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A DNA fragment containing the origin of replication of the *Escherichia coli* chromosome

(bidirectional replication/*dnaA* and *dnaC* initiation mutants/restriction mapping)

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ABSTRACT **A 38 kilobase pair region of the *Escherichia coli* K12 chromosome containing the replication origin has been physically mapped with restriction endonucleases *Eco*RI and *Hin*dIII. Replication starts within or very near a 1.3 kilobase pair *Hin*dIII fragment in the middle of this region and proceeds outward in both directions with apparently equal speed. This pattern was observed in both *dnaA* and *dnaC* temperature-sensitive (*ts*) initiation mutants at the start of the synchronous round of replication which occurs after downshift from the nonpermissive to the permissive temperature.**

The origin of replication of the *Escherichia coli* chromosome is located in the vicinity of the *ilv* operon (1–3), most likely between the *dnaA* and *bglA* genes (4, 5). This would place it at approximately 82 min on the revised genetic map of *E. coli* (6). From this site, replication proceeds in both directions around the large, circular chromosome and terminates within the region diametrically opposite the origin (1, 2, 7, 8).

The isolation of conditional mutants defective in initiation has led to the identification of several genes specifically involved in this process: *dnaA* and *dnaC* (see ref. 9 for review), *dnaI* (10), and *dnaP* (11). The *dnaH* initiation mutant described by Sakai *et al.* (12) apparently is a double mutant with the defect in initiation due to a *dnaA* lesion (13). The *dnaA* and *dnaC* loci were the first discovered and are the best characterized. Mutations affecting DNA chain elongation have also been mapped within the *dnaC* gene (14). These were earlier classified as *dnaD* mutations. The *dnaC* gene product thus appears to have two functions. When *dnaA* or *dnaC ts* (temperature-sensitive) initiation mutants are shifted to the nonpermissive temperature, a residual amount of DNA replication occurs which has been interpreted as completion of rounds of replication in progress at the time of the shift. Return to the permissive temperature results in initiation of a new round of replication (9, 15–21).

We report here the use of restriction endonucleases to analyze the region of the *E. coli* chromosome initially replicated upon downshift of a *dnaA* and a *dnaC ts* mutant to the permissive temperature. By this method, the origin of replication in this 4000 kilobase (kb) chromosome can be assigned to a site within or very near a 1.3 kb *Hin*dIII fragment.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. Temperature-sensitive initiation mutants PC2 (*dnaC2*) and PC5 (*dnaA5*) were derived from *E. coli* K12 strain DG75 (F⁻, *leu*-6, *thyA*47, *dra*-3, and *str*-153) by P. Carl (17, 22) and provided by him. Bacteria were grown in a shaking bath in Tris·HCl-buffered (pH 7.5) minimal medium containing per liter: 0.54 g of NaCl, 0.3 g of KCl, 1.1 g of NH_4Cl, 15 mg of $CaCl_2 \cdot 2H_2O$, 203 mg of $MgCl_2 \cdot 6H_2O$, 0.2 mg of $FeCl_3 \cdot 6H_2O$, 87 mg of KH_2PO_4, 22.7 mg of Na_2SO_4, and 12.1 g of Tris. The medium additionally contained 0.2% glucose, 0.4% casamino acids, and 40 μg of thymine per ml.

Radioactive Labeling of Origin Region and Isolation of DNA. To obtain synchronized initiation of replication, we grew cultures of PC2 and PC5 at 28°C to 0.25 OD_{600} and then shifted them to 40°. After 1 hr, the cultures were rapidly cooled to 28° and 10-ml aliquots taken at 1- or 2-min intervals for pulse labeling with 0.4 ml of [^{3}H]thymidine (0.5 mCi/ml and 60 Ci/mmol, Schwarz/Mann). Pulses were quenched by adding nonradioactive thymidine to 100 μg/ml and pouring the aliquot over ice in a centrifuge tube containing enough KCN to make its final concentration 10 mM.

Total cellular DNA was purified as follows: Pelleted cells were resuspended in 0.4 ml of 20% sucrose/100 mM NaCl/10 mM Tris·HCl (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·HCl (pH 8.2)/50 mM EDTA, and lysed with Sarkosyl (0.4 ml of a 2.5% solution). The lysate was heated to 65° for 5 min, cooled, diluted with water to 7.7 ml, and digested for 4 hr at 37° 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 hr at 20°. The DNA, which banded in the center of the gradient, was collected and dialyzed against 20 mM Tris·HCl (pH 7.8)/0.2 mM EDTA.

Restriction Endonuclease Digestion. Restriction endonucleases were purchased from Miles Laboratories and New England Biolabs. A standard reaction mixture was used for all digestions [20 mM Tris·HCl (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 a limit digest within a 4-hr incubation period at 37°. When the DNA was digested with more than one enzyme, all were added at the beginning of the digestion period.

Gel Electrophoresis and Fluorography. Limit digests were fractionated by electrophoresis on 18-cm-long slab gels of 0.6% agarose (Seakem) in an apparatus constructed according to Sugden *et al.* (23). 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 limit digest, adjusted to contain 10% sucrose, was placed in each well and 30 V were applied for 16 hr at room temperature.

Total DNA was visualized by immersing the gel in a solution containing 5 μg of ethidium bromide per ml for 15 min, followed by shortwave ultraviolet illumination. ^{3}H-Labeled DNA was detected by fluorography, using a modification of the method described by Bonner and Laskey (24) for polyacrylamide gels. The agarose gels were first dehydrated by soaking in ethanol, then immersed in acetone containing 10% wt/vol 2,5-diphenyloxazole (PPO). After equilibration, the gels were

Abbreviations: *ts*, temperature-sensitive; kb, kilobase.

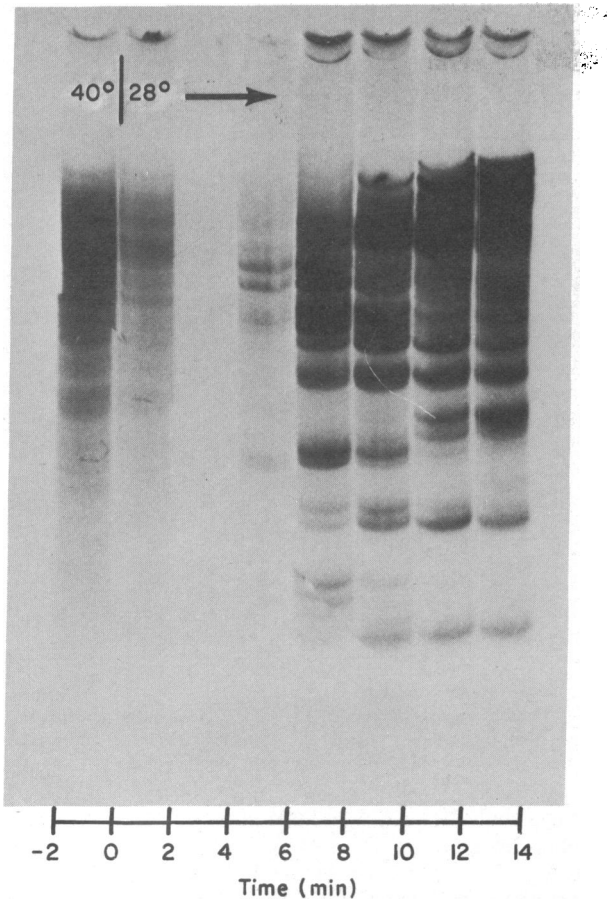

FIG. 1. Agarose gel electrophoresis of *Eco*RI restriction fragments of the *E. coli* PC5 chromosome pulse labeled with [^{3}H]thymidine during 2-min intervals immediately prior to and after downshift from 40° to 28°. Within the 0- to 6-min time period 3750, 1920, and 3550 cpm of ^{3}H were applied to the gel for each 2-min pulse, respectively. Otherwise 7500 cpm of ^{3}H were used.

placed between a sheet of Whatman 3 MM filter paper and one of thin Mylar plastic and dried under reduced pressure under a heat lamp. These gels were exposed to Kodak RP Royal X-Omat film at −70° for 2 days to 2 weeks, depending upon the ^{3}H input.

Isolation of DNA Restriction Fragments. Bands detected in preparative agarose gels by fluorography were cut out, the PPO was extracted with acetone, and the gel was dissolved in saturated KI at 37°. The agarose and DNA fragments were then separated in KI density gradients as described (25).

RESULTS

Initial Pattern of DNA Replication after a 40° to 28° Shift of *dnaA* and *dnaC ts* Mutants. At the beginning of a round of chromosomal replication induced in either *E. coli* PC5 (*dnaA*5) or PC2 (*dnaC*2) by temperature shift, we have observed that [^{3}H]thymidine is incorporated into the same few *Eco*RI restriction fragments and that the incorporation proceeds sequentially from one fragment to another as would be expected with initiation at a unique site and outward movement of the replication forks. The *dnaA* and *dnaC* mutants wre grown at 28°, shifted to 40° for 1 hr, then returned to 28° and pulse labeled with [^{3}H]thymidine for 1- or 2-min intervals. The 1-hr incubation at 40° was sufficient time for completion of rounds of replication in progress at the time of the shift up and yet not so long that the cells accumulate the capacity to initiate

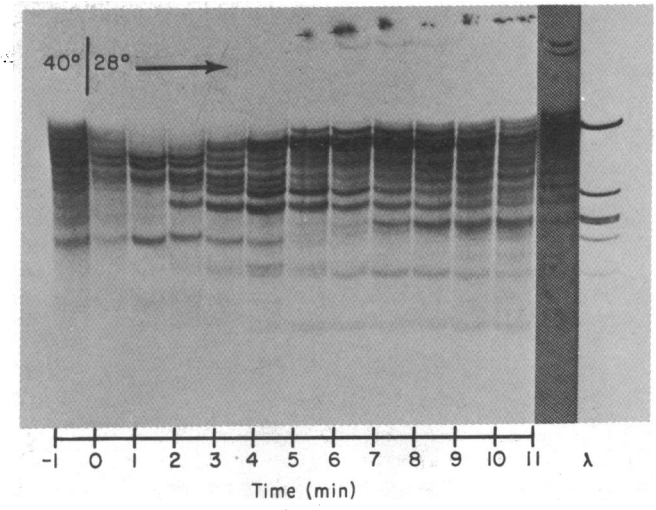

FIG. 2. Agarose gel electrophoresis of *Eco*RI restriction fragments of the *E. coli* PC2 chromosome pulse labeled with [^{3}H]thymidine during 1-min intervals immediately prior to and after downshift from 40° to 28°. Each sample contained 10,000 cpm of ^{3}H. The *Eco*RI fragments of phage λ were included as molecular weight standards.

more than one round of replication on average after the return to 28° (ref. 22, and personal observation). A 0.6% agarose gel containing the DNA fragments labeled in the *dnaA* mutant is shown in Fig. 1. While still at 40° and just prior to the downshift, a 2-min pulse with [^{3}H]thymidine produced a gel banding pattern indicative of label being incorporated over the entire chromosome, since essentially the same pattern was observed when total DNA in the gel was visualized by staining with ethidium bromide (not shown). This is most likely the result of DNA turnover associated with repair synthesis, and it continued at a reduced rate for the first minute or two after downshift to 28°. Within the following 2-min interval, insufficient [^{3}H]-thymidine was incorporated to detect clearly any bands in the gel. Thereafter label began to appear in an ordered manner in specific fragments, each increasing in activity to a maximum and then declining within a period of 6–8 min. This is consistent with initiation of replication at a unique site in all cells within a short time and synchronous progression of the replication forks around the chromosome.

With the *dnaC* mutant the same ordered pattern of replication was observed (Fig. 2) but without the 3- to 4-min delay in onset seen with *dnaA*5. There does appear to have been some selective incorporation of label into the earliest observed fragments even at 40°, just prior to the shift to 28°. Turnover of DNA within the origin region or initiation and incorporation during quenching of the sample on ice and KCN might explain this observation.

When feasible, equal amounts of radioactivity were placed in the gel slots to normalize any differences in specific radioactivity of the DNA fragments arising from nucleotide pool changes in the first few minutes of replication. During the first 6 min after downshift of the *dnaA* mutant, however, insufficient [^{3}H]thymidine was incorporated, and therefore the patterns from these intervals in Fig. 1 can only be compared qualitatively to the others.

Physical Mapping of Origin Region. The *Eco*RI fragments labeled at early times in Figs. 1 and 2 can be ordered in a linear sequence by using a second restriction enzyme (*Hin*dIII) to generate the necessary set of overlapping fragments. Overlaps between the *Eco*RI and *Hin*dIII fragments were established by comparing the products of a *Hin*dIII digestion of each isolated *Eco*RI fragment with the products of a *Eco*RI digestion

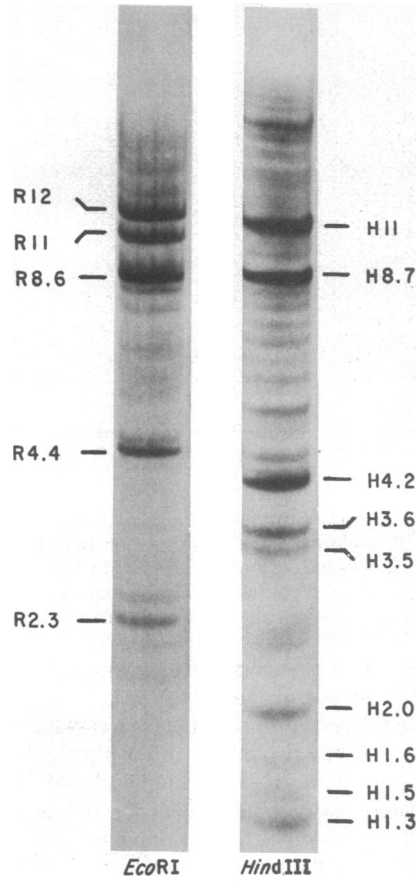

FIG. 3. A comparison of the *Eco*RI and *Hin*dIII restriction fragments labeled with [^{3}H]thymidine during the first 1.5 min after temperature downshift of *E. coli* PC2. In each case, 40,000 cpm of ^{3}H were applied to the gel. The fragments of highest specific radioactivity are designated by their size in kilobase pairs prefixed with R or H according to the restriction nuclease used.

of each isolated *Hin*dIII fragment. The *Eco*RI and *Hin*dIII fragments that overlap will produce one matching secondary cleavage product. *Eco*RI fragments that are completely contained within a *Hin*dIII fragment will not undergo secondary cleavage by *Hin*dIII and vice versa. Such fragments will, however, correspond to one of the secondary cleavage products of the fragment containing it.

Fig. 3 shows the *Eco*RI and *Hin*dIII primary digestion products from the *dnaC* mutant pulse labeled during the first 1.5 min after temperature downshift. In both cases, five prominent bands were observed in the gels. These have been designated according to their size in kilobase pairs and the restriction enzyme used: R representing *Eco*RI and H the *Hin*dIII nuclease. The *Eco*RI fragments of phage lambda DNA were used as molecular weight standards (26). Four less prominent *Hin*dIII fragments were also noted and included in the mapping experiments. These were H 1.5, H 1.6, H 3.5, and H 3.6. Together, the five *Eco*RI fragments have a combined molecular weight of 25.6×10^6, the nine *Hin*dIII fragments, one of 25.0×10^6.

In Fig. 4, the secondary cleavage products that resulted from digestion of each *Eco*RI fragment with *Hin*dIII and vice versa are shown and compared. Whenever secondary cleavage occurred, the total molecular weight of the products agreed closely with the molecular weight of the original fragment, indicating that each gel band contained only one labeled fragment. With two exceptions, the matching fragments in the two sets could be readily identified on the basis of their electrophoretic mobility. The exceptions were (*i*) the smallest fragment from R 11 for which there was no corresponding fragment and must therefore represent one end of the region being mapped and (*ii*) the presence of four fragments measuring 2.0 kb in length. To determine which of these four fragments matched one another and to further verify that the other matched pairs were identical, we digested each fragment with *Bam* H1, *Hpa* I, or *Hin*cII. The results are summarized in Table 1. Based on identical cleavage patterns, the 2 kb fragments from R 2.3 and H 4.2 could be matched, as could also the 2 kb fragment from R 8.6 with H 2.0. As expected, the other previously paired fragments showed identical cleavage patterns or lack of cleavage, as the case may be.

Table 1. Identification of matching *Eco*RI and *Hin*dIII secondary cleavage fragments by their pattern of digestion with *Bam* H1, *Hpa* I, and *Hin*cII

Fragment		Restriction products with*†		
Size*	Sources	*Bam* H1	*Hpa* I	*Hin*cII
6.4	R11/H11	—‡	2.7, 2.3	
			1.1	
5.6	R12/H8.7	3.5, 2.1		
4.4	R4.4/H11	—	3.2, 1.3	1.5, 1.0, 0.8
				0.4, 0.4, 0.3
3.6	R12/H3.6	—	—	2.1, 1.5
3.5	R11/H3.5	—	2.9, 0.7	1.4, 0.8, 0.5
				0.4, 0.2
3.2	R8.6/H8.7	1.8, 1.3	—	1.7, 1.2
2.2	R8.6/H4.2	1.4, 0.7	1.2, 1.0	1.0, 0.6
2.0	R8.6/H2.0	1.6, 0.4	—	1.5, 0.4, 0.2
2.0	R2.3/H4.2	—	—	0.4, 0.4, 0.3
				0.3, 0.2, 0.2
1.6	R12/H1.6	—	—	1.2, 0.4
1.5	R12/H1.5	—	—	1.1, 0.4
1.3	R8.6/H1.3	—	0.8, 0.6	0.7, 0.5
0.5	R2.3/H11	—	—	

* Expressed in kb.
† Only restriction products ≥0.2 kb are listed; smaller ones would not have been detected.
‡ A dash indicates that no cleavage was observed.

Based on these results, the restriction map in Fig. 5 can be drawn connecting all fragments into one continuous 38 kb segment of the chromosome. Only the location of the two *Hin*dIII fragments within R 8.6 and the three within R 12 cannot be definitely assigned, relative to one another, on the basis of these data. Recent mapping of *Hpa* I cleavage sites within R 8.6, however, shows that the positions of the H 2.0 and H 1.3 fragments are correct as drawn (Marsh, unpublished data). The tentative positions shown for the other *Hin*dIII fragments within R 12 are based entirely on the extent to which they are labeled with [^{3}H]thymidine during the 1.5-min pulse, as discussed in the following section. The unmatched secondary fragment from R 11 has been placed at the right of the map as the connecting link to the chromosome on one side. R 12 is at the left, but the unmatched fragment by which it is connected to the rest of the chromosome is apparently too small to have been detected in the agarose gels at the overall level of [^{3}H]-thymidine incorporation achieved in these experiments.

Location of Origin of Replication In or Near Fragment H 1.3. Because several minutes were required for all cells in a culture of the *dnaC* mutant to initiate replication after temperature downshift, a pulse with [^{3}H]thymidine during the first 1.5 min should create a gradient in the amount of label incor-

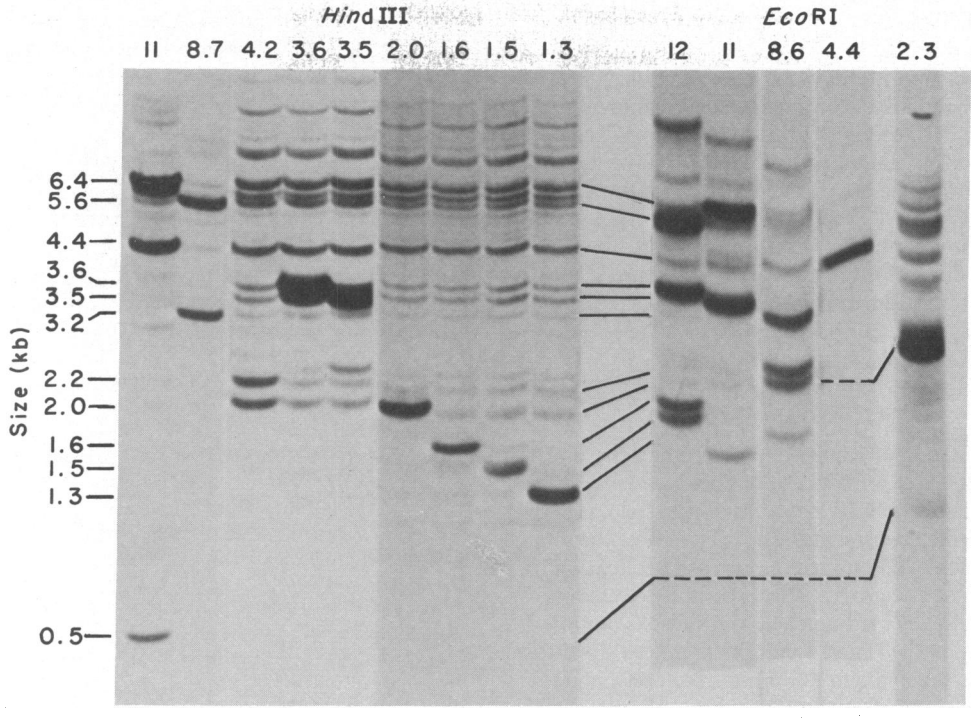

FIG. 4. Matching of the secondary cleavage fragments obtained by digestion of each *Hin*dIII fragment in Fig. 3 with *Eco*RI and vice versa. To facilitate their identification and matching, the secondary products were run on the agarose gels together with a small amount of DNA from *E. coli* PC2 that had been labeled with [^{3}H]thymidine for the first 4 min after downshift from 40° to 28° and digested with both *Eco*RI and *Hin*dIII nucleases. Bands representing identical fragments have been connected in this composite of several gels.

porated, extending outward from a maximum at the origin. When replication proceeds bidirectionally from the origin, as in *E. coli* (1, 2, 7, 8), the actual pattern of incorporation should resemble a pyramid with the origin at its apex.

To determine the specific radioactivity (cpm/kb) of each restriction fragment after 1.5 min of labeling, the bands in Fig. 3 were cut out and assayed in a liquid scintillation counter with a toluene-based scintillant. This was necessitated by the nonlinear response of the x-ray film during fluorography that resulted in a bias toward more heavily labeled bands (27). The secondary digestion products of individual *Eco*RI and *Hin*dIII restriction fragments were then analyzed to determine

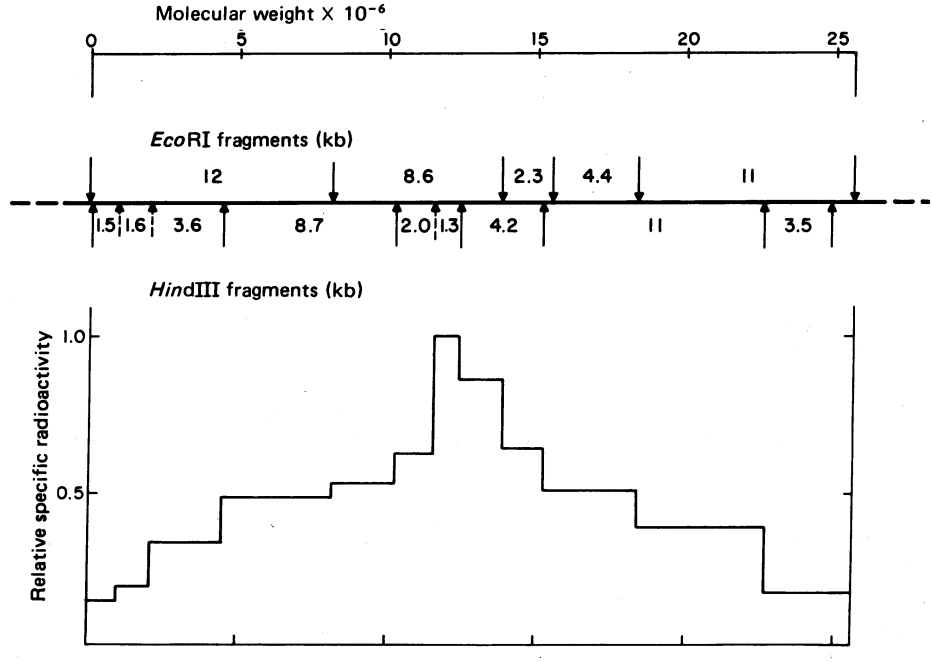

FIG. 5. *Eco*RI and *Hin*dIII restriction map of the *E. coli* K12 origin region, and below it, the relative specific radioactivity of each restriction fragment from *E. coli* PC2 pulse-labeled with [^{3}H]thymidine for the first 1.5 min after downshift from 40° to 28°. The shorter dashed arrows on the map indicate a tentative assignment of those restriction sites (see *text*). Relative specific activity of the fragments was determined as the cpm of [^{3}H]thymidine incorporated per kb normalized to that found in fragment H 1.3. Based on these values, the origin of replication should lie within or very near fragment H 1.3.

the relative distribution of label within the primary fragments. The results are shown in Fig. 5.

From the observed pyramid in specific radioactivity, it can be concluded that the replication origin is located within or very near fragment H 1.3 in the center of the 38 kb mapped region. From there, the two replication forks appear to have moved outward with the same speed over the entire mapped region, judging from the gradual decrease in incorporation of [^{3}H]-thymidine in both directions. No discontinuities were observed at the present stage of resolution. However, the *Hin*dIII fragments contained within R 12 that have been arranged according to their decreasing specific radioactivity may change relative positions.

DISCUSSION

With the synchronization of replication offered by the *dnaA* and *dnaC ts* mutants, PC5 and PC2, it has been possible to locate the *E. coli* origin of replication within or very near a small *Hin*dIII fragment which represents less than 0.04% of the total chromosome. The observed pattern of outward replication in both directions from this fragment with apparently equal speed corresponds well with the symmetrical grain tracks observed by Prescott and Kuempel (7) and Kuempel *et al.* (28) in autoradiographs of pulse-labeled chromosomes from exponentially growing cells or cells synchronized by amino acid starvation. It also agrees with the overall pattern of replication of the chromosome ascertained by comparison of marker frequencies in cells growing at different rates (1) and by the pattern of nitrosoguanidine mutagenesis during a synchronous round of replication (2).

When the origin-containing region was first mapped, we considered the possibility that it might not be located on the bacterial chromosome but rather represented a cryptic plasmid. This has been ruled out by our inability to detect any covalently closed circular DNA molecules in pulse-chased lysates in either alkaline sucrose gradients or CsCl/ethidium bromide density gradients. Furthermore, the same initial pattern of replication has been observed in temperature-shifted cells of the separate *dnaC ts* mutant CT28 isolated by Schubach *et al.* (21) and in a nontemperature-sensitive *E. coli* K12 strain following its release from amino acid starvation (Parks and Marsh, unpublished data).

No substantial incorporation of [^{3}H]thymidine into any specific fragments outside the 38 kb mapped region bounding the origin was observed in the initial stage of replication following downshift of either PC2 or PC5 to the permissive temperature. Both mutants are apparently blocked specifically at the origin.

The defect in both PC2 and PC5 is known to be thermally reversible. Initiation of replication occurs upon shift to the permissive temperature even in the presence of chloramphenicol or rifampicin (22). Thus, the observed rapidity of initiation upon downshift of these mutants might have been predicted despite the fact that overall incorporation of [^{3}H]-thymidine would indicate a longer delay. Earlier, Schubach *et al.* (21) reported evidence that CT28, like PC2, can complete initiation of replication during a period as brief as 3 min at 30°.

Unfortunately, initiation was not completely synchronous upon shift of either the *dnaA* or *dnaC* mutant to the permissive temperatures, as reflected in the 6–8 min required for all cells in either population to replicate any one restriction fragment. It was clear, however, that the *dnaA* mutant initiated replication 3–4 min after the *dnaC* mutant. Apparently this delay cannot be due to a longer half-time for renaturation of the *dnaA* gene product, as this would lead to a greater time spread in replication of each restriction fragment. This may therefore indicate that the *dnaA* gene product is involved at an earlier step in initiation than the *dnaC* gene product and that this step requires several minutes to complete.

The *Eco*RI and *Hin*dIII restriction map presented here places the origin of replication in *E. coli* within a region containing 1000–2000 base pairs. It should be possible to use additional restriction enzymes to more narrowly define the chromosomal site at which initiation of replication occurs.

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