC* region.

RESULTS

Initiation of chromosome replication in bacteria released from amino acid starvation.

When a required amino acid is restored to an *E. coli* auxotroph which has been starved long enough for preexisting replication forks to reach the chromosomal replication terminus, new rounds of replication are initiated at a time which varies from cell to cell, depending on the age of the cell at the onset of starvation. Older cells that were closer to initiation when the starvation was begun will initiate first, followed sequentially by the younger cells as they attain the requisite protein mass to *oriC* ratio. Reinitiation of replication in amino acid-starved cells will not display the synchrony found, for example, in temperature-sensitive *dnaA* or *dnaC* initiation mutants held for a time at the nonpermissive temperature and then shifted to the permissive temperature (16).

To observe the initial pattern of replication in cells released from amino acid starvation, we pulse labeled the leucine auxotroph DG75 with [^{3}H]thymidine for consecutive 5-min intervals upon restoration of leucine after a 2-h starvation. The DNA was digested with restriction endonuclease *Eco*RI and electrophoresed in an agarose gel to reveal the pattern of [^{3}H]thymidine incorporation shown in Fig. 1. For comparison, also shown are the *Eco*RI fragments labeled during the first 1.5 and 6 min of replication in the *dnaC* mutant PC2 synchronized by being held at 40°C for 1 h and then shifted to 28°C to initiate replication. PC2 is a derivative of DG75 (4).

We have previously shown that within the first 1.5 min after a shift of PC2 to the permissive temperature, the newly created replication forks move outward from each side of *oriC*, traversing the five *Eco*RI fragments labeled by size in Fig. 1 (16). The physical map of these fragments is shown in Fig. 2. Together, these fragments comprise about 40 kb of DNA. By 6 min postshift, [^{3}H]thymidine is incorporated into about 27 *Eco*RI fragments totaling 240 kb. In this total are included four pairs of overlapping fragments, as well as several others not resolved from neighboring fragments in the agarose gel shown in Fig. 1 but clearly visible with shorter exposure of the gel to X-ray film. Not included are fragments smaller than 2 kb, which could not be detected at the level of [^{3}H]thymidine incorporated in this experiment.

With DG75 released from leucine starvation, the first clearly discernible ^{3}H labeling of specific *Eco*RI fragments occurred within the second 5-min pulse, producing a banding pattern which was fainter but otherwise similar to the one observed within the first 6 min of replication in PC2. During the later [^{3}H]thymidine pulses of DG75, the gel banding pattern became increas-

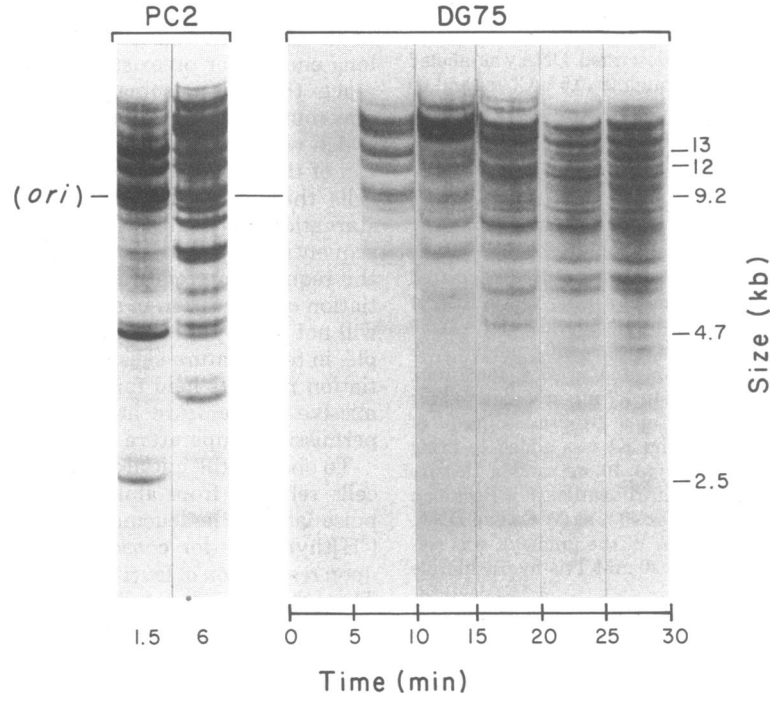

FIG. 1. *Fluorograph of an agarose gel in which ^{3}H-labeled EcoRI restriction fragments from E. coli PC2 and DG75 chromosomes were separated by electrophoresis. PC2 was grown at 28°C, shifted to 40°C for 1 h, and pulse labeled with [^{3}H]thymidine for the first 1.5 or 6 min upon return to 28°C. DG75 was grown at 37°C and pulse labeled for consecutive 5-min intervals after restoration of leucine to a culture that had undergone a 2-h leucine starvation. A sample of 6,000 cpm and a 2-week exposure were used with the first two pulses of DG75; otherwise, 40,000 cpm and a 3-day exposure were used.*

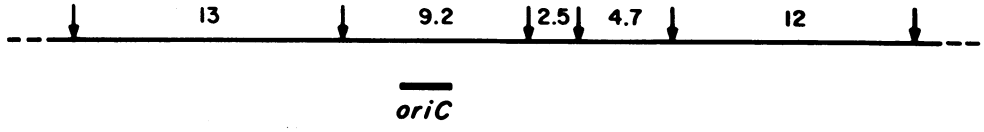

FIG. 2. *EcoRI restriction map of the oriC region of the E. coli K-12 chromosome. Redrawn from reference 15.*

ingly complex, as initiation was occurring in some cells while replication was already well advanced in others.

The high degree of correspondence between the initial pattern of labeling in DG75 released from amino acid starvation and in PC2 after temperature downshift is more clearly evident in densitometer tracings of fluorographs developed after a 1-day exposure of the agarose gel depicted in Fig. 1 for the PC2 DNA (Fig. 3a) and a 2-week exposure for the DG75 DNA (Fig. 3b). All bands labeled within the second 5-min pulse of DG75 were also labeled during the 6-min pulse of PC2 and in much the same relative proportion. Included was the 9.2-kb *Eco*RI fragment known to contain the origin of replication.

To determine whether partial replication of the origin-containing fragment had occurred during the amino acid starvation, the amount of [^{3}H]thymidine incorporated into the 9.2-kb fragment in PC2 and DG75 was compared with the amount incorporated into the neighboring 12- and 13-kb *Eco*RI fragments. This was accomplished by measuring the relative area under the corresponding peaks in Fig. 3. Although the response of the X-ray film to the amount of radioactivity in the agarose gel is not strictly linear, such a comparison is valid when similar amounts of radioactivity are present in the compared bands, as is the case here. The results (Table 1) demonstrate that, per unit length, [^{3}H]thymidine was incorporated to about the same extent into all three fragments during the first 6 min of replication in PC2. This is as expected since replication of these fragments is known to be complete by 6 min after shift of PC2 to the permissive temperature (16). In DG75 released from amino acid starvation, the

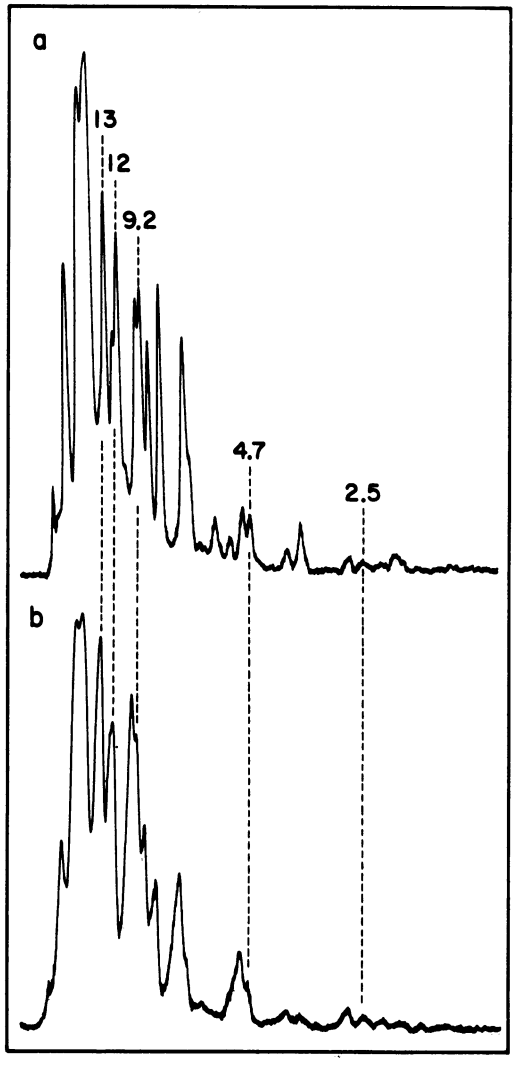

FIG. 3. *Densitometer tracings of fluorographs made from the agarose gel depicted in Fig. 1. (a) The lane containing EcoRI fragments from PC2 labeled with [^{3}H]thymidine for 6 min following the shift from 40 to 28°C was traced from a fluorograph developed after a 1-day exposure to the gel. (b) The lane containing the fragments from DG75 labeled during the second 5-min pulse with [^{3}H]thymidine following release from amino acid starvation was traced from a fluorograph developed after a 2-week exposure. Peaks which correspond to EcoRI fragments within the immediate origin region mapped in Fig. 2 are labeled according to size in kilobases.*

relative incorporation of [^{3}H]thymidine during the second 5-min pulse was slightly higher for the 9.2-kb origin-containing fragment than for either the 12- or 13-kb fragment. Given bidirectional replication from *oriC* and a polymerization rate of about 50 kb per min per replication fork, only those forks that were initiated during the last 0.5 min of the 5-min pulse would not have completely traversed the region containing these three fragments. This would account for the slightly higher ^{3}H incorporation into the central origin-containing fragment. If a significant portion of the origin-containing fragment had been replicated during the amino acid starvation, the relative amount of ^{3}H incorporated into the 9.2-kb fragment would have been reduced relative to the 12- and 13-kb fragments. Thus, these results demonstrate that amino acid starvation blocks initiation prior to the synthesis of any significant portion of the origin-containing *Eco*RI fragment.

Because all bands labeled in DG75 within 10 min after restoration of leucine can be correlated with fragments from the origin region of PC2, there is no evidence for replication of a segment of the chromosome at the terminus of replication immediately upon release from amino acid starvation. These results, therefore, do not support the hypothesis by Marunouchi and Messer (17) that amino acid starvation causes replication forks to halt with about 0.5% of the chromosome remaining unreplicated at the terminus of replication and that restoration of the amino acid leads to the immediate replication of the terminus region.

Attempt to specifically label the terminus of replication. To exclude the possibility that *Eco*RI fragments from the replication terminus may have been obscured in the agarose gel shown in Fig. 1 by similar-sized fragments from the origin region, we attempted to specifically label the terminus fragments by the procedure used by Marunouchi and Messer (17). The temperature-sensitive initiation mutant PC2 was starved for leucine for 2 h at the permissive temperature and then was shifted to 40°C to

TABLE 1. *Comparison of [^{3}H]thymidine incorporated into EcoRI fragments of the oriC region during initiation of replication*

*Eco*RI fragment size (kb)	Relative specific radioactivity[a] of *Eco*RI fragments from:	
	PC2[b]	DG75[c]
9.2	1.08	1.26
12	1.00	1.00
13	1.05	1.16

[a] Determined as area under the peak for each fragment in Fig. 3 divided by the size of the fragment and then normalized to the value obtained for the 12-kb fragment.

[b] From Fig. 3a. PC2 was labeled with [^{3}H]thymidine for 6 min following a shift to 28°C after 1 hr at 40°C.

[c] From Fig. 3b. DG75 was pulse labeled with [^{3}H]-thymidine for 5 min, starting 5 min after release from a 2-h leucine starvation.

block replication initiation. After restoration of leucine, the cells were immediately pulsed with [^{3}H]thymidine for 10 min. Very little label was incorporated into DNA, but the amount was sufficient to produce the gel banding pattern shown in Fig. 4b. No specific *Eco*RI fragments were labeled. Rather, the [^{3}H]thymidine appears to have been incorporated into all *Eco*RI fragments of the PC2 chromosome, yielding a pattern similar to one obtained by pulse labeling nonsynchronous, exponentially growing cells (Fig. 4c). An analogous banding pattern was also observed if a culture of unstarved PC2 was simply shifted to 40°C for 1 h to allow preexisting replication forks to reach the terminus region and then given a 10-min pulse with [^{3}H]thymidine while the culture was maintained at 40°C (Fig. 4a). We therefore conclude that this latter incorporation of [^{3}H]thymidine and the incorporation observed in PC2 which had been shifted to 40°C before release from starvation are due to a low level of repair-type synthesis occurring at random around the chromosome and not to replication specifically within the terminus region.

Initiation of replication in bacteria synchronized by consecutive amino acid and thymine starvations. To obtain synchronous initiation after amino acid starvation, investigators have frequently used thymine-requiring strains and included a period of priming for initiation after readdition of the amino acid, during which time thymine was removed from the medium. The rationale was that the resumption of protein synthesis would allow the cells to build initiation capacity but that actual DNA synthesis would be blocked by the absence of thymine. Following this protocol with *E. coli* DG75 at 37°C or PC2 at 28°C, we found that, when [^{3}H]thymidine was added together with thymine to allow resumption of replication, it was incorporated *simultaneously* into all restriction fragments within the 240-kb region which surrounds the origin and was *sequentially* replicated over a 6-min period in PC2 synchronized by temperature shift (Fig. 5b, c, and d). With pulses of [^{3}H]thymidine as short as 10 s, all fragments from this region were still labeled.

In contrast to this result, if the leucine-starved PC2 was shifted to 40°C for the thymineless priming step, [^{3}H]thymidine was incorporated sequentially from the origin when at the completion of priming the cells were returned to 28°C and pulse labeled (Fig. 5a and e). To assure that this difference in labeling was due to priming at the nonpermissive temperature and was not an artifact induced by the temperature shift, we examined the pattern of [^{3}H]thymidine incorporation in PC2 starved for leucine and

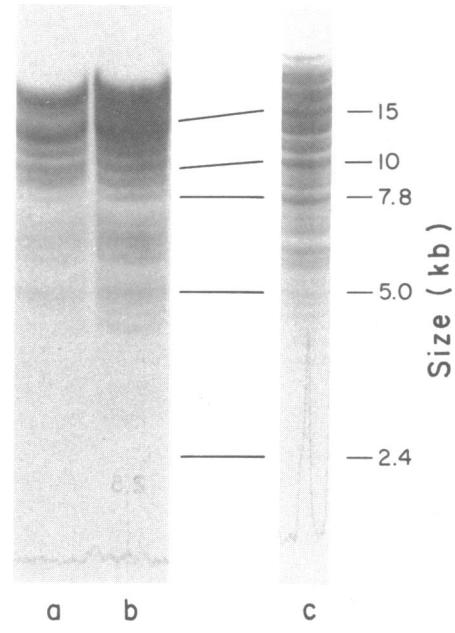

FIG. 4. *Fluorograph of two agarose gels containing EcoRI fragments from E. coli PC2 labeled with [^{3}H]thymidine for 10 min (a) after a 1-h shift up to 40°C and maintenance of the cells at 40°C for labeling and (c) during exponential growth at 28°C. (b) Cells growing at 28°C were starved for leucine for 2 h and then labeled immediately after a shift up to 40°C and restoration of leucine.*

primed at 28°C and labeled at 28°C after a 10-min incubation at 40°C. Figure 5f shows that all *Eco*RI fragments in the 240-kb region around the origin were simultaneously labeled. Thus, simultaneous labeling of the 240-kb region can be correlated with priming at 28°C, a condition in which initiation would be blocked only by removal of thymine from the medium as opposed to the additional lack of functional *dnaC* protein at 40°C. This leads us to conclude that amino acid starvation effectively blocks initiation but that the cells either maintain a sufficient residual pool of thymidine nucleotides or synthesize sufficient thymidine to allow a very limited amount of replication to occur during the priming step. Variation from cell to cell in the extent of this replication would account for the observed simultaneous labeling of all restriction fragments in the 240-kb region around the origin upon addition of the [^{3}H]thymidine.

One would predict that, when PC2 is starved for leucine and primed for replication in the absence of thymine at 28°C, sufficient replication will have occurred within the origin region during the priming step to allow replication of the chromosome to proceed despite a shift of the culture of 40°C before readdition of exogenous

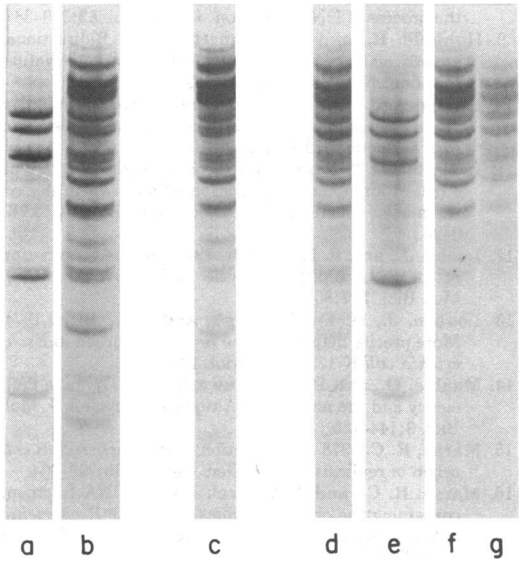

FIG. 5. *Fluorograph of an agarose gel containing EcoRI fragments from: (a and b) E. coli PC2 synchronized by temperature shift and labeled with [^{3}H]thymidine for 1.5 min (a) or 6 min (b) at the start of replication as in Fig. 1; (c) DG75 synchronized by a 2-h leucine starvation followed by 50 min of priming in thymineless medium and labeled for 1 min upon restoration of thymine to the medium; (d) PC2 grown at 28°C and labeled according to the same protocol as DG75 in (c); (e) PC2 grown and leucine starved at 28°C, shifted to 40°C for priming, and returned to 28°C for restoration of thymine and 1 min of labeling; (f) PC2 grown, starved, and primed at 28°C, shifted to 40°C for 10 min, and returned to 28°C for restoration of thymine and 1 min of labeling; and (g) PC2 grown, starved, and primed at 28°C then shifted to 40°C for restoration of thymine and 1 min of labeling.*

thymine. The gel banding pattern in Fig. 5g shows this to be true.

DISCUSSION

The principal means available for selectively blocking initiation of chromosome replication in *E. coli* are (i) inhibition of protein synthesis either by amino acid starvation or chloramphenicol treatment and (ii) temperature shift up of temperature-sensitive initiation mutants. An earlier analysis of [^{3}H]thymidine incorporation into *Eco*RI and *Hin*dIII fragments of the *E. coli* chromosome showed that temperature shift up of either the *dnaA* mutant PC5 or the *dnaC* mutant PC2 results in blockage of the initiation process before any significant amount of DNA is synthesized at the origin of replication (16). The present results show that this is also true for an amino acid-starved auxotroph. Neither set of experiments can rule out the possibility that a small segment of DNA is synthesized on one or both parental DNA strands at *oriC* before initiation is blocked since the *Eco*RI and *Hin*dIII fragments we have examined are relatively large, measuring from 1.4 to 9.2 kb within the immediate origin region. The level of [^{3}H]thymidine incorporation into these fragments compared to neighboring fragments, however, indicates that any such synthesis must occur over only a short section of any one of the fragments.

When temperature-sensitive mutants in the *dnaA* and *dnaC* genes were first isolated, one criterion for their classification as initiation mutants was the inability to initiate replication when cells that had been starved for an amino acid at the permissive temperature were shifted to the nonpermissive temperature before restoration of the amino acid (1, 4, 8, 10). This property could be taken as evidence for a step in initiation which is sensitive to amino acid starvation and precedes the action of the *dnaA* and *dnaC* gene products, or, alternatively, amino acid starvation could simply prevent the accumulation of sufficient *dnaA* or *dnaC* protein for initiation. In this case amino acid starvation and temperature shift up of the *dnaA* and *dnaC* mutants would block initiation at the same stage. A third possibility would be that, in amino acid-starved cells, initiation can proceed with the continued participation of the *dnaA* and *dnaC* proteins to the stage where some DNA has been replicated but conversion to the normal DNA elongation process is blocked. Apparent support for this third possibility could be found in the recent report by Nüsslein-Crystalla and Scheefers-Borchel (19) that, in cells synchronized by combination of amino acid starvation with priming in thymineless medium, replication had occurred within a 180-kb region around *oriC* before restoration of thymine to the medium. Our experiments confirm Nüsslein-Crystalla and Scheefers-Borchel's observation, but further show that this rather extensive replication occurs during the priming step and not while the cells are being subjected to amino acid starvation.

We believe the replication that occurs during the priming step is best explained by the presence of a residual pool of thymidine nucleotides. Nüsslein-Crystalla and Scheefers-Borchel (19) have offered, in addition, the suggestion that, "In the lack of thymine, forks are opened and move without TMP incorporation, but putative primers are synthesized along a rather long stretch of parental DNA. A thymidine pulse will rapidly label this whole stretch which will be finished to yield double stranded DNA...." More experiments are needed to clarify this point.

With regard to termination of chromosome replication, amino acid starvation appears to have no specific effect. A direct examination of the patterns of [^{3}H]thymidine incorporation into *Eco*RI fragments of the *E. coli* chromosome under conditions hypothesized by Marunouchi and Messer (17) to selectively label the terminus revealed only random incorporation of label. Had a unique terminal segment containing about 0.5% of the chromosome been labeled, the resultant 20 kb of [^{3}H]DNA should have been readily detected among *Eco*RI fragments of the chromosome. Our data thus corroborate Loehr and Hanawalt's (12) studies in which they were unable to detect a unique terminal segment.

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