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The absence of effect of *gid* or *mioC* transcription on the initiation of chromosomal replication in *Escherichia coli*

(DNA replication/*oriC*/transcriptional activation)

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ABSTRACT **Despite the widely accepted view that transcription of *gid* and *mioC* is required for efficient initiation of cloned *oriC*, we show that these transcriptions have very little effect on initiation of chromosome replication at wild-type chromosomal *oriC*. Furthermore, neither *gid* nor *mioC* transcription is required in cells deficient in the histone-like proteins Fis or IHF. However, *oriC* that is sufficiently impaired for initiation by deletion of DnaA box R4 requires transcription of at least one of these genes. We conclude that transcription of *mioC* and especially *gid* is needed to activate *oriC* only under suboptimal conditions. We suggest that either the rifampicin-sensitive step of initiation is some other transcription occurring from promoter(s) within *oriC*, or the original inference of transcriptional activation derived from the rifampicin experiments is incorrect.**

Initiation of replication of the *Escherichia coli* chromosome occurs at a unique site, *oriC*. Initiation depends primarily on DnaA protein, which binds to five 9-bp repeats within *oriC* and facilitates duplex melting at three A+T-rich 13-mers (see Fig. 1). DnaB helicase subsequently enters the opened duplex and leads to priming and chain elongation reactions resulting in bidirectional replication. *oriC* is a complex regulatory region containing recognition sites for many positive and negative regulatory proteins, including Fis, IHF, SeqA, and IciA, some or all of which are thought to help precisely regulate initiations occurring at *oriC* (see ref. 1 for review).

Early physiology experiments by Lark (2) and Messer (3) first suggested that RNA polymerase (RNAP) is somehow involved in initiation at *oriC*. This was inferred from findings that rifampicin (an inhibitor of RNAP) inhibits a new round of DNA synthesis at a time when protein synthesis is no longer required. Genetic evidence as well suggests a role of RNAP in initiation. Mutations in *rpoB* and *rpoC*, which encode the β and β' subunits of RNAP, respectively, have been shown to increase copy numbers of both the chromosome and chimeric plasmids (*oriC* plasmids) carrying both an *oriC* site and a ColE1-type replication origin (4, 5). Further, specific *rpoB* mutations have been shown to suppress the temperature sensitivity phenotype of certain *dnaA*(Ts) mutations (6). Despite the evidence suggesting an involvement of RNAP, a specific transcription event required for initiation has not been identified.

Of promoters possibly involved in transcriptional activation of initiation, one likely candidate is the promoter of *gidAB*. The *gid* promoter (P_{gid}) is situated just counterclockwise of *oriC* (Fig. 1) and transcription proceeds leftward away from *oriC*. *oriC* plasmids in which the *gid* promoter has been inactivated

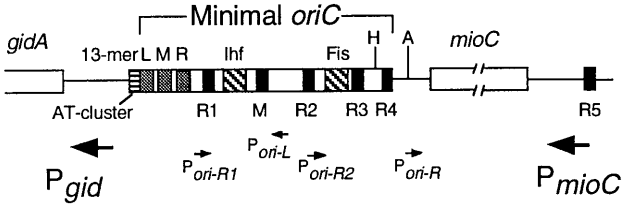

FIG. 1. The minimal *oriC* and surrounding transcription. The position of the six DnaA boxes R1–R5 and M; 13-mer repeats L, M, and R; A+T-rich cluster; and binding sites for IHF and Fis proteins are indicated. Large arrows represent location and direction of major promoters near *oriC*. Small arrows represent weaker promoters within *oriC*. H, *Hin*dIII (+244); A, *Acc*I (+285) (ref. 1 and references therein).

exhibit decreased transformation efficiency and stability as well as decreased replication *in vitro* (7, 8). The twin-domain model of Liu and Wang (9) predicts that an actively transcribing RNAP generates local domains of increased negative supercoiling behind it and positive supercoiling in front of it. It has been postulated that transcription from P_{gid} facilitates duplex opening in the 13-mer region by increasing the local negative supercoiling. This idea is supported by the findings that *gid* or *kan* transcription stimulates initiation of an *oriC* plasmid only when transcription is oriented away from *oriC* (7) and that P_{lac} transcription entering *oriC* is inhibitory (10). In an alternative model, helix destabilization may be propagated from an R-loop formed in the vicinity of *oriC*, to the 13-mer region, thus activating initiation (11, 12). Also implicated in initiation control is transcription originating from the *mioC* promoter (P_{mioC}). *mioC* is located clockwise of *oriC* (Fig. 1), and transcription proceeds leftward either reading through or occasionally terminating within *oriC* (13). Mutation of the *mioC* promoter decreases copy number and stability of minichromosomes (14, 15).

A remarkable feature of P_{mioC} and P_{gid} is their periodicity within the cell cycle (16, 17). It was found that just prior to initiation *gid* transcription peaks and *mioC* transcription is shut off, whereas just after initiation *mioC* inhibition is relieved and *gid* transcription declines. These findings support the idea that *gid* transcription is activating and *mioC* transcription is inhibitory. On the other hand, it is not clear whether these promoters are regulated to control initiation or as a consequence of initiation.

Until recently, all knowledge of the cis requirements for *oriC* initiation has been based almost exclusively on information

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Abbreviations: RNAP, RNA polymerase; IPTG, isopropyl β-D-thiogalactoside.

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obtained from cloned *oriC*, using either minichromosomes or *oriC* plasmids. However, recent findings have made it obvious that conditions on the chromosome are not matched by plasmids, and therefore, cloned *oriC* sites do not represent reliable models of chromosomal initiation. For instance, while inactivation of the *mioC* promoter on a minichromosome has been shown to result in a dramatic decrease in replication activity, this same mutation, when placed on the *E. coli* chromosome, had little or no effect on initiation (18). We recently found that the deletion of DnaA box R4 (Fig. 1), which completely blocks initiation of an *oriC* plasmid, is tolerated on the chromosome (19). To examine the effects of *oriC* modifications on the chromosome, we have developed a genetic system by which *oriC* modifications are systematically transferred from an *oriC* plasmid onto the *E. coli* chromosome (19). Using this system, we have investigated the contributions of *gid* and *mioC* transcription to initiation in their native location. We also examined the effects of *gid* and *mioC* transcription in cells that have been compromised for initiation by mutation of the genes encoding Fis and IHF proteins, or by deletion of the DnaA box R4. We show that disruption of transcription from P_{gid} and P_{mioC} has a very modest effect on initiation, even in the absence of Fis or IHF protein. In the absence of DnaA box R4, however, the presence of at least one of these transcriptions becomes essential for growth.

MATERIALS AND METHODS

Media and Growth Conditions. Cells were grown at 37°C, with aeration by shaking, in LB medium (20) supplemented with 0.1% glucose except for *rnhA* mutants, which were grown in CAA medium (21). Antibiotics were added at the following concentrations: ampicillin, 40 μg/ml; chloramphenicol, 50 μg/ml; tetracycline, 20 μg/ml; and kanamycin, 55 μg/ml.

***E. coli* strains and plasmids.** Strains are listed in Table 1. All *oriC* modified strains were constructed by using an *in vivo* replacement system previously described (19). Briefly, mutated *oriC* sites were transferred from an *oriC* plasmid onto the chromosome of a specialized λ transducing phage (λ10.2) by a double-crossover event, then from the phage onto the AQ7664 chromosome by a second double-crossover event. The exchange of wild-type *oriC* with the *oriC* mutations was confirmed by Southern blot hybridization (21) for all clones. For flow cytometric analysis, *oriC* modifications (and *asnA101*::*cat*) were transferred from AQ7664 into AQ9555 by phage P1 transduction, by selecting for chloramphenicol resistance and screening for kanamycin sensitivity. Modified *oriC* configurations on the chromosome were reconfirmed by Southern blot hybridization for all transductants. Promoter mutant derivatives of *fis* and *himA* mutant strains were created by moving *fis*::*kan* and *himA*::*tet* alleles into promoter mutant strains by P1 transduction.

Table 1. *E. coli* strains

Strain	Genotype	Ref. and/or source
AQ634	F⁻ *ilv metB his-29 pro trp9605 thyA deoB* (or -C)	26
AQ2178	AQ634 *polA1*	26
AQ3529	*supF* (λ*c*I857 *S*am7)	Lab collection
AQ7626	*supF* (λ*c*I857 *S*am7/λ10.2)	Lab collection
AQ7664	AQ634 *gidA95*::*kan*	19
AQ7996	*fis*::*kan*	As WM2016 (27)
AQ7998	*himA*::*tet*	As WM2017
AQ9552	Wild type	As MG1655 (28)
AQ9555	AQ9552 *gidA95*::*kan*	19
AQ9648	AQ9552 *asnA101*::*cat*	This work
AQ9652	AQ9552 $P_{gid\text{-}103}$ *asnA101*::*cat*	This work
AQ10033	AQ634 *rnhA224 gidA95*::*kan*	19
AQ10293	AQ9552 $P_{mioC112}$ *asnA101*::*cat*	This work
AQ10614	AQ9552 $P_{gid\text{-}103}$ $P_{mioC112}$ *asnA101*::*cat*	This work
AQ11296	AQ10033 $P_{gid\text{-}103}$ $P_{mioC112}$ *asnA101*::*cat*	This work
AQ11297	AQ10033 *oriC207*::*bla* $P_{gid\text{-}103}$ $P_{mioC112}$ *asnA101*::*cat*	This work

All *oriC* plasmids used are derivatives of pDB101 (Fig. 2*A*) (19). pDB103 carries a mutation ($P_{gid\text{-}103}$) in the −10 region of the *gid* promoter (Fig. 2*B*) that was constructed by digestion with *Hin*fI, brief digestion with mung bean nuclease, and religation (7). pDB109 was constructed by deletion of the *Bgl*II(+38)–*Acc*I(+285) *oriC* fragment of pDB101, and insertion of a *bla* gene into the deleted region (19). pDB112, which carries $P_{mioC112}$, an *Eco*RV(+722)–*Mlu*I(+777) deletion in the *mioC* promoter (Fig. 2*B*), was constructed by replacing the *Hin*dIII(+244)–*Bgl*II(+2006) fragment of pDB101 with the same from pALO47 (15). pDB113 carries both *gid* and *mioC* promoter mutations and was constructed by replacing the *Sma*I(−47)–*Xho*I(+417) fragment of pDB112 with the *Sma*I(−47)–*Xho*I(+417) fragment from pDB103. pDB123 contains deletions in the open reading frames of *gidA* and *mioC* resulting in inactive gene products, without affecting promoter activity. To create the 305-bp *mioC* deletion (Δ*mioC121*), pDB101 was digested with *Xho*I and *Eco*RV, filled in with Klenow enzyme, and religated to form pDB121. pDB121 was then digested with *Sna*BI and *Sac*II, blunt-ended, and religated to create the 253-bp *gidA* deletion (Δ*gidA122*), resulting in pDB123. The DnaA box R4 deletion (*oriC226*) was constructed by deleting the *oriC* domain between the *Hin*dIII (+244) and *Acc*I (+285) sites, followed by Klenow enzyme treatment and religation, just like the construction of the previously reported R4 deletion (*oriC207*::*bla*) (19) except for omission of the insertion of a *bla* gene in the deleted region. pRNH-Km is a derivative of pHK (22) carrying a *lacZ′*–*′rnhA* fusion which encodes a fusion protein that has near normal RNase HI activity (23). Construction of pLacI^q, which contains a *lacI*^{q} gene fragment, was previously described (23).

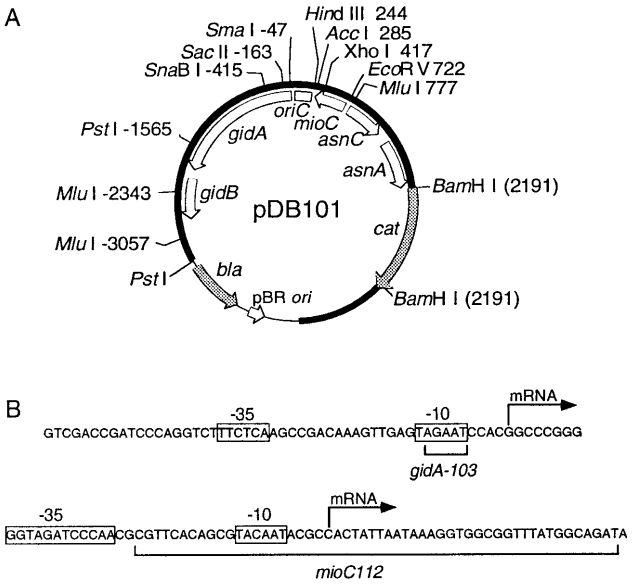

FIG. 2. Structure of *oriC* plasmid and promoter sequences. (*A*) The plasmid (pDB101) from which other *oriC* plasmids are derived is shown with relative restriction sites and genetic map (open arcs). Thick (solid) and thin arcs denote the *oriC* region and vector sequences, respectively. Shaded arcs denote antibiotic markers. All *oriC* coordinates are according to refs. 29 and 30. (*B*) DNA sequences of the *gid* (7) and *mioC* (14) promoter regions with promoter mutations are shown. The −10 and −35 consensus homologies and transcriptional start sites are indicated.

***oriC* Plasmid Copy Number Determination.** AQ2178 cells harboring each *oriC* plasmid were grown to approximately 2×10^8 cells per ml in LB + glucose in the presence of chloramphenicol (50 μg/ml). A 5-ml sample of the culture was collected and total DNA was extracted as previously described (21). The DNA ($\approx$9 μg) was digested with *Pst*I, electrophoresed in a 0.8% agarose gel, blotted onto a nylon filter, and hybridized with a [^{32}P]dCTP-labeled probe consisting of a 714-bp *oriC* region fragment from *Mlu*I(−3057) to *Mlu*I(−2343). The blot was then exposed to a PhosphorImager screen (Molecular Dynamics) and the relative band intensities were quantified.

Flow Cytometry. Cell preparation and flow cytometry with an Argus flow cytometer (Skatron, Lier, Norway) were performed essentially as previously described (24, 25).

RESULTS

Effects of Promoter Mutations on Cloned *oriC*. Promoter deletion mutations (Fig. 2*B*) were introduced into pDB101 (Fig. 2*A*), a derivative of pBR322 carrying a 6.6-kb *oriC* fragment. *gid* and *mioC* promoter mutations have been reported to reduce the frequency of replication initiated from cloned *oriC* (7, 8, 14, 15). It is known, however, that the origin activity of cloned *oriC* is affected by various factors such as the type of cloning vectors, the cloning sites, the orientation of *oriC* with respect to the vector, and the size of cloned fragments (refs. 1, 18, and 19 and references therein). We therefore reexamined the effects of promoter mutations on our plasmids that contain relatively long chromosomal sequences at both sides of *oriC*. The examination took advantage of the fact that the replication origin of pBR322, but not *oriC*, requires the *polA* gene product (DNA polymerase I) for initiation. Thus, derivatives of pDB101 can transform and be maintained in *polA* mutant cells when they contain active *oriC*.

We created deletions in the promoter regions of *gid* and *mioC* (Fig. 2*B*), and the inactivation of promoter activities was confirmed by a greater than 500-fold drop in β-galactosidase activity expressed from *gidA–lacZ* and *mioC–lacZ* fusion plasmids in which the promoters were mutated. As shown in Table 2, pDB112 carrying the *mioC* promoter mutation ($P_{mioC112}$) alone exhibited no detectable decrease in the efficiency of transformation of *polA* mutant cells compared with pDB101. The copy number of pDB112 in the mutant cells, however, was reduced from that of pDB101 (Fig. 3). pDB103 carrying the *gid* promoter mutation ($P_{gid\text{-}103}$) alone, on the other hand, showed a decreased transformation efficiency, and the copy number of this plasmid was further reduced from that of pDB112. Simultaneous inactivation of both promoters (pDB113) had an even greater effect than $P_{gid\text{-}103}$ alone; only a fraction of cloned *oriC* initiated plasmid replication in *polA* mutant cells. These results are in a general agreement with a current view of the importance of these transcriptions on cloned *oriC*: (*i*) Cloned *oriC* requires *gid* transcription for efficient initiation; and (*ii*) *mioC* transcription, although less significant in the presence of *gid* transcription, does compensate for the absence of it. The residual *oriC* activity observed with pDB113 may depend on other transcriptions detected within and/or near *oriC* (Fig. 1).

Table 2. Transformation frequencies of *polA1* cells with *oriC* plasmids

Plasmid	*mioC*	*gid*	Ratio of transformants ($polA^-/polA^+$) relative to pDB101
pDB101	+	+	1.00
pDB112	*112*	+	1.09 ± 0.12
pDB103	+	*103*	0.32 ± 0.02
pDB113	*112*	*103*	0.08 ± 0.01
pDB123	△	△	0.83 ± 0.09

+, Wild type; △, gene deletion mutant.

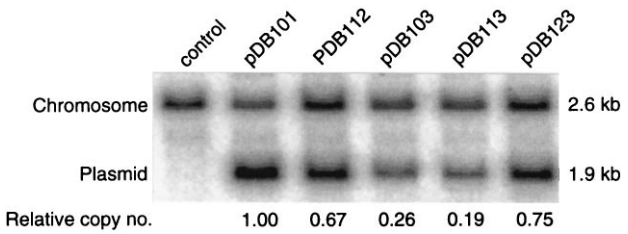

FIG. 3. Copy numbers of *oriC* plasmids carrying promoter mutations. *polA1* cells (AQ2178) were transformed with the indicated modified *oriC* plasmids and grown in the presence of chloramphenicol (50 μg/ml). Total DNA was extracted from exponentially growing cells and digested with *Pst*I and electrophoresed in a 0.8% agarose gel. DNA was blotted, and probed with a 714-bp *Mlu*I(−2343) to *Mlu*I(−3057) ^{32}P-labeled *oriC* DNA fragment. Band intensities were quantified by using a PhosphorImager, and the plasmid-to-chromosome ratio relative to pDB101 is indicated under each lane. For relevant genotypes of plasmids, see Table 2. Control lane is untransformed AQ2178.

To examine the unlikely possibility that the initiation defects of the promoter mutant plasmids were caused by reduced levels of *gid* and *mioC* gene product (neither of which have any known function), we constructed a gene deletion mutant plasmid (pDB123) carrying large deletions in the open reading frames of both *gidA* and *mioC*, leaving the promoter regions intact. This plasmid had a transformation efficiency (Table 2) and copy number (Fig. 3) severalfold higher than pDB113, suggesting that cloned *oriC* requires *gid* and *mioC* transcription *per se*, for efficient replication, rather than their gene products. The slightly decreased replication of pDB123 compared with the wild-type plasmid (pDB101) may be due to the decreased distance between *oriC* and the *mioC* promoter in the gene deletion mutant plasmid.

Promoter Mutations Have Little Effect on Initiation at Chromosomal *oriC*. Using a genetic system previously described (19), we replaced the wild-type promoters on the chromosome with modified ones from the *oriC* plasmids described above. Growth characteristics of the resulting promoter mutants were then examined by flow cytometry. Analysis of exponentially growing cells showed that the DNA/mass ratio and doubling times of the promoter mutant cells were

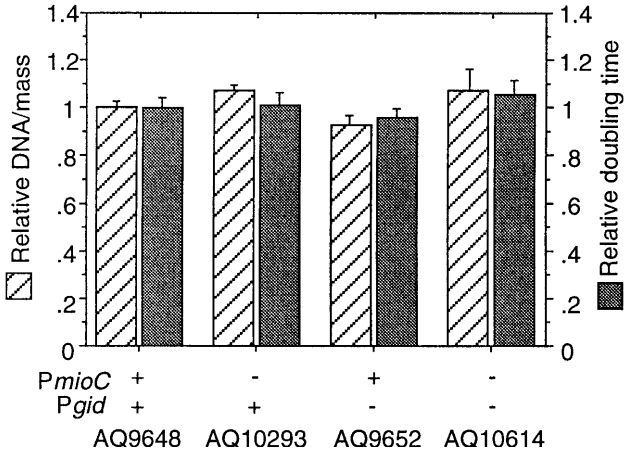

FIG. 4. DNA synthesis and growth rates of promoter mutants. Cells were grown in LB plus glucose at 37°C with aeration. DNA/mass values were obtained from flow cytometric analysis of exponentially growing cells. Doubling times were determined from cell counts with a particle counter. Values are averages of three independent analyses plotted relative to AQ9648 (18.8-min doubling time). Error bars indicate the standard deviation.

comparable to those of wild type (Fig. 4), suggesting that initiation from chromosomal *oriC* is not affected by either promoter mutation. Notably, the absence of *gid* transcription appears to cause a slight decrease in DNA/mass, whereas inhibition of *mioC* transcription may slightly increase DNA content. However, while these divergences are generally repeatable, they are within the range of experimental error and may be insignificant. As mentioned, the cell cycle-specific regulation of *gid* and *mioC* transcription suggests that these transcriptions affect the precise timing of initiation. Flow cytometry revealed that inhibition of *gid* and/or *mioC* transcription resulted in no detectable loss of the high degree of initiation synchrony exhibited by wild-type cells (data not shown). We conclude that these transcription defects have no effect on initiation, at least under otherwise optimal conditions for initiation.

***fis* or *himA* Mutations Do Not Enhance the Effect of the Promoter Mutations on Chromosomal Initiation.** For replication of cloned *oriC*, *gid* and *mioC* transcriptions become essential under nonoptimal conditions such as lack of histone-like proteins (11, 31). We introduced *gid* and *mioC* promoter mutations onto the chromosome of *fis*::*kan* and *himA*::*tet* mutant cells, which are deficient in Fis and IHF, respectively, and the resulting mutants were examined for growth characteristics by flow cytometry. Surprisingly, *fis*::*kan* and *himA*::*tet* mutations had little effect on requirements of chromosomal *oriC* for *gid* or *mioC* transcription. DNA/mass ratios (Fig. 5) were not significantly affected by either *gid* or *mioC* promoter mutations. Note that the slight effects on DNA synthesis caused by inhibition of *mioC* and *gid* transcription (Fig. 4) are generally reflected in *fis* and *himA* mutants. Exacerbation of the severe asynchrony phenotype of *fis* and *himA* mutants (32) was not detected when the promoter mutations were introduced (data not shown). Interestingly, cell size was significantly increased in *fis*::*kan* cells carrying either $P_{gid\text{-}103}$ or $P_{mioC112}$ mutation, and cells with all three mutations exhibited extreme filamentation (data not shown).

Transcription from Either *gid* or *mioC* Is Essential for Initiation with a Suboptimal *oriC* Sequence. Previously we showed that cells carrying a deletion of DnaA box R4 on the chromosome are viable (19). These cells replicate inefficiently as indicated by decreased DNA/mass and presence of the asynchrony phenotype. We considered that local transcription might be required to activate this truncated *oriC*. To test this hypothesis we created *oriC* plasmids carrying a deletion of DnaA box R4 (*oriC207*::*bla*) combined with either or both $P_{gid\text{-}103}$ and $P_{mioC112}$. Transfer of the R4 mutated *oriC* combined with a single promoter mutation onto the chromosome was successful. Cells carrying a P_{gid} promoter mutation exhibited severely retarded growth (48.6-min doubling time) as indicated by a more than 2-fold increase in doubling time compared with *oriC207*::*bla* alone (23.2 min). The absence of *mioC* transcription had a much smaller effect on the ΔR4 mutant (32.4-min doubling time).

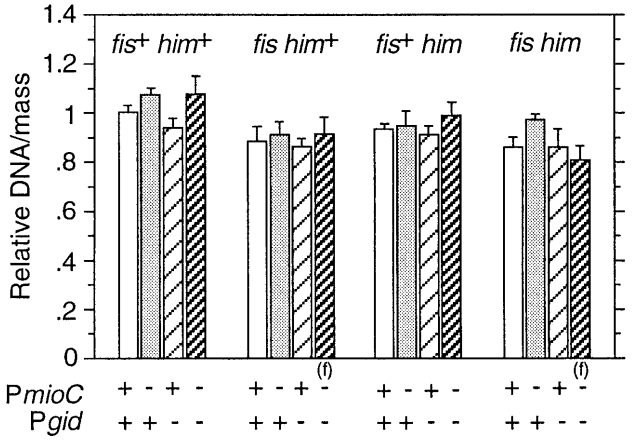

FIG. 5. DNA synthesis of *fis*::*km himA*::*tet* cells carrying promoter mutations. Cells were grown and DNA/mass values were obtained as in Fig. 4. An (f) indicates that these cells exhibit extreme filamentation during exponential growth. Values shown are averages of three independent analyses. Error bars indicate the standard deviation.

Several attempts to transfer the triple mutation *oriC207*::*bla* $P_{gid\text{-}103}$ $P_{mioC112}$ from *oriC* plasmid onto the chromosome were fruitless, suggesting that *E. coli* could not accommodate such an extensive *oriC* modification. In *rnhA224* mutant cells (AQ10033), an alternative replication system, constitutive stable DNA replication (cSDR) is activated and thus *oriC* defects are suppressed (33). Using this strain, we successfully transferred the triple mutant *oriC* onto the chromosome. We could transfer the mutant *oriC* into an *rnhA224* mutant by P1 transduction but not into wild-type cells, further confirming the lethality of the triple mutant. To verify the dependence of the growth of the resulting *rnhA224 oriC207*::*bla* $P_{gid\text{-}103}$ $P_{mioC112}$ mutant (AQ11297) on cSDR, the mutant strain was transformed with pRNH-Km, which produces an active RNase HI protein from a *lac* promoter. The transformants did not grow in the presence of 5 mM isopropyl β-D-thiogalactoside (IPTG), whereas the vector plasmid (pHK) had no effect on cell growth (Fig. 6). Furthermore, a DnaA box $R4^+$ counterpart (AQ11296) was not sensitive to IPTG. We conclude that the *oriC* triple mutant is inviable in the presence of RNase HI. In addition, the $OriC^-$ phenotype of this strain could not be rescued by introduction of a plasmid (pDB109) carrying wild-type *mioC* and *gid* genes (data not shown). This suggests that the R4 deletion mutant requires an activating transcription event *per se*, rather than *gid* or *mioC* gene product.

To rule out the possibility that any transcription reading through the *bla* terminators (transcription is oriented leftward into *oriC*) is responsible for the *oriC* lethality of the *oriC207*::*bla* $P_{gid\text{-}103}$ $P_{mioC112}$ mutant, we constructed an identical R4 deletion mutant except without *bla* gene insertion. When combined with both *gid* and *mioC* promoter mutations, this construct strictly required the presence of an *rnhA224* mutation for growth (data not shown).

DISCUSSION

Much evidence exists suggesting a role of *gid* and *mioC* transcription in the initiation of replication. *oriC* plasmids and

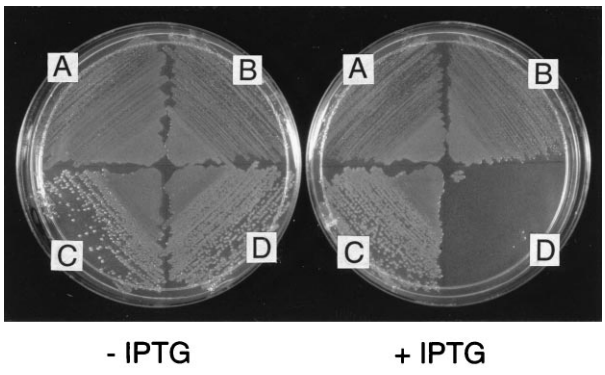

FIG. 6. Lethal effect of expression of an $rnhA^+$ gene on *rnhA224* $P_{mioC112}$ *oriC207*::*bla* $P_{gid\text{-}103}$ mutant. Cells were transformed with pRNH-Km (carries *lacZ*::*rnhA* gene fusion) or pHK (vector) and selected for kanamycin resistance. Transformants were then transformed with $pLacI^q$, selecting for tetracycline resistance. Equal amounts of cells were spread on minimal plates with or without 5 mM IPTG as indicated. (*A*) *rnhA224* $P_{mioC112}$ $P_{gid\text{-}103}$/pHK, $pLacI^q$. (*B*) *rnhA224* $P_{mioC112}$ $P_{gid\text{-}103}$/pRNH-Km, $pLacI^q$. (*C*) *rnhA224 oriC207*::*bla* $P_{mioC112}$ $P_{gid\text{-}103}$/pHK, $pLacI^q$. (*D*) *rnhA224 oriC207*::*bla* $P_{mioC112}$ $P_{gid\text{-}103}$/pRNH-Km, $pLacI^q$.

minichromosomes have been shown to be very sensitive to mutations in these promoters. In concordance with previous findings, our data indicate that an *oriC* plasmid requires transcription from both promoters for efficient replication. Transcription occurring from P_{gid} was more important, although a plasmid carrying mutations in both promoters (pDB113) was still able to replicate; initiating at about 8–19% efficiency compared with a wild-type plasmid (Table 2, Fig. 3). In spite of the fact that promoter inactivation had a significant effect on replication of an *oriC* plasmid, we found that initiation at chromosomal *oriC* did not require transcription from either promoter. Blocking these transcriptions had almost no detectable effect on the rate of growth or DNA synthesis (Fig. 4). This is in agreement with the recent finding that inactivation of the *mioC* promoter has no effect on initiation of chromosome replication (18). Findings that *oriC* plasmids and minichromosomes are much more sensitive to promoter mutations is likely a reflection of the small size of plasmids relative to the chromosome and hence a decreased ability to balance superhelical changes over a much shorter domain.

Transcription from *mioC* was suggested to regulate the timing of initiations by inhibiting additional *oriC* firings after negative factors such as sequestration have become exhausted (16, 17). Inconsistent with this suggestion, the present and previous (18) data show no defects in initiation synchrony of P_{mioC} mutants. Our data are in agreement with recent findings that replication initiation in synchronized *dnaC2*(Ts) mutants is unaffected by the absence of *mioC* transcription (34). In addition, we found that inhibition of *gid* transcription had no effect on initiation synchrony. As suggested, the periodicity of these transcriptions within the cell cycle may be a consequence of initiation, rather than a regulator of it (34).

We also found that inhibition of *mioC* and *gid* transcription does not affect growth in the absence of the two histone-like proteins Fis and IHF. Whereas DNA synthesis was slightly lower in *fis* and *himA* mutants, inactivation of *mioC* and *gid* transcription had little or no additional effect (Fig. 5). This is surprising, given that a minichromosome carrying an inactive *mioC* promoter cannot replicate in the absence of IHF protein (31). In contrast, transcription from either P_{gid} or P_{mioC} became obligatory when the right-most DnaA box, R4, was deleted (Fig. 6). This is, to our knowledge, the first observed case of transcriptional activation of chromosomal *oriC*. The DnaA box ΔR4 mutant requires transcription from at least one of the promoters, possibly due to a reduced ability to melt the 13-mer region when fewer DnaA-binding sites are available. This hypothesis is consistent with findings that overexpression of DnaA protein results in significant replication in the presence of rifampicin (35, 36). We propose that *gidA* and *mioC* transcription becomes essential only when *oriC* is under suboptimal conditions. Consistent with this proposal, replication of an *oriC* template *in vitro* also requires RNAP, but only under conditions that make unwinding of the origin difficult (e.g., extreme concentrations of HU protein, reduced negative superhelicity, or reduced temperature) (11, 12). Furthermore, phage λ DNA replication *in vitro* has been found to require transcription when HU protein is present (37). More recent studies indicate that transcription initiated either upstream or downstream of oriλ can activate initiation of λ DNA replication, most likely by enhancing localized negative supercoiling in the A+T-rich region of the origin (S.-H. Chung and R. McMacken, personal communication).

Our findings, however, do not readily explain the sensitivity of wild-type cells to rifampicin (2, 3). *gid* and *mioC* transcription represents the bulk of RNAP activity around *oriC*, but the possibility still exists that one or more of the several other promoters detected within or in the close vicinity of the minimal *oriC* (Fig. 1) may be responsible for transcriptional activation of *oriC*. Having eliminated the two prime suspects of transcription representing the rifampicin sensitive step in initiation, we should now begin to seriously reexamine the original inference of transcriptional activation. In an *in vitro* initiation system, either RNAP or DnaG primase (which is insensitive to rifampicin) is capable of activating replication. However, DNA synthesis in both systems is sensitive to rifampicin, suggesting a second unknown capacity of rifampicin (38). It is known that incubation of cells with rifampicin results in a decrease in the sedimentation rate of nucleoids, suggesting a decrease in the overall extent of supercoiling of the nucleoids (39, 40). We suggest the possibility that rifampicin, which shuts down all transcriptions on the chromosome, brings about a drastic global change in the nucleoid structure, severely altering the topological structure of *oriC*. In other words, the rifampicin effect may be nonspecific, and not due to the inhibition of a particular promoter such as P_{gid} or P_{mioC}.

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