

mioC Transcription, Initiation of Replication, and the Eclipse in *Escherichia coli*

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Received 21 December 1995/Accepted 3 April 1996

The potential role of *mioC* transcription as a negative regulator of initiation of chromosome replication in *Escherichia coli* was evaluated. When initiation was aligned by a shift of *dnaC2*(Ts) mutants to nonpermissive temperature (40°C), *mioC* transcript levels measured at the 5' end or reading through *oriC* disappeared within one mass doubling. Upon return to permissive temperature (30°C), the transcripts reappeared coordinately about 15 min after the first synchronized initiation and then declined sharply again 10 min later, just before the second initiation. Although these observations were consistent with the idea that *mioC* transcription might have to be terminated prior to initiation, it was found that the interval between initiations at permissive temperature, i.e., the eclipse period, was not influenced by the time required to shut down *mioC* transcription, since the eclipse was the same for chromosomes and minichromosomes which lacked *mioC* transcription. This finding did not, in itself, rule out the possibility that *mioC* transcription must be terminated prior to initiation of replication, since it might normally be shut off before initiation, and never be limiting, even during the eclipse. Therefore, experiments were performed to determine whether the continued presence of *mioC* transcription during the process of initiation altered the timing of initiation. It was found that minichromosomes possessing a deletion in the DnaA box upstream of the promoter transcribed *mioC* continuously and replicated with the same timing as those that either shut down expression prior to initiation or lacked expression entirely. It was further shown that *mioC* transcription was present throughout the induction of initiation by addition of chloramphenicol to a *dnaA5*(Ts) mutant growing at a semipermissive temperature. Thus, transcription through *oriC* emanating from the *mioC* gene promoter is normally inhibited prior to initiation of replication by the binding of DnaA protein, but replication can initiate with the proper timing even when transcription is not shut down; i.e., *mioC* does not serve as a negative regulator of initiation. It is proposed, however, that the reappearance and subsequent disappearance of *mioC* transcription during a 10-min interval at the end of the eclipse serves as an index of the minimum time required for the establishment of active protein-DNA complexes at the DnaA boxes in the fully methylated origin region of the chromosome. On this basis, the eclipse constitutes the time for methylation of the newly formed DNA strands (15 to 20 min at 30°C) followed by the time for DnaA protein to bind and activate *oriC* for replication (10 min).

Initiation of chromosome replication in *Escherichia coli* involves the interplay of several molecular interactions at the origin of replication, *oriC*. One of the essential protein components of the interaction is DnaA, which associates in its active ATP-bound form with four 9-bp recognition sequences within *oriC* (reviewed in reference 43). The level of DnaA activity appears to be limiting for initiation under normal steady-state growth conditions (23), and this activity level is apparently established by a combination of the abundance of the protein and its interactions with several negative modulators (9, 11, 16, 17, 25). There are also histone-like proteins that associate with *oriC* and that may be involved in initiation, such as Hu, Fis, and IHF (38), which affect the conformation of the region. The latter two bind to *oriC* in a cell cycle-dependent manner (7). There is at least one protein, IciA, that can act directly at *oriC* to inhibit initiation of replication (14). Thus, setting the stage for initiation during steady-state growth necessitates, at the very least, the appearance of the pertinent positive elements and release of any inhibitors in an appropriate sequence.

There is also transcription in the vicinity of *oriC* that seems to participate in this positive-negative interplay during initia-

tion. The *gidA* gene, located to the left of *oriC*, is transcribed leftward away from *oriC* and plays a positive role in initiation (1, 2, 18, 32). The *gidA* gene activates replication from *oriC* on plasmids, possibly through production of negative superhelicity behind the polymerase to facilitate unwinding of the duplex in preparation for initiation (1, 2). The *mioC* gene, located on the right side of *oriC*, is also transcribed leftward, with a significant fraction of the transcripts entering and passing through *oriC* (15, 31, 37, 40). It has recently been shown that transcription of *mioC* is shut down prior to initiation (33, 47), consistent with suggestions that transcription entering *oriC* from the right inhibits initiation (24, 46) and must be shut off before initiation can take place. This potential negative role for *mioC* is not at odds with the finding that chromosomes and minichromosomes lacking *mioC* transcription initiate replication with the same cell cycle timing as those that contain the transcripts (20, 22), since *mioC* transcription may normally be inhibited before, or coincident with, the actual start of replication at *oriC* (47).

On the basis of the preceding analysis, the potential negative role of *mioC* transcription in initiation might only be detectable under conditions in which the activators for initiation are not limiting, such as during the eclipse period. The eclipse period is the time after initiation when *oriC* is refractory to further initiation, even when all activators, such as DnaA, are present in excess. For instance, when a thermoreversible temperature-sensitive initiation-defective mutant is held at non-

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permissive temperature for at least two mass doubling times and then returned to permissive temperature (e.g., 30°C), the first two rounds of replication are initiated 25 to 30 min apart, which defines the eclipse (8, 10, 13, 41). The duration of this interval between the first and second initiation events is unaffected by inhibition of protein synthesis, so all proteins required for initiation are present and the delay must therefore be related to alternative requirements for initiation. The period of hemimethylation of adenine in GATC sequences in *oriC* can account for 10 to 20 min of the eclipse (5, 33, 35, 47), depending on the temperature, since the origin is refractory to initiation of replication when it is hemimethylated and presumably sequestered immediately after it replicates (4, 5, 9, 25, 28–30, 35, 39, 44), and increased *dam* methylase shortens the eclipse (29). However, hemimethylation cannot account fully for the 25- to 30-min eclipse at 30°C. Our data indicate that transcription of *mioC* resumes about 15 min into the eclipse and then ceases again before the end of the eclipse (47). Thus, the eclipse period could consist of the sum of the time for *oriC* to become fully methylated and the time for *mioC* transcription to be halted. The purpose of this study was to examine whether *mioC* transcription contributes to the eclipse or behaves in any way as a negative regulator of initiation of replication.

MATERIALS AND METHODS

Bacteria, growth conditions, and radioactive labeling. The strains employed were *E. coli* K-12 PC2 (*dnaC2 thyA47 leu-6 deoC3 Str^r*) and K-12 PC5 (*dnaA5 thyA47 leu-6 deoC3 Str^r*) (6) and K-12 ALO659 (*mioC54 leu-6 thi-1*) and K-12 LJ24 (*leu-6 thi-1*) (22). The *dnaC2* allele was introduced into strains ALO659 and LJ24 by P1 transduction. The minichromosomes were pAL4 (Δ *mioC*) and pAL49 (*mioC⁺*) (20) and pTOA27 (*pmioC* Δ DnaA box) (34). Cultures were grown in minimal salts medium (13) supplemented with 0.1% glucose plus 0.2% Casamino Acids (Difco Laboratories). When necessary, thymine or thiamine was added at 10 μ g/ml. For temperature shift experiments with *dnaC2* mutants, the cultures were grown for three to five doublings at 30°C (measured by A_{450} or A_{600}), shifted to 40°C for approximately two doublings (60 or 90 min, depending on the strain), and then returned to 30°C (13). For analysis of *E. coli* K-12 PC5 *dnaA5* cells grown at intermediate temperature, the cultures were incubated at 33°C until a concentration of approximately 2×10^8 cells per ml was reached, at which point chloramphenicol was added to a final concentration of 200 μ g/ml. For measurement of DNA replication, 0.5-ml samples were removed from the cultures and placed in a test tube in a shaking water bath at the growth temperature along with 5.0 μ Ci of [³H]thymidine (80 Ci/mM; Amersham Life Sciences) for 5 min, and uptake was determined as described previously (47). Bacterial concentrations were determined with a model ZB Coulter electronic particle counter. Absorbances of the cultures were measured with a Milton Roy Spectronic 601 spectrophotometer.

Quantitative S1 nuclease transcription assays. Isolation of total cellular RNA and the S1 nuclease protection assays were performed as described in detail previously (47). The probes used in the S1 nuclease assay were synthetic oligonucleotides, purchased from Keystone Scientific (Menlo Park, Calif.) or Oligos Etc. (Wilsonville, Oreg.), which complemented the transcripts of interest. Crude oligonucleotides were purified by polyacrylamide gel electrophoresis on an 8% acrylamide–9 M urea gel. The probes were designed so that S1-protected fragments after hybridization to total cellular RNA would be a minimum of 10 nucleotides shorter than the undigested probe to prevent interference in the subsequent analysis. This was achieved by overlapping the transcriptional start site and/or by tailing the 3' end of the probe with a mismatch to the transcript. The probes for *gidA*, *mioC*, *dnaA*, and *rpoA* transcripts have all been described previously (47). They were 5' end labeled with polynucleotide kinase (U.S. Biochemicals) and [γ -³²P]ATP (>3,000 Ci/mM; NEN). To quantitate transcripts, 10 μ g of total cellular RNA was hybridized with 0.3 to 0.6 ng of 5' ³²P-end-labeled oligonucleotide (300,000 to 500,000 cpm). In some experiments, the assay also contained a probe complementary to the transcripts from the *rpoA* gene as a control for a gene whose transcription does not fluctuate in the cell cycle (47). After S1 digestion, the protected fragments were electrophoresed on an 8% denaturing polyacrylamide gel. Since the lengths of the complementary sequences of the probes differed, ranging from 35 (*rpoA*) to 65 (*mioC*) nucleotides, the protected fragments migrated to different positions on the gels. Densitometric quantitation of the S1 nuclease assay was conducted by scanning of the autoradiographs into an Apple Macintosh IIfx computer with a Microtek flat-bed scanner with Adobe photoshop and quantitation with NIH Image 1.52. Because of the limited linear response range of the film and the variation in intensity of the signals produced by the different probes used simultaneously in the S1

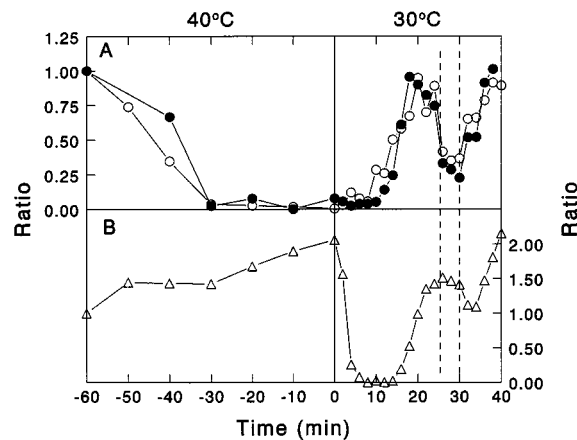


FIG. 1. Transcript levels of *mioC* and *gidA* during a temperature shift of PC2 *dnaC2*. PC2 *dnaC2* cells growing exponentially at 30°C in glucose-Casamino Acids minimal medium were shifted to 40°C for 60 min and then returned to 30°C. At intervals, 10 μ g of total cellular RNA was isolated, hybridized to ³²P-labeled oligonucleotide probes, and assayed by S1 protection. The resultant autoradiograms were quantitated by densitometry, and the intensities of the signals are expressed as ratios to the intensity of the *rpoA* protected signal, normalized to 1.0 at the shift to 40°C (–60 min). (A) Relative transcript levels of *mioC* at the 5' end (○) and reading through *oriC* (●). (B) Relative transcript levels of *gidA* (△). The *gidA* and *mioC* read-through transcripts were measured with the same probe in the same experiment. The interrupted vertical lines indicate the approximate average time of the second synchronized initiation of replication after the return to 30°C (Fig. 2).

transcription assays, multiple exposures of most autoradiograms were scanned to allow accurate quantitation.

Minichromosome replication. For measurement of minichromosome replication, 1.0-ml samples were removed at intervals during the course of the temperature shift experiments and pulse-labeled with 10 μ Ci of [³H]thymidine per ml for 3 min. After labeling, 100 μ l of each of the 1.0-ml samples was placed in 5.0 ml of 10% ice-cold trichloroacetic acid for use in measurement of [³H]thymidine uptake into chromosomal DNA, while the remainder of each sample was quickly chilled in an ice bath. For analysis of radioactivity incorporated into the plasmid DNA, whole-cell lysates were prepared as described by Projan et al. (36), except that lysozyme was used at 25 μ g/ml to lyse the cells (20). The samples were electrophoresed in a 1% agarose slab gel (FMC Bioproducts, Rockland, Maine) at 75 V for 18 to 20 h in Tris-borate-EDTA buffer (20). Gels were prepared for fluorography, dried, and exposed to Kodak X-Omat AR X-ray film at –70°C for 14 to 25 days. Densitometric quantitations were performed as described above. Quantitation of plasmid DNA contents was also performed in the same manner but on negative photographs of ethidium bromide-stained agarose gels. Relative plasmid copy numbers were estimated by correcting the ratios of band densities for plasmid size differences.

RESULTS

Transcription of *mioC* and *gidA* after alignment of initiation of chromosome replication in *E. coli* PC2 *dnaC2*(Ts). The potential involvement of *mioC* gene transcription in the duration of the eclipse period was evaluated initially by correlating transcript levels with initiation timing during temperature shifts of *E. coli* PC2 *dnaC2*(Ts) mutants. Exponentially growing cultures were shifted from 30°C (permissive temperature) to 40°C (nonpermissive temperature) for 60 min and then back to 30°C, and samples were taken at intervals. The transcript levels were quantitated on the basis of protection of ³²P-labeled complementary oligonucleotides from digestion by nuclease S1 after hybridization with RNA extracted from the samples. As seen in Fig. 1A and previously (47), *mioC* transcripts measured at either the 5' end or reading through *oriC* disappeared by 30 min at 40°C. They reappeared after the shift to the permissive temperature and then decreased and increased a second time. The transcript levels fluctuated with the same timing whether measured before entry into or upon exit

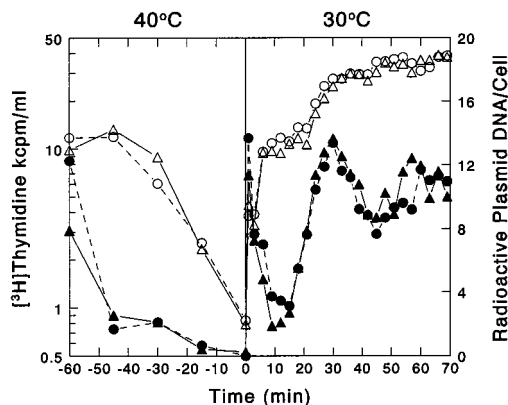


FIG. 2. Chromosome and minichromosome replication during a temperature shift of PC2 *dnaC2*. The cultures were pulse-labeled with [³H]thymidine for 3 min at the indicated times. For analysis of minichromosome replication, whole-cell lysates were prepared, and radioactive plasmid DNA was isolated by agarose gel electrophoresis and quantitated by densitometry of the resultant fluorograms. Δ and \blacktriangle , [³H]thymidine incorporated into total DNA and minichromosome DNA, respectively, in PC2(pAL49). \circ and \bullet , [³H]thymidine incorporated into total DNA and minichromosome DNA, respectively, in PC2(pAL4).

from *oriC*. The decrease in *mioC* transcripts between 20 and 30 min after the return to 30°C indicates that *mioC* transcription was shut down before initiation of the second round of replication at 25 to 30 min. On the other hand, *gidA* transcripts decreased abruptly after the shift back to the permissive temperature, before reappearing again to reach a maximum when *mioC* transcript levels were at a minimum (Fig. 1B). A reasonable inference from these data is that *mioC* transcription must be shut off prior to the second initiation and thus that the eclipse might be shortened in the absence of this transcription.

Initiation from *oriC* after alignment of chromosome replication in *E. coli dnaC2*(Ts) in the presence and absence of *mioC* transcription. As a first test of the possibility that *mioC* transcription influences eclipse duration, the timing of replication of minichromosomes which possess (pAL49) or lack (pAL4) resident *mioC* gene transcription was assayed in temperature shift experiments with PC2. During the course of the shift, samples were pulse-labeled with [³H]thymidine, and then uptake into both total DNA and minichromosome DNA was measured. After the shift back to 30°C, pAL4 and pAL49 replicated in bursts, with maxima immediately after the shift and at 30 min, which were coincident with each other and with the midpoints of the steps in total [³H]thymidine uptake (Fig. 2). Since the peaks in minichromosome replication and the steps in [³H]thymidine uptake represent the average times for initiation of replication, the results indicate that chromosomal and minichromosomal eclipse periods were indistinguishable and that the eclipse for minichromosomes was unaffected by *mioC* transcription. It is possible, however, that the timing of minichromosome replication could be controlled by the chromosome, irrespective of the status of *mioC* gene expression on the plasmid. Accordingly, the potential involvement of *mioC* transcription in the length of the eclipse period was further investigated by comparing the duration of the eclipse in ALO659 *dnaC2*, which possesses a deletion in the *mioC* promoter (22), with that in its isogenic *mioC*⁺ parent, LJ24, in the same temperature shift protocol. *mioC* transcripts (or any transcripts emanating from the upstream *asnC* promoter [18]) were not detectable in ALO659 *dnaC2*, but *gidA* transcript levels fluctuated in the same manner in LJ24 and ALO659 (Fig. 3A and B). Furthermore, *mioC* levels in LJ24 and *dnaA* levels in

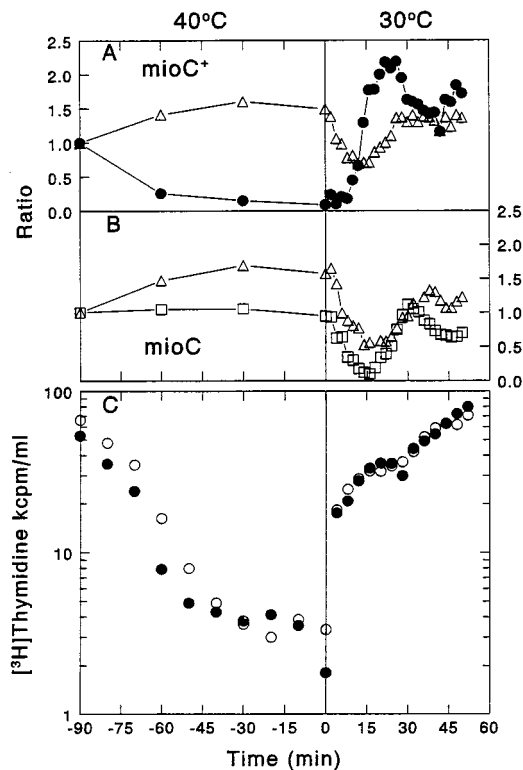


FIG. 3. Transcription and replication during temperature shifts of *dnaC2* mutants with and without a functional *mioC* promoter. LJ24 *dnaC2* or ALO659 *dnaC2* cells growing exponentially at 30°C in glucose-Casamino Acids minimal medium were shifted to 40°C for 90 min and then returned to 30°C. (A) *gidA/rpoA* (Δ) and *mioC* read-through/*rpoA* (\bullet) transcript ratios in LJ24 *dnaC2*. (B) *gidA/rpoA* (Δ) and *dnaA* (\square) transcript ratios in ALO659 *dnaC2*. (C) [³H]thymidine incorporated into LJ24 *dnaC2* (\circ) and ALO659 *dnaC2* (\bullet) cells which had been pulse-labeled for 3 min at the times indicated.

ALO659 fluctuated as in PC2 (Fig. 1 and reference 47), except that they were slightly less pronounced. The similarity of the fluctuations in the transcript levels in the three strains, as well as the [³H]thymidine uptake measurements (Fig. 3C), confirms that the eclipse was unaffected by the presence or absence of transcription from *mioC*.

Initiation of replication from *oriC* during continuous *mioC* transcription. In spite of the finding that *mioC* transcription did not influence the duration of the eclipse, it is evident that transcription from this gene is nonetheless inhibited prior to initiation of replication. It remains conceivable that *mioC* transcription must always be inhibited prior to initiation of replication but that completion of the eclipse is set by another process that takes the same time or longer to finish than *mioC* transcript shutdown. If that were the case, total absence of *mioC* transcription would not be anticipated to shorten the eclipse, but prolonged expression would be expected to lengthen it. Accordingly, experiments were performed to determine the effects of continual *mioC* transcription on initiation of replication from *oriC*.

The first set of experiments employed minichromosome pTOA27, which contains a deletion in the DnaA box upstream of the *mioC* promoter (34). Since transcription of the gene is under negative control by DnaA, it would be anticipated that the level of expression would be higher on this plasmid. Furthermore, if the sharp changes in expression from the normal gene and its shutoff prior to initiation were indeed consequences of DnaA protein binding, then these characteristics

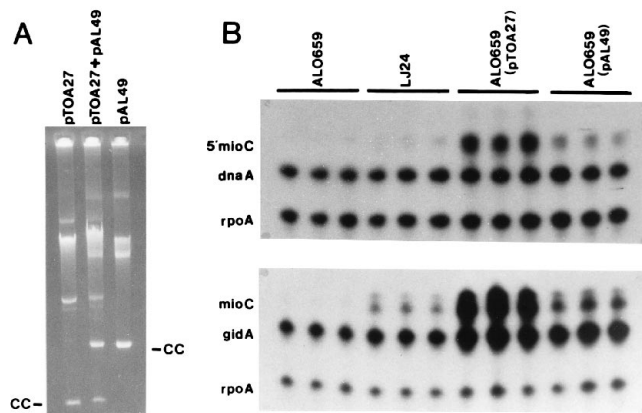


FIG. 4. Comparative plasmid DNA content and transcript levels in ALO659 *dnaC2* containing pTOA27 or pAL49. (A) Cultures of ALO659 *dnaC2* cells containing pAL49 or pTOA27 were grown to an A_{600} of 0.65, and the minichromosome DNA from 40-ml samples was electrophoresed in 0.7% agarose and stained with ethidium bromide. Lanes 1 and 3 show DNA isolated from the individual cultures containing either pAL49 or pTOA27. Lane 2 shows DNA from a sample consisting of a mixture of 20 ml from each of the two cultures. CC designates the closed circular monomer forms of the plasmids. (B) Cultures of LJ24 and ALO659 cells (with and without pTOA27 or pAL49, respectively) were grown exponentially to an A_{600} of 0.65. Total RNA was extracted and assayed by S1 nuclease protection in triplicate for transcript levels of *mioC* at the 5' end, *dnaA* and *rpoA* (upper gel) and transcript levels of *mioC* reading through *oriC*, *gidA*, and *rpoA* (lower gel).

should be absent during replication of this minichromosome. Figure 4 shows a comparison of the relative quantities of plasmid DNA and *mioC* transcripts in identical samples taken from exponential-phase cultures of ALO659 harboring pTOA27 or pAL49. Quantitation of the film indicates that the copy number of pTOA27 was twofold lower than that of pAL49 (Fig. 4A), whereas the *mioC* transcript level was fivefold higher (Fig. 4B). This is consistent with the absence of a negative regulatory control for *mioC* transcription in pTOA27.

The relationship between *mioC* transcription and replication of pTOA27 was determined by again aligning chromosome replication with temperature shifts. As seen in Fig. 5A, *mioC* transcription continued essentially unaltered at 40°C in ALO659 *dnaC2* containing pTOA27 in which the only transcription

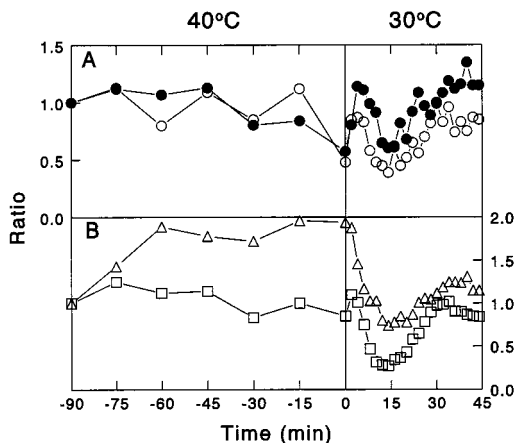


FIG. 5. Transcript levels of *mioC*, *gidA*, and *dnaA* during a temperature shift of ALO659 *dnaC2* cells containing pTOA27. (A) Ratios of transcript levels of *mioC* at the 5' end (○) and reading through *oriC* (●) to *rpoA*. (B) Ratios of transcript levels of *gidA* (△) and *dnaA* (□) to *rpoA*.

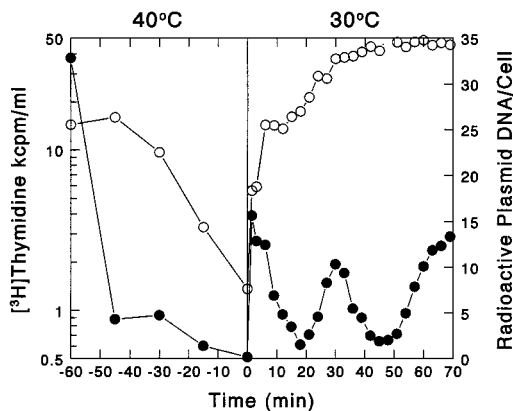


FIG. 6. Chromosome and minichromosome replication during a temperature shift of PC2 *dnaC2* cells containing pTOA27. The experiment was performed as described in the legend to Fig. 2. ○, ^3H in total DNA; ●, ^3H in pTOA27 DNA.

from *mioC* must initiate from plasmid-borne copies. The decrease in transcripts shortly after the shift to 30°C, with a minimum at about 15 min, is presumed to be due to hemimethylation and/or sequestration of the region, since the pattern mimicked the results for *gidA* and *dnaA* transcripts which fluctuated normally (Fig. 5B). Figure 6 shows that minichromosome pTOA27 replicated in bursts upon the return to 30°C, similar to that shown in Fig. 4 for pAL4 and pAL49, in spite of the continued presence of *mioC* transcription before and during initiation of replication. The same results were observed in ALO659 *dnaC2* (data not shown). Thus, the results show that a functional DnaA box was required for repression of *mioC* transcription prior to initiation of replication, but that the minichromosome pTOA27 replicated with normal timing in the absence of this repression.

Transcription from *mioC* during induction of initiation in a *dnaA5* mutant at intermediate temperature. As a final test of the involvement of *mioC* transcription in initiation, transcription and initiation in a *dnaA*(Ts) mutant induced to initiate replication by addition of chloramphenicol to a culture growing at an intermediate temperature were compared. It has been shown that addition of chloramphenicol to temperature-sensitive *dnaA* mutants grown at a temperature intermediate between permissive and nonpermissive causes an abrupt stimulation in initiation of chromosome replication (12, 19, 26, 48). Almost immediately after addition of this inhibitor of protein synthesis, RNA polymerase is redistributed to stable RNA promoters (42) and could result in the increased availability of RNA polymerase to function at promoters in the origin region, including *mioC*. To determine whether this was the case, chloramphenicol was added to a culture of PC5 *dnaA5* cells grown at 33°C, and the effects on transcription were assayed. Upon induction of initiation by the inhibitor, there was an abrupt increase, not decrease, in *mioC* transcription (Fig. 7), again consistent with initiation in the presence of *mioC* transcription.

DISCUSSION

Proposals for the possible involvement of *mioC* in initiation control have been ubiquitous. Its presence increases minichromosome copy number and stability (21, 34, 45) and stimulates minichromosome replication when *gidA* is absent (32), but it is unlikely to have a role as an activator under normal growth conditions, because both minichromosomes and chromosomes initiate replication normally when *mioC* transcription is absent

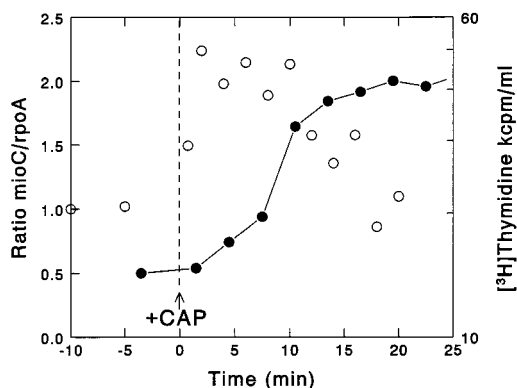


FIG. 7. [³H]thymidine incorporation and *mioC* transcript levels after addition of chloramphenicol to PC5 *dnaA5* cells growing at 33°C. Chloramphenicol, at a final concentration of 200 μg/ml, was added to a culture growing at 33°C. At the times indicated, the culture was pulse-labeled with [³H]thymidine for 3 min, and a 10-ml aliquot was removed for measurement of *mioC* transcript levels reading through *oriC* ●, ³H in total DNA; ○, *mioC/rpoA* transcript ratio.

(20, 22). The most likely role had been considered to be that of a negative effector. This conclusion was initially based on indirect evidence that high-level leftward transcription into *oriC* from a *lac* promoter inhibits minichromosome replication (46), that transcription into *oriC* interferes with replication in vitro (24), and that *mioC* transcription is normally shut down just prior to initiation of replication (33, 47). Although this inhibitory effect must not limit initiation during steady-state growth, for the reasons indicated above, it could come into play when a surfeit of activators were present, such as during the eclipse. As shown in this paper and previously (47), transcription in the vicinity of *oriC* and in the *dnaA* gene resumes about 10 min before the end of the eclipse, presumably because of completion of methylation and release of sequestration. These observations, plus the observation that *mioC* transcription shuts down again at the end of the eclipse, led to the idea that the eclipse could constitute the time to methylate *oriC* plus the time to shut *mioC* transcription off again once it resumed (47).

In this study, we tested this potential negative role of *mioC* in the eclipse and in initiation of chromosome replication in general. It was found that the eclipse period was not shortened for chromosome or minichromosome replication when *mioC* transcription was absent. It was further shown that a minichromosome which contains a deletion in the DnaA box upstream of the *mioC* promoter, pTOA27, did not cease *mioC* transcription prior to initiation but still replicated with normal timing. Thus, the striking cycle-dependent periodic repression of *mioC* transcription passing through *oriC* is a consequence of the binding of DnaA protein to the DnaA box upstream of the -35 sequence in the *mioC* promoter, but the repression is not necessary for initiation or normal initiation timing. However, the results do not rule out the possibility that *mioC* transcription could affect either the proportion of minichromosomes that initiate replication each cycle or minichromosome stability in light of the lower copy number of pTOA27.

In further support of this conclusion, *mioC* transcription was enhanced rather than inhibited when initiation of chromosome replication was induced with chloramphenicol in a *dnaA5* mutant growing at a semipermissive temperature. One of the explanations for the chloramphenicol-induced initiation of chromosome replication in *dnaA*(Ts) mutants grown at a semipermissive temperature is increased transcription in the *oriC* region (19, 26, 49). It has since been shown that transcriptional activation increases minichromosome replication in vitro (3) and that

transcription from the *gidA* promoter to the left of *oriC* enhances minichromosome replication and copy number (1, 2, 18, 32). Since *gidA* transcription was also elevated upon addition of chloramphenicol and initiation was induced in mutants lacking *mioC* transcription (data not shown), *gidA* transcription could be responsible for induced initiation. Nevertheless, it is evident from the results that initiation can take place in the presence of *mioC* transcription.

We conclude that the 10-min period of *mioC* transcription at the end of the eclipse is a manifestation of the cause of the delay in initiation rather than the cause itself. This interval constitutes the lag between methylation of the region, presumably required for optimal *mioC* promoter activity (41), and the formation of the complex between DnaA and its box in the *mioC* promoter region suitable for the shutoff of *mioC* transcription. When viewed in this way, the sequence of events in the eclipse is as follows. Upon a shift to 30°C, replication of the origin region and the consequent production of a hemimethylated and sequestered state result in the simultaneous removal (or inactivation) of DnaA protein bound to all DnaA boxes in the region (11, 27) and repression of transcription, including continued repression of *mioC*. Upon completion of methylation in 15 to 20 min, DnaA, which is present in excess throughout the eclipse, reassociates in active form with the boxes. Since 10 min are required postmethylation to shut down *mioC* transcription, it is proposed that this is also the time interval required to assemble active protein-DNA complexes at the DnaA boxes in *oriC* to produce the origin conformation required for initiation. Thus, the last portion of the eclipse is not the time required to shut off *mioC* transcription specifically but rather the minimum time for construction of functional DnaA-DNA complexes.

ACKNOWLEDGMENTS

We thank Eric Boye for supplying the strains ALO659 and LJ24 and Toshio Ogawa for the plasmid pTOA27. We also thank Ping Zhou for excellent technical assistance.

This work was supported by Public Health Service grant GM-26429 from the National Institute of General Medical Sciences.

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