Tracking bacterial DNA replication forks *in vivo* by pulsed field gel electrophoresis

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ABSTRACT

The location of chromosomal DNA replication forks was identified in synchronously replicating *E. coli* cultures by pulse labeling DNA at specific times with ^{14}C-thymidine and following incorporation of radionucleotide into genomic *Not* I restriction fragments. This technique could be used to characterize chromosomal DNA replication, to characterize mutations which affect this process, to identify the location of DNA replication origins and termini as well as aid in the construction of macrorestriction maps. Here, we further characterize the DNA replication mutations *divE* and *dnaK* and preliminarily characterize the genomic organization of *E. coli* isolate 15.

INTRODUCTION

The analysis of various types of mutations and detailed *in vitro* experiments, usually on plasmids, have revealed many features of bacterial DNA replication (for example, see 1–3). In *E. coli* K12, DNA replication initiates at a single, specific site called *oriC* (4, 5) located at 84 min on the genetic map (6) and proceeds bidirectionally towards a termination region located 180 degrees from the initiation site at about 35 min (7, 8). The initiation of DNA replication depends on *de novo* protein and RNA synthesis (9–12) and appears to be modulated by methylation (for review see 13). Interaction between the replication origin and the cell membrane has also been postulated to be involved in regulation (14, 15).

However, the characterization of DNA replication on intact bacterial chromosomes is difficult and laborious. For example, the first demonstration that $dnaA_{ts}$ mutations affected DNA initiation required a series of hybridization experiments to show that sequential DNA synthesis occurs from *oriC* when cells are shifted from the non-permissive to permissive temperature (16). This same kind of laborious approach was used to characterize DNA replication termination (7, 8).

Digestion of *E. coli* K12 chromosomal DNA with the restriction enzyme *Not* I generates about 20 fragments ranging in size from 20 kb to 1,000 kb (17). These fragments can be fractionated by pulsed field gel (PFG) electrophoresis (18) and have been aligned along the chromosome to create a low resolution restriction map (19). This *E. coli* physical map now allows molecular studies to be conducted directly and easily on the chromosome (For example see 20).

Here, we describe the application of PFG electrophoresis and large DNA technology to studying *E. coli* chromosomal DNA replication *in vivo*. Chromosomal DNA replication forks were followed in synchronous cultures using a DNA pulse labeling procedure.

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Table I. *E coli* strains and clones.

E. coli strains

Name	Derived from	Genotype	Reference
EMG2	K12	(l, F^+)[1]	38, 39
AQ2	15 T^-(15)[2]	*thy*$^-$, *trp*$^-$, *met*$^-$, *arg*$^-$, *thr*$^-$, *r15m15* (λ^-· P15)	23
CRT46	K12(CRT34)	*dnaAts*46, *thy*$^-$, *thr*$^-$, *leu*$^-$, *thi*$^-$, *ilv*$^-$, *lac*$^-$, *mal*$^-$ (λ^-)	40
N42	K12(AT713)	*divE*$_{ts}$ (tsC42), *cysC39, argA21, lysA10, malA1, xyl4, rpsL9, met*$^-$	21
groPC756	K12(C600)	*dnaK756, thr*$^-$, *leu*$^-$ (λ^-)	22

Clones

			EMG2 *Not* I Fragment[3]		
Name	Gene	Min[4]	Name	Size (kb)	Source
pSY317	*oriC*	84	L	203	41
pHA5	*crp*	73	A	1000	42
divE231	*divE*	22	J[5]	210	31
pDNAJ-A	*dnaJ*	0	B	360	43
pMC1871	*lacZ*	8	D	275	44

[1] This strain is the original *E. coli* K12 wild type and by definition contains no mutations.
[2] *E. coli* isolate 15 has two restriction-modification systems, r15m15 on the chromosome and *Eco* P15 on the endogenous P15 plasmid (45).
[3] See reference 19.
[4] See reference 6.
[5] This is assumed based on the genetic position.

Chromosomal DNA replication was synchronized, *in vivo*, by incubating *E. coli* cells containing a *dnaA*$_{ts}$ mutation at the restrictive temperature or by starving cells for amino acids. The *dnaA*$_{ts}$ mutation is a mutation that affects DNA initiation (3). A temperature shift to the non-permissive temperature followed by a shift back to the permissive temperature aligns DNA replication forks at *oriC*. Since *de novo* protein synthesis is required for initiation of each new round of replication, amino acid starvation also aligns DNA replication forks at *oriC*. In both cases, DNA replication synchrony is maintained for at least one round of replication. Thus, pulse labeling of DNA with ^{14}C-thymidine during the course of such experiments leads to the incorporation of radioactive label into specific chromosomal restriction fragments. Autoradiography of PFG fractionated pulse labeled genomic *Not* I restriction fragments will then reveal the location of the fork at the time of labeling.

This simple technique could be used to study the chromosomal DNA replication mechanism, to characterize DNA replication mutations, to identify DNA replication origins and termini, and to order megabase restriction fragments around the chromosome. Here we have used it to characterize the effect of the *divE* (21) and *dnaK* (22) mutations on DNA replication and to tentatively compare the *Not* I physical map of *E. coli* K12 strain

EMG2 with *E. coli* isolate 15 (23). A similar, but more laborious approach, has been used to map the chromosomal DNA replication origin and terminus in Mycoplasma (24).

MATERIALS AND METHODS

Materials

E. coli strains and clones are described in Table I. M9 medium (25) contained 0.4% glucose, while other supplements were added as described below. ^{14}C-thymidine was obtained from New England Nuclear and had a specific activity of >50 mCi/mmole.

Methods

DNA replication was synchronized in *E. coli* strain AQ2 using amino acid starvation. Cultures were grown for several generations to a cell density of 1 X 10^8 cells/ml at 37 °C in M9-glucose medium that contained 100 μg/ml arginine, 50 μg/ml methionine, 50 μg/ml tryptophan, 50 μg/ml threonine, 1 μg/ml thiamine and 4 μg/ml thymine. The cells were collected by centrifugation, washed once with M9 basal medium without amino acid, and finally resuspended in M9-glucose medium that contained only 2 μg/ml thymine and 2 μg/ml thiamine. After amino acid starvation for 70 min, amino acids were added back to the same concentration as the prestarvation culture.

Temperature shift experiments were carried out by first growing cells at 30°C in M9-glucose medium containing 0.4% casamino acids, 1 μg/ml thiamine, and 1 μg/ml thymine to a density of 0.7−1 X 10^8 cells/ml and then shifting the culture to 42°C for 55 min. The culture was then returned to 30°C for further incubation. Cell growth was monitored throughout the shift experiments by optical density measurements. Previous work correlated optical density changes with shut off of DNA synthesis in the *divE* and and *dnaK* mutant strains (Ohki, M. unpublished results).

Usually cells were pulse labeled for 5 min with ^{14}C-thymidine at a concentration of 2 μCi/ml. Labeling of thymine prototrophs with ^{14}C-thymidine was done in the presence of 180 μg/ml deoxyadenosine. After labeling, the cells were incubated for an additional 30 min in the presence of excess (60 μg/ml) cold thymidine and 20 μg/ml chloramphenicol.

Not I fragments obtained by *in situ* digestion of chromosomal DNA purified in agarose were fractionated on a PFG Pulsaphor apparatus (Pharmacia-LKB) as described (17, 26, 27). PFG running conditions were 25 sec pulse times, 330 V, 40 hr run times at 15°C. Gels were dried on Whatmann 3MM filter paper *in vacuo* and subjected to autoradiography using Kodak AR X-ray film typically for 30 to 50 days.

RESULTS AND DISCUSSION

Partial Characterization of the E.coli isolate 15 chromosome

Our first goal was to establish the validity of the described approach. In order to do this, chromosomal replication was followed under conditions and in *E. coli* strains that were extremely well characterized. In the course of this work, fragmentary information accumulated on the chromosomal organization of *E. coli* isolate 15 (see below). This information, summarized in Table II, is a sound foundation for constructing a complete *Not* I physical map of this organism. This approach is an extremely powerful method of obtaining a quick tentative overview of a chromosome that can enormously aid subsequent map construction (see below).

E. coli strain AQ2 (isolate 15) was chosen for these experiments because of its multiple

Table II. Partial characterization of the *E. coli* strain AQ2 (isolate 15) genome.

Not I Fragment Number[1]	kb	Time (min)[2]	Location of genes[3]
1	868	21	*crp*
2	360	37[4]	
3	350	31	*lac*
4	330	21	
5	315	34[4]	
6	291	11	
7	286	47[4,5]	*divE* , *ter*[6]
8	276	31	
9	267	16	
10	252	1	*oriC*
11	218	34[4]	
12	184	37[4 7]	
13	182	31	
14	165	34[7]	
15	165	11	
16	104	31	
17	90	37[4,5]	*dnaJ*
18	87	21	
19	49	16	
20	44	16	
21	39	16	
22	34	16	
23	29	16	
	4,835		

[1] Fragment numbers and sizes are taken from Figure 1.
[2] Shown is time ^{14}C-thymidine is first detected in fragment in Figure 2. In some cases (16 and 26 min) an average of two time points is indicated since the stoichiometry of the labeling indicated a fragment was labeled at the end of the earlier time point.
[3] Determined by hybridization experiments.
[4] Fragments last to be labeled when DNA synthesis shuts down (See Figure 2, lane C).
[5] Fragment 7 (rather than fragments 6 or 8) and fragment 17 (rather than 18) are identified in lane C of Figure 2 by taking into account the parallel shifts of other fragments in lanes A-C.
[6] The location of the DNA replication terminus is identified as the last to be labeled fragment (see Figure 2, lane I) after the resumption of DNA synthesis.
[7] Labeling times are somewhat ambiguous for the following pairs of fragments: (a) 12 and 13, and 14 and 15. Shown is the most likely assignment.

amino acid requirements, its previous extensive use in characterizing *E. coli* DNA replication (10, 11) and the fact that its *Not* I fragments were easily resolved by PFG. However, it was difficult to order the *Not* I fragments of *E. coli* strain AQ2 by simple comparison to the known physical map of *E. coli* K12 (14) since the pattern of *Not* I fragments from *E. coli* strains EMG2 (the original K12 wild type) and AQ2 detected by ethidium bromide staining are quite different (Figure 1). This is not surprising in view of the fact that these are independently isolated *E. coli* strains. When the *Not* I fragment sizes are added up a genome size of 4.8 Mb is obtained for *E. coli* isolate 15 (Table II). This is slightly higher than that of *E. coli* K12 (19).

To aid the interpretation of the labeling experiments described below several *Not* I

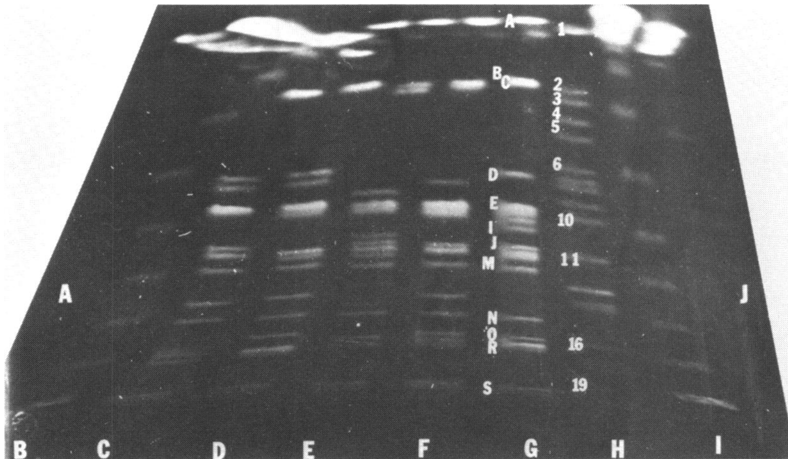

Figure 1. Comparison of *Not* I genomic fragments from different *E. coli* strains. Chromosomal DNA was purified in agarose, digested with the restriction enzyme *Not* I and the resulting fragments fractionated with PFG as described in Materials and Methods. Lane C−groPC756, D- N42, E−K7052 (λ*dnaK*), F−CRT46, G−EMG2, and H−AQ2. The differences in *Not* I fragments detected between strains EMG2 and AQ2 are discussed in Results. Some of the differences detected in the other strains can be understood from their genotypes. For instance, the fragment appearing between fragments N and M in lanes C, D and F is a smaller fragment K due to the absence of λ prophage in these strains. Fragment I, in lanes A−F, is probably increased in size because of variation in size in the *rac* locus (Smith, C.L., unpublished observations). Besides the absence of F^+ plasmid and therefore the absence of fragment Q, other minor fragment size changes are not readily interpreted from the known genotypes. *E. coli* strain K7052(λ*dnaK*) was not used in this study. Size standards are lanes A and J− chromosomal DNAs from *S. cerevisiae* strain YN295 from Ron Davis and lanes B and I−annealed λ phage size standard (48.5 kb monomer).

fragments were identified by hybridization to cloned *E. coli* genes. On the genetic map of *E. coli* K12, the genes *crp, lac, divE, oriC* and *dnaJ* are located at 73, 8, 22, 84 and 0 min, respectively (6). These genes are located on *Not* I fragments that are 1000, 360, 210, 203, and 390 kb in size, respectively in EMG2 (19). In *E. coli* strain AQ2, hybridization experiments (not shown) located these genes on *Not* I fragments 1, 3, 7, 10 and 17, respectively. Interestingly most of these fragment sizes (868, 360, 286, 252, and 90 kb, respectively) and the terminus fragment (286 kb in *E. coli* AQ2 and 230 kb in *E. coli* EMG2; see below) are similar to the *Not* I fragment sizes located in the equivalent regions of the *E. coli* K12 chromosome. For instance, it appears that in both isolates the same 20% of the chromosome is devoid of *Not* I sites (i.e. compare 1000 kb with 868 kb). In addition, the experiments described below will show that the DNA replication origin and terminus as well as the genes described above appear to be arranged in a similar manner in the two isolates (K12 and 15).

Identifying DNA replication origins and termini

Initiation of DNA replication is coupled to *de novo* protein synthesis (9−12). This means that initiation of each new cycle of DNA synthesis is inhibited when cells are deprived of required amino acids or when protein synthesis is inhibited with antibiotics such as chloramphenicol. However, under these conditions ongoing rounds of DNA replication are completed (10). When required amino acids are added back to starved cells (or chloramphenical removed from inhibited cultures), DNA synthesis initiates synchronously from the authentic replication origin. Such synchronous cultures were used to examine

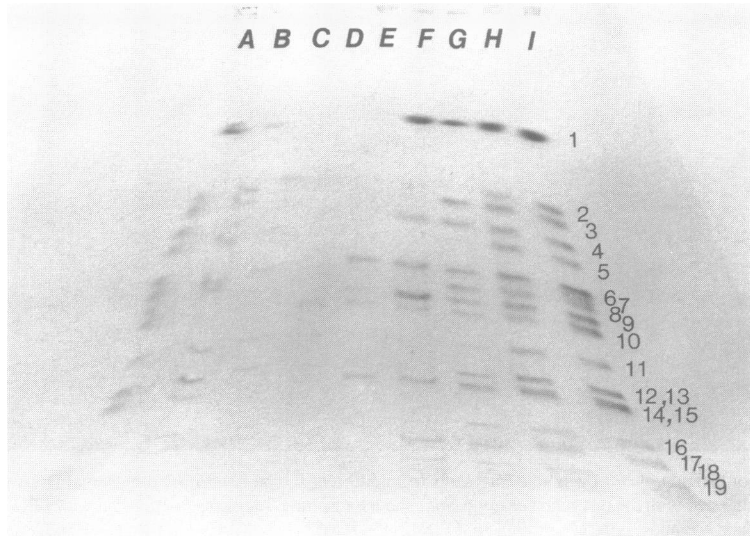

Figure 2. PFG analysis of DNA synthesis in *E. coli* strain AQ2 during and after amino acid starvation. Samples were pulse labeled with ^{14}C-thymidine, and chromosomal DNA was purified as described in Materials and Methods. Aliquots were labeled with ^{14}C- thymidine at 2 min (lane A), 40 min, (lane B) and 65 min (lane C) during amino acid starvation, and at 1 min (lane D), 11 min (lane E), 21 min (lane F), 31 min (lane G), 37 min (lane H), and 47 min (lane I) after addition of amino acids.

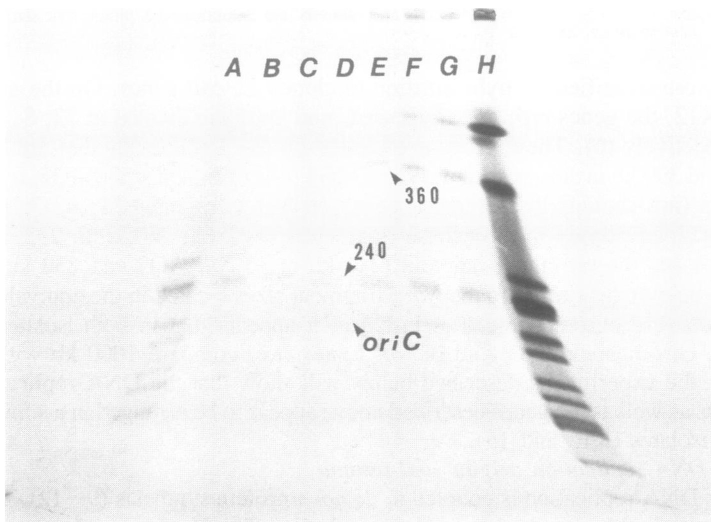

Figure 3. PFG analysis of DNA synthesis in *E. coli* strain CRT46 containing the *dnaA*$_{ts}$ mutation temperature sensitive to DNA replication initiation. Temperature shift, pulse labeling, and DNA purification were carried out as described in Materials and Methods. Aliquots were labeled with ^{14}C-thymidine at 30 min (lane A), 50 min (lane B) and 60 min (lane C) during incubation at 42°C and at 5 min (lane D), 20 min (lane E), 30 min (lane F) and 40 min (lane G) after return to the permissive temperature. Lane H represents a sample uniformly labeled at 30°C for 1 hr.

chromosomal DNA replication with PFG electrophoresis.

E. coli strain AQ2, derived from isolate 15, was pulse labeled with ^{14}C-thymidine at various times during amino acid starvation and after the start of synchronous DNA synthesis. Intact chromosomal DNA was prepared in agarose and digested with the restriction enzyme *Not* I. The resulting *Not* I fragments were fractionated by PFG electrophoresis. Exposure of this gel to X-ray film revealed the time-dependent incorporation of ^{14}C-thymidine into various *Not* I fragments (Figure 2). This data is summarized in Table II. Immediately after deprivation of required amino acids, almost all *Not* I fragments (>20) were evenly labeled by pulse labeling with ^{14}C-thymidine for 2 min (Figure 2, lane A). However, as time passed the number of *Not* I fragments labeled with ^{14}C-thymidine decreased. Only five fragments (2, 5, 7, 11 and 12 or 13) were predominantly labeled just before DNA replication ceased (Figure 2 lane C). These fragments represent the entire set of fragments that were labeled after 34 min following the resumption of DNA synthesis (Figure 2, lanes G-I). These fragments represent 30% (1.5 Mb) of the chromosome. The fragment of this group (fragment 7) that contained the terminus region could be identified as the very last fragment labeled after the resumption of DNA synthesis (Figure 2, lane I).

Not I fragments labeled early after DNA replication initiated were different from those detected in the late stages of residual DNA synthesis (Figure 2 and Table II). Immediately after restoration of DNA synthesis, label was exclusively found in *Not* I fragment 10 (lane D). Soon afterward label was found in two other fragments: 6 and 14 (or 15). The initial time of appearance of label into all *Not* I fragments is summarized in Table II. In a few cases, definitive assignments cannot be made because two fragment migrated very closely in the PFG experiment (see footnotes in Table II). Despite this problem, fragments can be grouped together based on the initial time that label appears in them as follows: 1 min—fragment 10; 11 min—fragment 6 and 14 (or 15); 16 min—fragment 9 and 19–23; 21 min—fragments 1, 4, 18 and 21; 31 min—fragments 3, 8, 13 (or 12) and 16; 34 min—fragments 5, 11 and 15 (or 14); 37 min—fragments 2, 12 (or 13) and 17; 47 min—fragment 7.

The *divE* gene has been genetically mapped at 22 min near the terminus region of the *E. coli* K12 chromosome which is located between 28 min and 35 min (28). This gene hybridized to *Not* I fragment number 7 when it was used as a hybridization probe with *E. coli* AQ2 DNA. This fragment was in the group of last labeled fragment during shut off and was the last fragment to be labeled following resumption of DNA synthesis (Figure 2, lane C and lane I). In contrast, the *oriC* containing fragment was the first to be labeled after the resumption of DNA synthesis. The remaining fragments identified by hybridization experiments and presumed to be between *oriC* and *ter* had intermediate labeling times. Thus, these results validate this approach for identifying restriction fragment around the origin and terminus of DNA replication.

The time that elapses before the appearance of label in particular *Not* I fragments represents the time required for the replication fork to traverse the chromosome from the replication origin. Labeling of fragment 1 (hybridizing to the *crp* genes) began at 25 min. This was the same time as fragment 17 (or 18) detected by *dnaJ*. The map distances in *E. coli* K12 between *oriC* and *crp* (11 min) and between *oriC* and *dnaJ* (6 min) are similar even though these genes face each other with *oriC* between them. This suggests that DNA replication is also bidirectional in *E. coli* isolate 15 and the alignment of these genes in *E. coli* strain AQ2 is almost identical with that in the *E. coli* K12 EMG2 genome.

Assuming that the AQ2 chromosome is arranged in a manner similar to that of *E. coli*

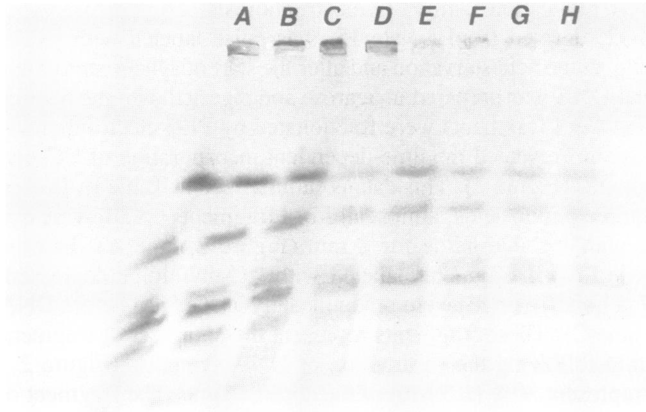

Figure 4. PFG analysis of DNA synthesis in *E. coli* strain N42 containing the *divE42* mutation. DNA pulse labeling was carried out at various times after shift to the restrictive temperature (42°C) and subsequent growth at the permissive temperature (30°C). All methods are identical with those described in the legend to Fig. 2, except that the pulse labeling time was for 3 min instead of 5 min. Aliquots were labeled with ^{14}C-thymidine at 0.5 min (lane A), 20 min (lane B), 40 min (lane C) and 60 min (lane D) after shift up to 42°C, and at 5 min (lane E), 15 min (lane F), 25 min (lane G) and 35 min (lane H) after shift down to 30°C.

K12 it is possible to predict on the basis of gene assignment the order of appearance of the five *Not* I fragments as follows: $10 < 1 < 3 < 17 < 7$. The most probable ordering by time of pulse labeling is the same as predicted from gene order. This further suggests that these chromosomes are arranged in a similar manner. However, only a complete restriction map of AQ2 would allow a definitive comparison of *E. coli* isolates 15 and K12.

The 230 kb *Not* I fragment 10, containing *oriC* (see below), begins to be pulse labeled two to four minutes after the addition of amino acids to starved cells and continues to be labeled throughout the course of the experiment. This suggests initiation of DNA replication after restoration of required amino acids is heterogeneous, consistent with observations by Lark and Renger (10).

Synchronizing DNA replication in dnaAts mutants

A number of genes involved in chromosome replication have been identified. The *dnaA* gene is known to participate specifically in initiation of DNA replication but not in its elongation (3). When a culture of an *E. coli* strain having the *dnaA*46 mutant is transferred to the restrictive temperature, the rate of DNA synthesis gradually decreases until synthesis finally stops. The slow stop reflects heterogenous termination of ongoing rounds of DNA replication and blockage of new rounds of DNA replication. Upon return to the permissive temperature, arrested cells synchronously initiate DNA synthesis from the origin of replication (16).

We analyzed DNA synthesis in *E. coli* strain CRT46, containing the $dnaA_{ts}$ mutation, during the course of temperature shifts up and shifts down. Our goal was to establish that PFG could be used as a simple and persuasive technique to characterize DNA replication mutations and to determine whether such defects reside in the DNA initiation elongation

process. DNA replication was followed in the $dnaA_{ts}$ cells by pulse labeling with ^{14}C-thymidine at various stages during the course of a shift up and down experiment (Figure 3). This *E. coli* strain, CRT 46, has many overlapping *Not* I fragments. Thus, the resolution of the labeling pattern in *E. coli* K12 strain was worse than that in the *E. coli* strain AQ2. Nevertheless, a sequential pattern of labeling of *Not* I fragments was observed following resumption of DNA synthesis. The sequential labeling of specific *Not* I fragments was shown above to be indicative of synchronous DNA replication from the replication origin.

E. coli strain CRT46 is closely related to *E. coli* EMG2 genetically (see Table I). Thus, it is not surprising that the genomic *Not* I restriction fragment patterns are very similar for these two organisms (compare lanes F and G in Figure 1). Hence, although many fragments were not resolved in the experiment shown in Figure 3, it was possible to predict the approximate location of fragments labeled at some times. For instance, introduction of label into the *oriC* containing fragments and a fragment about 240 kb in size occurred soon after the culture was returned to the permissive temperature (Figure 3, lane D). The latter fragments presumably corresponds to *Not* I fragment H (240 kb in size; see reference 21) in *E. coli* EMG2 which is close to the origin. Label began to accumulate in a 360 kb band after 20 min (lane E). This correlates with the expected location of the two overlapping *E. coli* EMG2 *Not* I fragments, B and C. Fragment B is next to fragment H (240 kb) in the *E. coli* EMG2 genome and covers between min 93 and 0 min on the genetic map. Fragment C covers from 5 to 14 min in *E. coli* EMG2. These results provide further evidence that the *Not* I fragment labeling patterns observed at the beginning, intermediate and late stages after the onset of chromosomal DNA synthesis correctly reveal the location of the DNA replication fork.

The effect of the divE and dnaK mutations on DNA replication

The *divE* gene is essential for cell growth, and it appears to regulate protein synthesis at specific stages in the cell cycle (29). Nucleotide sequence determination of the cloned wild-type gene revealed that the gene product is a $tRNA^{ser}$ (30). Synthesis of certain proteins, such as succinate dehydrogenase and β-galactosidase, halts immediately after transfer of a culture from the permissive temperature to the restrictive temperature in *E. coli* cells containing *divE* mutations (21, 29). Synthesis of these proteins only occurs at specific stages during the replication cycle (31). The time course of DNA synthesis in *E. coli* strains containing *divE* mutants, after transfer to 42 C, closely resembled that seen with *dnaA* mutants: DNA synthesis ceased after a 1.5 fold increase in total mass at the restrictive temperature. This raised the possibility that the *divE* gene was directly involved in DNA synthesis as well as protein synthesis. PFG analysis of DNA synthesis in *E. coli* strain N42, containing the *divE*42 mutation, was examined to determine whether this involvement was at the initiation of DNA synthesis (Figure 4).

In Figure 4, lanes A to C contain chromosomal DNA from an *E. coli* strain $divE_{ts}$ mutant that has been pulse labeled with ^{14}C-thymidine at various stages during residual DNA synthesis. Lanes D to H represent DNA synthesis at different stages after return of the culture to the permissive temperature. The DNA pulse labeling pattern during shift up and down was different from that observed in the *dnaA* mutant strain. No sequential events were detected. This indicates that the effect on the *divE* mutation on DNA replication is not restricted to initiation of DNA replication. Cessation of DNA synthesis in the *divE* mutant might occur through an indirect effect on the elongation process or through an effect on both the initiation and elongation of DNA replication.

Although the *dnaK* gene is essential for initiation of l DNA replication (32), its function

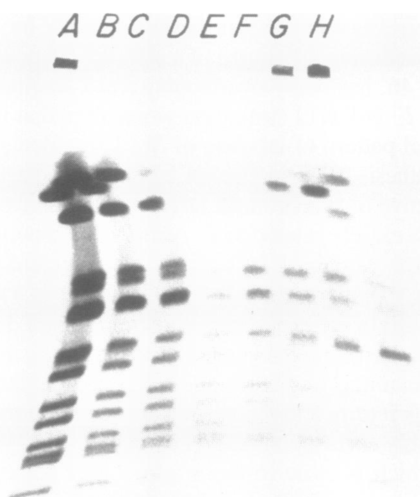

Figure 5. PFG analysis of DNA synthesis in *E. coli* strain groPC756 containing the *dnaK*756 mutation. DNA pulse labeling was carried out at various times after shift to the restrictive temperature (42°C) and subsequent growth at the permissive temperature (30°C) as described in Materials and Methods. Shown are aliquots labeled with ^{14}C-thymidine at 5 min (lane A), 25 min (lane B), and 50 min (lane C) after shift up to 42°C and at 5 min (lane D), 15 min (lane E), 25 min (lane F), 35 min (lane G) and 45 min (lane H) after shift down to 30°C.

is poorly understood (33). The gene codes for a major heat shock protein of about 70 kilodaltons (34) which appears to be associated with RNA polymerase (35). The effect of a *dnaK* temperature sensitive mutation on *E. coli* chromosomal DNA replication was assessed as described above for the *dnaA* and *divE* mutations . The results show a sequential labeling of specific *Not* I fragments following a return to the permissive temperature after incubation for 50 min at the restrictive temperature (Figure 5, lanes F, G and H). This indicates that shift of an *E. coli* strain containing this mutation to the restrictive temperature aligns the chromosome replication fork at the origin of replication and suggests that the *dnaK* gene is involved in initiation of chromosomal DNA replication. The data shown in Figure 4 also reveals that chromosomal replication may terminate abortively when this strain is shifted to the restrictive temperature. However, more experiments are needed to see whether this result is significant.

After this work was completed, Sakakibara (36), while searching for temperature sensitive defects suppressed by an *rnh-* (RNase H) mutation, isolated a new temperature sensitive *dnaK* mutation that specifically affected initiation of chromosomal replication. He had reasoned that unknown initiation proteins might be identified in this way because such suppression had been noted previously for $dnaA_{ts}$ mutations. Since the *dnaK* mutation used in our studies was not selected in such a manner, it appears that inhibition of DNA replication is a general characteristic of *dnaK* mutations.

RNA synthesis at *oriC* is closely tuned to the intracellular levels of DnaA protein (12) while activation of DnaA protein requires membrane attachment (37). Suppression of both $dnaA_{ts}$ and $dnaK_{ts}$ mutations by a defect in RNase H activity may be through stabilization of *oriC* transcripts. The effect of the DnaK protein on *oriC* transcription or DnaA protein-

membrane interaction has not yet been explored, nor has the possible interaction between DnaA protein with the DnaK protein.

SUMMARY

The construction of a complete genomic restriction map can be aided by the tentative ordering of fragments using the pulse labeling experiments shown here. For instance, physical map construction in bacteria is most efficiently accomplished using partial genomic restriction enzyme digestions fractionated by PFG electrophoresis (19, 20). Hybridization of such a gel with a single copy probe identified a series of fragments which extend bidirectionally away from the complete digestion fragment homologous to the probe. Overlapping data from a series of experiments can then to used to construct a physical map. In *E. coli* isolate 15, *Not* I restriction fragments may be tentatively assigned to chromosomal regions on the basis of labeling time and presumed size of region (based on comparison with *E. coli* K12). This allows partial digestion products to be tentatively predicted. Hence, one may be able to minimize partial digestion experiments by judicially choosing probes and PFG running conditions.

PFG electrophoresis and large DNA technology now allow molecular studies to be conducted on whole chromosomes. Thus, molecular studies on chromosome function, such as DNA replication, which formerly required characterization on small cloning vehicles can now be examined directly on the intact chromosome. It has enabled us to identify another component of chromosomal DNA replication initiation machinery. It is reasonable to expect that as such studies proceed they may reveal new and even unexpected features of the behavior of bacterial chromosomes.

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