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The Synchrony Phenotype Persists after Elimination of Multiple GATC Sites from the *dnaA* Promoter of *Escherichia coli*

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To examine whether methylation of the GATC sites present in the *dnaA* promoter region is responsible for the strict temporal coordination of initiation events at *oriC* as measured by the synchrony of initiation, we introduced point mutations eliminating three (TGW1) and five (TGW2) of the six GATC sites present in the *dnaA* promoter region. All of the strains containing these mutations, including the one with five GATC sites eliminated, initiated chromosomal replication synchronously.

The initiation of DNA replication at two or more origins is strictly coordinated such that all origins fire simultaneously and do so once per cell cycle (28). Methylation of GATC sites located at *oriC* and *dnaA* has been implicated in the strict temporal regulation of initiation timing in *Escherichia coli* (1). The transfer of a methyl group from *S*-adenosylmethionine to the adenosine present in 5′-GATC-3′ sites of newly synthesized DNA is catalyzed by deoxyadenosyl methyltransferase or Dam. Most GATC sites become fully methylated 2 to 5 min following the passage of the replication fork. GATC sites present in *oriC* and the *dnaA* promoter, however, experience a delay in methylation following initiation at *oriC* (6). In exponentially growing cells, the genes of the *dnaA* operon are coordinately expressed from the $dnaAp_2$ promoter (24), and decreased *dnaA* expression is observed during this period of hemimethylation (31).

This strict temporal coordination of initiation events and expression of *dnaA* is disrupted in *dam* mutant strains. Asynchronous initiation timing is observed in *dam* cells, and initiation events occur randomly throughout the cell cycle (1, 2). Expression from the $dnaAp_2$ promoter in cells grown at 37°C is 2.7- to 4.5-fold lower in dam^+ than in *dam* mutant strains (3). Mutations in *seqA* greatly reduce the delay in methylation of *oriC* and the *dnaA* promoter after initiation (22), and DnaA protein levels are twofold higher in cells carrying a *seqA2* mutation (32).

SeqA binds to *oriC* in vitro (29), to *oriR* of plasmid P1 (4), to the bacteriophage λ p_R promoter (30), and to synthetic oligonucleotides containing multiple GATC sites (4) and may negatively regulate initiation of replication (22). Binding of SeqA to hemimethylated DNA requires the presence of two GATC sites (5) that can be as far apart as 31 bp (18). GATC sites are overrepresented in *oriC* and the *dnaA* promoter, and many are separated by less than 31 bp. If GATC sites occurred randomly in the *E. coli* genome, they would occur, on average, once every 256 bp in the *E. coli* chromosome, assuming a GC content of 50%. However, the *dnaA* promoter region contains 6 GATC sites (12) (Fig. 1); 11 are present in the minimal 245-bp *oriC* gene (20, 33).

The *E. coli* K-12 *dnaA* promoter sequence was compared to those of closely related bacteria (Table 1). A BLAST search was conducted against the microbial database at the National Center for Biotechnology Information with the *dnaA* sequence from *E. coli* K-12. Except for two *Yersinia pestis* strains, all hits for the bacterial strains presented in Table 1 had the expect values of zero. The *dnaA* promoter sequences that were obtained from hits with low expected values, and therefore a high degree of similarity to the *dnaA* gene, were uploaded into the Macromolecular Structural Analysis Resource Core, San Diego State University. The GCG (Genetics Computer Group) Wisconsin package of sequence comparison tools was used to perform an alignment of the *dnaA* sequences. An alignment of the uploaded sequences was performed with the GCG program PILEUP, and the output was then converted with the GCG program PRETTY. Along with the DnaA box (data not shown), the GATC sites located in the −10 and −35 sites of the $dnaAp_2$ promoter are 100% conserved. The most proximal of the three GATC sites located downstream of the $dnaAp_2$ start site is more frequently conserved than the GATC site located in the $dnaAp_1$ promoter.

Of the six GATC sites present in the *dnaA* promoter, those in the −10 and −35 sequences of the $dnaAp_2$ promoter are conserved in all of the gram-negative bacteria examined in Table 1 and are present in a consensus sequence for σ^{70} transcription factors (14, 15). Strains with the −10 and −35 GATC sites eliminated, alone and in combination, from the $dnaAp_2$ promoter demonstrated synchronous initiation events (12). DnaA protein levels in strains with the −10 and −35 GATC sites eliminated from the $dnaAp_2$ promoter were similar to those in the parental MG1655 strain, in agreement with experiments with a *dnaA*-chitobiase reporter construct integrated at *attB* on the *E. coli* chromosome (19). The amount of *dnaA* expression is twofold lower in dam^+ than in *dam* mutant

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FIG. 1. The *dnaA* promoter sequence.

strains, which was thought to result from the release from sequestration that occurs through the binding of SeqA to hemimethylated GATC sites (3). However, when DnaA protein levels were compared in *dam*$^+$ and *dam* mutant strains possessing a *dnaAp*$_2$ promoter with the −10 and −35 GATC sites eliminated, the effect was attenuated. The −10 and −35 GATC sites in the *dnaAp*$_2$ promoter appear not to be necessary for the strict coordination between *dnaA* expression and initiation timing (7). However, SeqA binding to GATC sites downstream of the p_I and p_{aQ} promoters in bacteriophage λ has been implicated in transcriptional regulation (30), so we investigated the effect of removing the GATC sites downstream of the *dnaAp*$_2$ promoter.

There are three additional GATC sites downstream of the *dnaAp*$_2$ promoter that are close enough to one another to permit SeqA binding (Fig. 1), and these GATC sites were eliminated in this study by the methods developed by Kedar et al. (19). Two strains, TGW1 and TGW2, were constructed. The TGW1 strain has three GATC-to-GATG mutations introduced downstream of *dnaAp*$_2$ (Fig. 2) but is otherwise identical to KGC1 (19). The *dnaA* promoter in the TGW2 strain has five of six GATC sites altered (Fig. 2). The sixth GATC site is located upstream of the *dnaAp*$_2$ promoter and would not have a neighboring GATC site close enough to permit SeqA binding (5, 18).

Plasmids and strains were constructed essentially as previously described (19). The pTGW1 and pTGW2 plasmids are derivatives of pKGC3 (8,558 bp) and pKGC4 (8,558 bp), respectively (Table 2). A PCR method of site-specific mutagenesis was used to introduce three C-to-G point mutations and thus eliminate the GATC sites downstream of the *dnaAp*$_2$ promoter. The mutations were introduced into pKGC3 and

TABLE 1. Conserved GATC sites in the *dnaA* promoter

Strain	Presence of conserved GATC site at:					
	dnaAp$_1$	*dnaAp*$_2$ (−35)	*dnaAp*$_2$ (−10)	+6	+19	+25
E. coli K-12 *dnaA* promoter	+	+	+	+	+	+
E. coli CFT073	+	+	+	+	+	+
E. coli O157:H7	+	+	+	+	+	+
E. coli O157:H7 EDL933	+	+	+	+	+	+
Shigella flexneri 2a strain 301	+	+	+	+	+	+
Shigella flexneri 2a strain 2457T[a]	+	+	+	+	+	+
Salmonella enterica serovar Typhimurium	+	+	+	+	+	−
Salmonella enterica serovar Typhi TY2	+	+	+	+	+	−
Klebsiella pneumoniae	+	+	+	+	−	−
Yersinia pestis CO92	−	+	+	+	−	−
Yersinia pestis KIM	−	+	+	+	−	−
Erwinia chrysanthemi 3937	−	+	+	−	−	−
% Conserved	75	100	100	92	66	50

[a] The superscript T indicates the type strain.

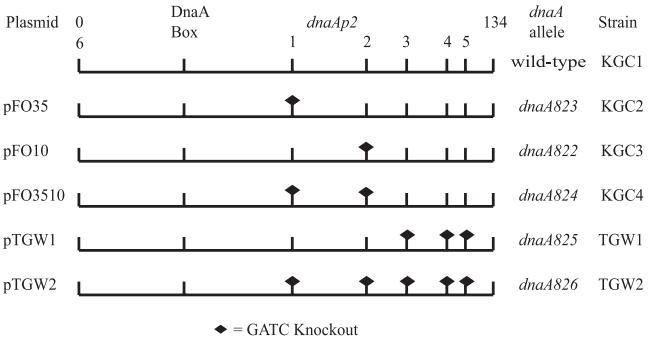

FIG. 2. Strains containing GATC mutations in the *dnaA* promoter. The first nucleotide of each GATC site and in the DnaA box (represented at the extreme left) are indicated with respect to the relative position to each other and to the 100-bp fragment representing the *dnaAp*$_2$ promoter (drawn to scale). The GATC sites present in the *dnaA* promoter sequence are numbered. The *dnaAp*$_2$ transcriptional start site is present between GATC sites 2 (−10) and 3. The plasmids and the strains ultimately constructed are to the left and extreme right, respectively. Black diamonds indicate the first G in a GATC site that was eliminated by site-specific mutagenesis.

pKGC4 by the four-primer method (17, 23). The mutations were present in oligonucleotide primers that were introduced into amplified PCR products, which were ligated into plasmids pKGC3 (7,421 bp) and pKGC4 (7,421 bp). The resulting plasmids, pTGW1 (8,558 bp) and pTGW2 (8,558 bp), respectively, were used to integrate the mutated *dnaA* promoter into the chromosome of a *polA107* (16) mutant in a two-step process. The plasmids contain the *dnaA* promoter region and part of the *dnaA* coding region inserted between the Omega cassette (25, 26) and *sacB*. The Omega cassette provides resistance to streptomycin and spectinomycin. The enzyme levansucrase is encoded by the *Bacillus subtilis sacB* gene, and its expression in the presence of 5% sucrose results in the accumulation of levans, which is ultimately lethal in *E. coli* (7, 8, 27). The pTGW1 and pTGW2 plasmids carry a ColE1-derived origin that requires a functional DNA polymerase I for replication and cannot be maintained in a *polA* mutant (9, 11). Integration of the plasmid, carrying the mutated *dnaA* promoter, into the chromosome by homologous recombination was selected with spectinomycin. In the second step, excision of the plasmid backbone was selected by addition of 5% sucrose to the culture medium.

The *polA107* (16) mutant strain was transformed with either pTGW1 or pTGW2 and incubated at 30°C overnight in 4 ml of SOC buffer containing thymine (25 μg/ml) and spectinomycin (50 μg/ml). The cultures were diluted 1:50 in low-salt LB–5% sucrose–5 mM $CaCl_2$–thymine (25 μg/ml)–spectinomycin (50 μg/ml) and incubated overnight at 30°C. The cultures again were diluted 1:50 in low-salt LB–5% sucrose–5 mM $CaCl_2$–thymine (25 μg/ml)–spectinomycin (50 μg/ml) and incubated overnight at 30°C. The mutated *dnaA* promoter region was then moved by P1 transduction into MG1655. Transductants were selected on low-salt LB agar plates containing 5% sucrose and spectinomycin (50 μg/ml) and replica plated to LB agar plates containing ampicillin (100 μg/ml) to screen for sensitivity to ampicillin, resulting in strains TGW1 and TGW2 (Table 2).

The resulting Ap^s Sp^r strains have three of six (TGW1) or

TABLE 2. Strains used in this study

Strain	Genotype	Source or reference
DH5α	*supE44 lacU169* [ϕ80 *lacZ*ΔM15] *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Stratagene
JZ294	F⁻ *polA1 argH hsdR rpsL thyA36*	13
JG108	*metE70 thyA deoC2 lacZ lacY14*(Am) *rhaS rpsL* λ⁻	Cathy Joyce
JZ365	JG108 *polA12*::Tn*10*	Cathy Joyce
JZ366	JG108 *polA107*::Tn*10*	Cathy Joyce
JZ1101	W3110 *tnaA262*::Tn*10*	Charles Yanofsky
MG1655	λ⁻ F⁻	Mary Berlyn
TGW1	MG1655 with three GATC sites downstream of $dnaAp_2$ eliminated	This study
TGW2	MG1655 with five of six GATC sites in *dnaA* promoter region eliminated	This study

five of six (TGW2) GATC sites eliminated from the *dnaA* promoter (Fig. 2). PCR of chromosomal DNA, followed by sequencing of the PCR product, verified the absence of intact GATC sites. To demonstrate integration at the wild-type chromosomal location of the Omega cassette (Sp^r) carrying the mutated *dnaA* promoter, a *tnaA262*::Tn*10* mutant was transduced with bacteriophage P1 lysates prepared on either TGW1 or TGW2. Transductants were selected on spectinomycin (50 μg/ml)-containing agar plates, replica plated onto agar plates containing both spectinomycin (50 μg/ml) and tetracycline (25 μg/ml), and screened for loss of tetracycline resistance, thereby verifying linkage between the *dnaA* (Sp^r) and *tnaA*::Tn*10* (Tc^r) loci.

The initiation of DNA replication is precisely coordinated, occurring at a specific time, once per cell cycle, at all origins in the cell simultaneously (28). If initiation is inhibited with rifampin and ongoing rounds of replication are allowed to complete, wild-type cells with coordinated and synchronous initiation of all chromosomes contain 2^n (n = 0, 1, 2, 3, 4) chromosomes when examined by flow cytometry (10, 19, 28). Defects in the timing of initiation result in cells that contain

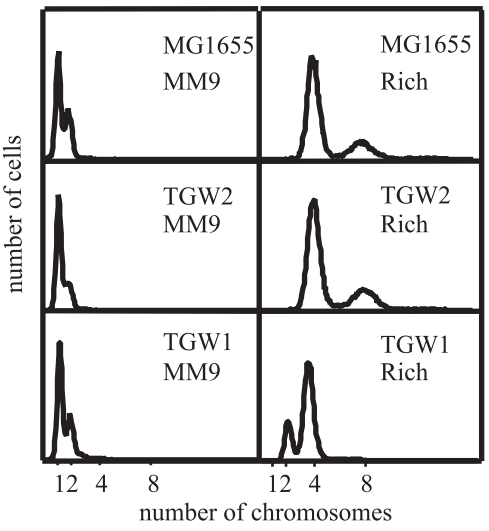

FIG. 3. Strains with three (TGW1) or five (TGW2) GATC sites eliminated from the *dnaA* promoter initiate DNA replication synchronously. Cells growing exponentially in M9 enriched (rich) or M9 minimal (MM9) medium were treated with rifampin and cephalexin for 4 h. Samples were removed, fixed, stained, and analyzed by flow cytometry as described previously (19).

"irregular" numbers of completed chromosomes, such as three, five, six, seven, etc. (27). Also, cells that contain chromosomes unable to complete replication because of a DNA lesion or blockage in replication after rifampin treatment show broad peaks of DNA where the amount of DNA per cell does not correspond to the amount of DNA in a single completed chromosome or in multiple numbers of completed chromosomes (10). We examined initiation timing and the completion of chromosomal replication in strains TGW1, TGW2, and MG1655 by flow cytometry (19) in both minimal and enriched media after treatment with rifampin to inhibit initiation and cephalexin to inhibit cell division (Fig. 3). As shown in Fig. 3, the strains with either three (TGW1) or five (TGW2) GATC sites eliminated in the *dnaA* promoter region contained 2^n completed chromosomes, the phenotype of a wild-type *E. coli* strain such as MG1655. Strain TGW1, with three of the GATC sites eliminated, grew more slowly than either TGW2 or MG1655 (Table 3). Also, strain TGW1 grown in enriched medium had a reduced number of completed chromosomes after rifampin and cephalexin addition. With fewer initiation events occurring at a lower growth rate, the number of completed chromosomes is expected to be lower. The number of chromosomes per cell after runout (4 h of treatment with rifampin and cephalexin) depends on the number of replication cycles per chromosome (n), where n equals C/τ, τ is the generation time in minutes, and C is the C period in minutes; the C period is the duration of chromosomal replication. When cells grow more slowly (τ increases), n decreases, resulting in fewer chromosomes per cell after inhibition of initiation. Al-

TABLE 3. Cell cycle parameters for GATC *dnaA* mutants

Growth condition and strain	τ[a]	DNA/cell[b]	Mass/cell[c]
M9 minimal medium			
MG1655	60	1.00	1.00
TGW2	60	1.00	1.00
TGW1	75	0.94	0.93
M9 enriched medium			
MG1655	32	1.00	1.00
TGW2	34	1.00	0.98
TGW1	52	0.80	0.88

[a] τ, generation time (min).
[b] DNA/cell, mean value of fluorescence in exponentially growing cells compared to MG1655.
[c] Mass/cell, mean value of light scattering in exponentially growing cells compared to MG1655.

though the number of completed chromosomes in TGW1 cells observed by flow cytometry was lower in enriched M9 medium than with MG1655 cells, the number of completed chromosomes in TGW1 was 2^n (two and four chromosomes), indicating synchronous initiation at all DNA replication origins in each cell.

The methylation state of the GATC sites in the *dnaA* promoter appears to be of little consequence to initiation timing, because elimination of the two GATC sites in the $dnaAp_2$ promoter (19), the three GATC sites downstream of the $dnaAp_2$ promoter (TGW1, this study), or all five GATC sites (TGW2, this study) did not result in initiation timing defects detectable by flow cytometry (Fig. 3). Either proper initiation timing does not require cell cycle-dependent *dnaA* expression, or cell cycle control of *dnaA* expression is maintained by a mechanism other than sequestration. The concentration of the active ATP-bound form of DnaA protein may be the critical event regulating initiation events, and this concentration may be regulated in a cell cycle-dependent manner that is independent of the sequestration of the *dnaA* promoter (21).

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