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Localizing the Replication Origin Region on the Physical Map of the *Mycoplasma capricolum* Genome

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Four lines of evidence argue that the replication origin of the *Mycoplasma capricolum* genome lies within the 46-kb *Bam*HI fragment bordered by two *Bam*HI sites of the total of nine *Bam*HI sites that have been located on the physical map (M. Miyata, L. Wang, and T. Fukumura, FEMS Microbiol. Lett. 79:329–334, 1991). First, this fragment lost its labeling in preference to other fragments when log-phase cultures were incubated in the presence of chloramphenicol for various times to inhibit the initiation of new rounds of replication and then further incubated with radioactive dTMP to allow DNA elongation to continue. Second, the relative frequencies of various restriction fragments of the genome DNA from exponentially growing cells decreased with increasing distance from the putative origin. Third, preferential labeling occurred when radioactive dTMP was added to cultures of a DNA elongation-defective, temperature-sensitive mutant with a simultaneous temperature downshift. Fourth, the *M. capricolum* homolog of the *dnaA* gene, which is located near the replication origin in many other bacteria, was found in the 46-kb fragment.

Mycoplasmas are the simplest, wall-less, self-replicating single-cell organisms, consisting of only a minimum set of subcellular structures for growth and reproduction (21), and their genomes are extremely small (17). Mycoplasmas are therefore attractive for use in the study of essential cellular mechanisms, and many molecular biological studies have been reported (5, 12, 22). However, there have been few reports concerning DNA replication (7, 19).

Analysis of chromosomal replication in a new organism may begin with attempts to map the replication origin and to determine the directionality of replication fork movement from the origin, a strategy that proved fruitful for *Escherichia coli* (9), *Bacillus subtilis* (26), and other bacteria (4).

Mycoplasma genome DNA, unlike that of the eubacteria, can be specifically labeled by [^{32}P]dTMP, because mycoplasmas generally incorporate nucleoside-5′-monophosphates as precursors of triphosphates (13), and all restriction fragments of whole DNA genome can be fractionated by field inversion gel electrophoresis (FIGE) (17, 18). These two special features can be advantageous and led us to use the following techniques: (i) chloramphenicol (CM) was used to inhibit de novo protein synthesis to stop the initiation of DNA replication (10, 15), (ii) the relative frequencies of restriction fragments of the genome DNA from exponentially growing cells were estimated (1, 3, 25); and (iii) a DNA replication-defective, temperature-sensitive (ts) mutant was used (8). We also tried to estimate the location of the origin by locating the *dnaA* gene homolog of the *Mycoplasma capricolum* genome, as the *dnaA* gene is found close to the origin in some eubacterial genomes (14).

Our results showed that the replication origin of *M. capricolum* is located in the 46-kb region on the physical map that we constructed earlier (11).

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MATERIALS AND METHODS

Strains and plasmids. *M. capricolum* ATCC 27343 and its ts mutant were grown in modified Edward medium (MEM) (23). Derivative M13 harboring the *dnaA* gene homolog of the *M. capricolum* genome was supplied by M. Q. Fujita, Osaka University Medical School (7).

Reagents. [^{32}P]dTMP was prepared by the following methods. [α-^{32}P]dTTP (10 μM) was hydrolyzed with 3.1 mg of phosphodiesterase I per ml in 10 mM Tris-HCl (pH 8.8) at 37°C for 10 min. The reaction was stopped by the addition of 11 mM EDTA. Phosphodiesterase I was denatured by being heated at 95°C for 3 min and removed by centrifugation at $(1.4 \times 10^4) \times g$ for 10 min. The [^{32}P]dTMP produced was checked by polyethyleneimine-cellulose thin-layer chromatography (20), and about 90% of the [α-^{32}P]dTTP was found to have been converted to [^{32}P]dTMP. [α-^{32}P]dTTP and phosphodiesterase I were purchased from the Radiochemical Centre, Amersham, United Kingdom, and Sigma Chemical Co., St. Louis, Mo., respectively.

Radiolabeling of DNA. Radiolabeling of mycoplasma DNA was carried out by the addition of 25 μCi of [^{32}P]dTMP per ml (250 nM) to a culture. Incorporation was stopped by the addition of 5 volumes of an ice-chilled solution consisting of 20 mM Tris-HCl (pH 7.6), 0.25 M NaCl, and 10 mM EDTA. The procedures for the purification and nuclease digestion of DNA and the separation of DNA fragments by FIGE have been described elsewhere (11). For autoradiography, the agarose gels were treated with 7% trichloroacetic acid at room temperature for 30 min, washed with water, and dried at 65°C in a vacuum dryer. The gels and the filters were exposed to preflashed X-ray film. For quantitative analysis with a Bio Image Analyzer BAS 2000 (Fuji Photo Film, Tokyo, Japan), the gels were exposed to a Fuji imaging plate.

Southern hybridization analysis. DNA probes for Southern hybridization were synthesized by the random primer method (6). Hybridization was carried out as described elsewhere (24).

Isolation of ts mutants defective in DNA synthesis. Log-phase cells of the wild-type strain were washed and suspended in phosphate-buffered saline (PBS). Mutagenesis

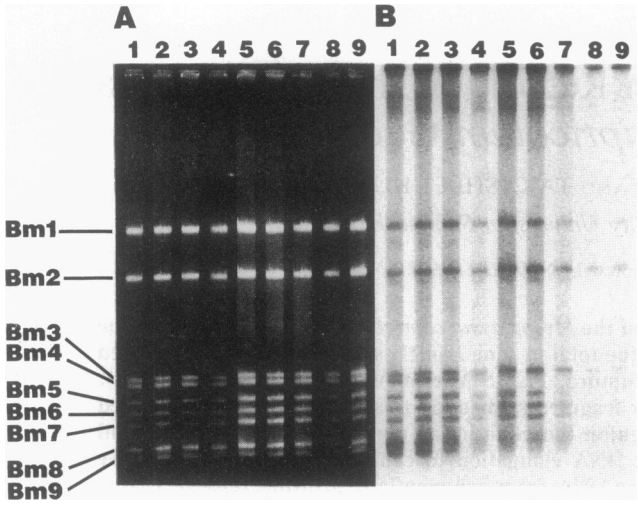

FIG. 1. Inhibition of initiation of DNA replication by CM. To the wild-type strain culture grown at 33°C to the mid-log phase was added 80 μg of CM per ml, and the culture was incubated for various times (0, 10, 20, 30, 40, 60, 80, and 100 min [lanes 2 to 9, respectively]); next, [^{32}P]dTMP was added to the culture, and incubation was continued at 33°C for 15 min. To the culture in lane 1, [^{32}P]dTMP was added before CM. (A) Ethidium bromide-stained FIGE gel. (B) Autoradiogram of the FIGE gel shown in panel A.

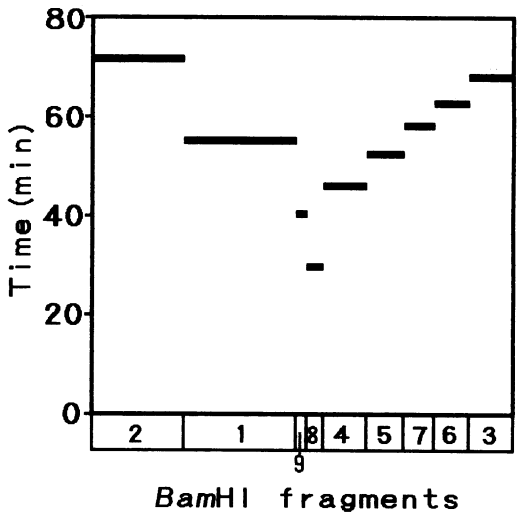

FIG. 2. Quantitative illustration of Fig. 1B. The radioactivity of the bands in Fig. 1B was quantified by use of an image analyzer, and the time needed for radioactivity in a band to reach 25% of that in the corresponding band in lane 1 of Fig. 1B after CM addition was calculated for each band. On the abscissa, the *Bam*HI fragments are arranged in the same order as in the physical map (see Fig. 5).

was conducted by incubation of the cells with 50 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml at 37°C for 40 min. After being washed with PBS, the treated cells were grown in MEM at 33°C for 12 h. Enrichment of ts mutants defective in DNA synthesis was performed by the methods of Bonhoeffer and Schaller (2) with slight modifications. The culture was transferred to 41°C, incubated for 70 min, and further incubated for 10 h after the addition of 2 mM 5-bromouracil. The cells harvested from the culture were washed, suspended at a depth of 5 mm in PBS, and exposed to UV light for 3 min at 0°C with agitation. The UV light source was a 200-V Hg lamp at a distance of 40 cm, with short-wavelength light (<300 nm) being removed with a filter (UV-D35; Toshiba, Tokyo, Japan). The UV light-treated cells were grown in MEM at 33°C for 12 h. The cells were spread on MEM agar plates and incubated at 33°C for 5 days. Colonies that appeared on the plates were replica plated, and the replica plates were incubated at 41°C for 2 days. Colonies that grew on the master plates at 33°C but not on the replica plates at 41°C were picked (about 100 colonies). After unstable strains were discarded, about 20 stable ts mutants remained.

RESULTS

Inhibition of DNA initiation by CM. The wild-type strain was grown to the mid-log phase, incubated in the presence of 80 μg of CM per ml for various times (0 to 100 min), and then labeled with [^{32}P]dTMP for 15 min at 33°C. Purified DNA was digested with *Bam*HI and subjected to FIGE. The gel was stained with ethidium bromide (Fig. 1A) and then subjected to autoradiography (Fig. 1B). As shown in Fig. 1B, all nine fragments in lanes 1 (CM was added after incorporation was stopped) and 2 (0 min) were fully radiolabeled. Bm8 (see the legend to Fig. 5 for an explanation of fragment designations), Bm9, and Bm4 were partially labeled in Fig. 1B, lane 3, scarcely labeled in lane 4, and not labeled at all in lanes 5 to 9. Bm2 and Bm3 remained radiolabeled even after 80 min, as can be seen in Fig. 1B, lanes 8 and 9. We noted a smear of radioactive material at about the same electrophoresis distance as those of Bm8 and Bm9 on the autoradiogram. It was thought to be attributable to short DNA fragments (20 to 40 kb) produced by the shearing force of pipetting for suspending cells, whose membranes are weak in the presence of mechanical forces. The inhibition of protein synthesis by CM made the cells less resistant to mechanical forces (data not shown). We quantified the radioactivity incorporated in the *Bam*HI bands in Fig. 1B by using an image analyzer and calculated the time needed for radioactivity in a band to reach 25% of that in the corresponding band in lane 1 of Fig. 1B. The shortest time, 29.6 min, was for Bm8, and the time increased with the distance of the fragment position from Bm8, with the longest, 71.6 min, being for Bm2, which was at the farthest position from Bm8 (Fig. 2).

Relative frequencies of DNA fragments in the genome. To estimate the site of the replication origin region, we examined the relative frequencies of the DNA fragments of the genome in mid-log-phase cells of the wild-type strain. Cells grown at 33°C were further incubated with added [^{32}P]dTMP for 240 min. The cells were then embedded in agarose blocks, and the DNA was cleaved with *Bam*HI-*Xho*I-*Mlu*I as reported previously (11). The radioactivity incorporated into the restriction fragments was measured with an image analyzer. The relative frequency was calculated by dividing the radioactivity of a certain band by its DNA size and normalized to the minimal value of one arbitrary unit. The highest value (5.0) was shown by Bm8. The ratio decreased with the distance of the fragment position from Bm8, and the lowest value (1.0) was shown by the larger subfragment of Bm2 produced by *Xho*I (Fig. 3). We ensured that the incorporated radioactivity of each band in the electrophoresis gel was higher than 1.2×10^4 cpm per band. A linear correlation was confirmed to exist between the amount of sample used and the radioactivity of each band in the gel.

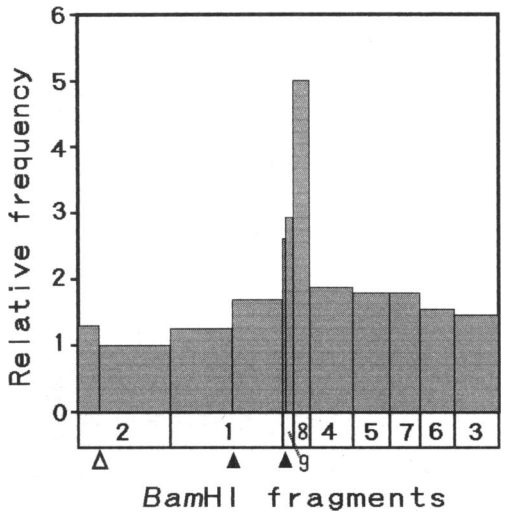

FIG. 3. Relative frequencies of *Bam*HI-*Xho*I-*Mlu*I fragments of genome DNA from log-phase cells of the wild-type strain. To a mid-log-phase culture grown at 33°C was added [^{32}P]dTMP, and incubation was continued for 240 min. The radioactivity incorporated into the restriction fragments was determined as described in the text. The relative frequency was calculated as described in the text and normalized to the minimal value of one arbitrary unit. The *Xho*I and *Mlu*I cleavage sites are shown by open and closed triangles, respectively. The *Bam*HI fragments are arranged in the same order as in the physical map (see Fig. 5).

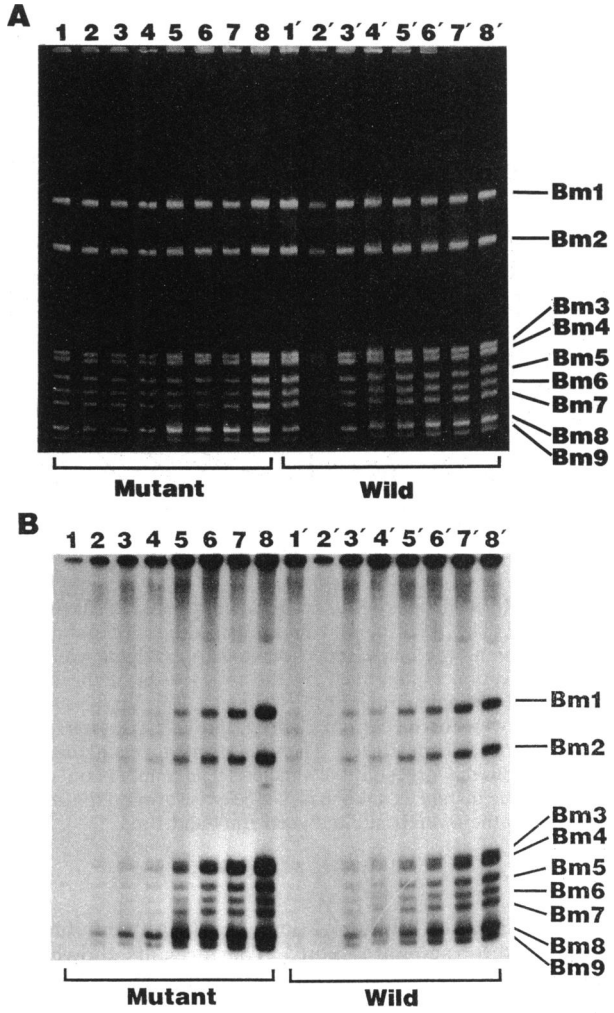

FIG. 4. Labeling of genome DNAs of the ts mutant and the wild-type strain with [^{32}P]dTMP after a temperature downshift from 41 to 33°C. Cultures grown at 33°C were incubated at 41°C for 90 min and then at 33°C with [^{32}P]dTMP for various times (0, 5, 10, 15, 20, 40, 60, and 80 min [lanes 1 to 8 for the ts mutant and lanes 1′ to 8′ for the wild-type strain, respectively]). The incorporation was stopped, and the cells were harvested. Preparation of *Bam*HI fragments and FIGE were carried out as described previously (11). (A) Ethidium bromide-stained FIGE gel. (B) Autoradiogram of the FIGE gel shown in panel A.

Measurements were made with five separate cultures, and the mean value was calculated. The standard deviations fell within 17%.

Screening and characterization of a ts mutant defective in elongation. To find a ts mutant defective in initiation among the 20 ts mutants described in Materials and Methods, we carried out the following screening. Mid-log-phase cultures grown at 33°C were incubated at 41°C for 90 min and then at 33°C with [^{32}P]dTMP for various times. DNAs from the cultures were digested with *Bam*HI and subjected to FIGE. One of the ts mutants showed an incorporation pattern that was clearly different from that of the wild-type strain (Fig. 4); i.e., in the wild-type strain, all the *Bam*HI bands were gradually radiolabeled during incubation, while in the ts mutant, intense radiolabeling of Bm8 increased for only up to 15 min. The intense radiolabeling spread to some other bands during incubation; i.e., the densities of Bm4 and Bm9 increased from 15 to 40 min of incubation (lanes 4 to 6). The order in which the bands became dense agreed with the order of the fragments in the physical map (Fig. 5).

To confirm whether the ts mutant was defective in initiation, we examined the pattern of incorporation of [^{32}P]dTMP into *Bam*HI fragments from ts mutant cells labeled at 41°C for 15 min after incubation for various times at 41°C (0, 10, 20, 30, 40, and 50 min). The incorporation of radioactivity into all nine *Bam*HI fragments occurred only at 0 min; none was observed in any *Bam*HI fragment after 10 min. These results indicated that the ts mutant was defective in elongation (data not shown).

The growth rates at 33°C of the ts mutant and the wild-type strain were almost the same throughout their growth phases. The doubling time for both strains at the mid-log phase was about 100 min.

Specific labeling of the replication origin region. Mid-log-phase cultures of the ts mutant and the wild-type strain grown at 33°C were incubated successively at 41°C for 90 min, at 33°C with added [^{32}P]dTMP for 15 min, and at 41°C for various times (0 to 45 min). Figure 6 shows the radiolabeling patterns for *Bam*HI fragments from both strains. When the ts mutant was used, preferential radiolabeling of Bm8 was observed on the electrophoresis gel at 10 min after the temperature downshift and progressed with incubation time for up to 20 min. In addition to Bm8, Bm9 was radiolabeled to some extent.

Localization of the *dnaA* gene homolog. Restriction fragments from wild-type strain genome DNA were hybridized with a probe prepared against the cloned *dnaA* gene homolog of *M. capricolum* (7). As shown in Fig. 7, of the *Bam*HI fragments, only Bm8 hybridized with the probe (lane 3), and of the *Apa*I fragments, only Ap2 did so (lane 6). Bm8 was cleaved into 12-kb (Bm8S) and 34-kb (Bm8L) fragments by

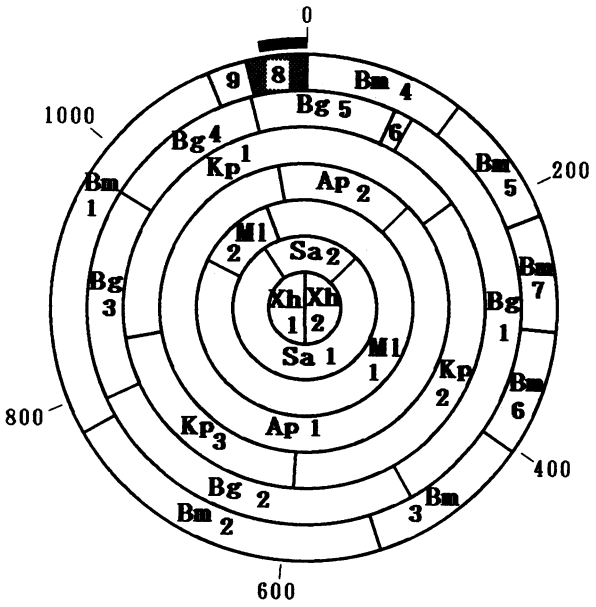

FIG. 5. Physical map of the *M. capricolum* genome. The hatched area is Bm8. The thick curved line outside the circle shows the region containing the *dnaA* gene homolog. A fragment yielded by nuclease digestion is identified by the two letters of the enzyme name and the ordinal number of the fragment. Bm, Bg, Kp, Ap, Ml, Sa, and Xh were derived from *Bam*HI, *Bgl*I, *Kpn*I, *Apa*I, *Mlu*I, *Sal*I, and *Xho*I, respectively. The fragments in each digest are numbered from the largest to the smallest. The numbers outside the circle show the distances in kilobases clockwise around the genome, starting from the *Bam*HI site between Bm4 and Bm8.

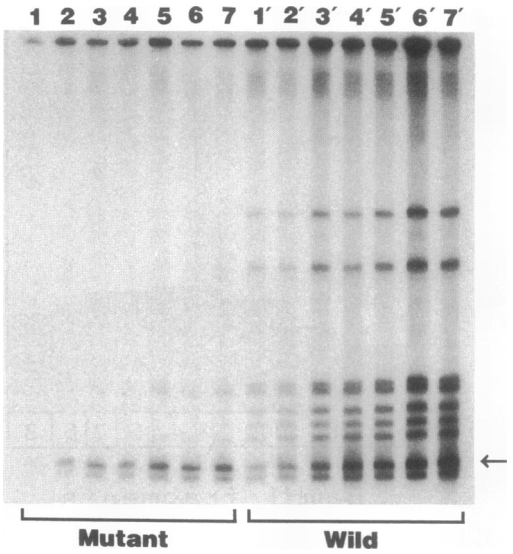

FIG. 6. Specific labeling of the replication origin. Cultures of the ts mutant and the wild-type strain grown at 33°C were incubated successively at 41°C for 90 min, at 33°C with [^{32}P]dTMP added for 15 min, and at 41°C for various times (0, 5, 10, 15, 20, 25, and 45 min [lanes 1 to 7 for the ts mutant and lanes 1′ to 7′ for the wild-type strain, respectively]). Preparation of *Bam*HI fragments and FIGE were carried out as described previously (11). The arrow indicates the position of Bm8.

additional digestion with *Apa*I (lane 2). The probe hybridized with the 34-kb fragment (Bm8L). These results showed that the *dnaA* gene homolog is present in the 34-kb region in Bm8.

DISCUSSION

In eubacteria (10, 15), the initiation of DNA replication is coupled to de novo protein synthesis, and new rounds of replication are inhibited when protein synthesis is blocked with some antibiotics. Therefore, in this study of the *M. capricolum* genome, we used CM, which is known to be harmful to mycoplasmas (19), to inhibit the initiation of new rounds of replication (Fig. 1). The cessation of radioactivity incorporation commenced in Bm8, spread via Bm5, Bm6, Bm7, and Bm1, and arrived at Bm2 (Fig. 1 and 2). These results suggest that initiation occurred at the replication origin located within Bm8, elongation proceeded bidirectionally, and termination occurred within Bm2.

For *E. coli* (1, 3) and *B. subtilis* (25), hybridization and transformation have been used in marker frequency analysis. However, these methods cannot be used to study mycoplasmas, for which no proper marker genes have been identified. Therefore, we estimated the relative fragment frequencies from the amounts of incorporated radioactivity. DNA fragments prepared by cleavage with *Bam*HI-*Xho*I-*Mlu*I were used instead of those prepared by cleavage with only *Bam*HI to determine the precise relative frequencies. Of the 12 *Bam*HI-*Xho*I-*Mlu*I fragments, Bm8 was found most frequently. The frequencies of the other fragments decreased in the order of increasing distance from Bm8 (Fig. 3), a result indicating that the initiation reaction commenced in Bm8 and that the elongation reaction proceeded bidirectionally. However, we found that the relative frequency of Bm8 was as high as 5.0 and that a steep decline in frequencies was seen on both sides of Bm8. These data suggest that the chromosomes in the log-phase cells possessed multiple forks that were concentrated around Bm8 and arrested for a certain time on both sides of Bm8. In this experiment, we took all possible care to obtain the correct relative frequencies; that is, we conducted the experiment under conditions yielding a linear correlation between the amounts of samples

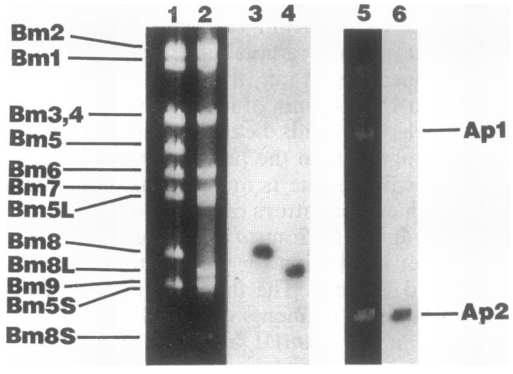

FIG. 7. Southern hybridization analysis of genome DNA with a *dnaA* gene homolog probe. Genome DNA of the wild-type strain was digested with *Bam*HI (lanes 1 and 3), *Bam*HI-*Apa*I (lanes 2 and 4), and *Apa*I (lanes 5 and 6) and then subjected to FIGE. Ethidium bromide-stained gels are shown in lanes 1, 2, and 5. Autoradiograms of the blotted membranes are shown in lanes 3, 4, and 6. The pulse time for FIGE varied linearly from 1 to 6 s in lanes 1 and 2 and from 3 to 20 s in lane 5.

applied and the radioactivities of their bands. However, since the relative frequencies were obtained by dividing the radioactivity by the size of the corresponding fragment, it is undeniable that there was a tendency to magnify the difference in the ratios calculated. This problem may be solved by detailed marker frequency analysis with cloned DNA fragments. Although the problem of the high relative frequency of Bm8 remained unsolved, the ordered arrangement of the relative frequencies shown in Fig. 3 suggests that Bm8 contains the replication origin and that replication proceeds bidirectionally.

When the elongation-defective ts mutant was transferred from 41 to 33°C, concentrated DNA synthesis commenced in Bm8 and spread to other regions; in addition, some synthesis was observed in each *Bam*HI fragment from the beginning (Fig. 4A). These results can be interpreted as follows. The elongation reaction was interrupted at various points in nonsynchronized cells by the temperature upshift from 33 to 41°C. However, the initiation reaction, which was not interrupted at 41°C, occurred while the cells were incubated for 90 min, without transfer to the elongation reaction. When the cells were returned to 33°C, they commenced new DNA synthesis from the origin and resumed the elongation reaction at the respective replication point.

Since the ts mutant was defective in elongation, DNA synthesis could be commenced or stopped by a temperature downshift or upshift, respectively. We carried out pulse-labeling of a fragment containing the putative origin. The radiolabeling was almost totally restricted to Bm8, but slight radiolabeling of Bm9 was also observed (Fig. 5). The gradual and slow increase in the radiolabeling of Bm8 observed in lanes 1 to 4 suggested that the time needed for the recovery of elongation activity after the temperature downshift varied among the cells. The fact that the elongation reaction continued almost exclusively in Bm8 for up to 20 min at 41°C suggested that a certain time was needed for the factor involved in elongation to be converted to the inactive form. Despite the time lags occurring in the cessation and resumption of DNA replication, as stated above, it is not unreasonable to infer that the replication origin lies in Bm8.

In the course of isolating ts mutants, we used an enriching method based on the fact that bacteria that have incorporated 5-bromouracil into their DNAs become very sensitive to UV light (2). *M. capricolum* cells took up 5-bromouracil well and became 15 to 20 times more sensitive (data not shown). The efficiency of this enrichment, estimated from replica plating, was about 10-fold.

We assume that the ts mutant undergoes a mutation in some reactions involving the ligation of Okazaki fragments (16) for two reasons. First, although the new incorporation of [^{32}P]dTMP into the genome (namely, into restriction fragments) ceased at about 10 min after the temperature upshift from 33 to 41°C, the incorporation of the radioactivity into the trichloroacetic acid-insoluble fraction continued for 90 min (data not shown). Second, in cells grown with [^{32}P]dTMP at 33°C, the accumulation of radiolabeled DNA fragments, which were 200 to 300 bases or bp long, was detected by electrophoresis with acrylamide gels (data not shown).

A homolog of an *E. coli dnaA* gene has been cloned from *M. capricolum*, and some *dnaA* box-like motifs have been found in the vicinity of the *dnaA* structural gene by Fujita et al. (7). Our demonstration of the occurrence of the *dnaA* gene homolog in the 34-kb region in Bm8 supports the possibility that the region contains the replication origin.

All the experiments carried out in this study indicate that the replication origin of *M. capricolum* is located in Bm8. Further studies are being conducted to determine the precise location of the replication origin.

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