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Initiation of Heat-Induced Replication Requires DnaA and the L-13-mer of *oriC*[▽]

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An upshift of 10°C or more in the growth temperature of an *Escherichia coli* culture causes induction of extra rounds of chromosome replication. This stress replication initiates at *oriC* but has functional requirements different from those of cyclic replication. We named this phenomenon heat-induced replication (HIR). Analysis of HIR in bacterial strains that had complete or partial *oriC* deletions and were suppressed by F integration showed that no sequence outside *oriC* is used for HIR. Analysis of a number of *oriC* mutants showed that deletion of the L-13-mer, which makes *oriC* inactive for cyclic replication, was the only mutation studied that inactivated HIR. The requirement for this sequence was strictly correlated with Benham's theoretical stress-induced DNA duplex destabilization. *oriC* mutations at DnaA, FIS, or IHF binding sites showed normal HIR activation, but DnaA was required for HIR. We suggest that strand opening for HIR initiation occurs due to heat-induced destabilization of the L-13-mer, and the stable oligomeric DnaA–single-stranded *oriC* complex might be required only to load the replicative helicase DnaB.

The initiation of chromosome replication is the central event in the bacterial cell cycle and the step that controls the frequency with which chromosomes are replicated. Initiation is a precisely timed event that occurs once during the cell cycle. In *Escherichia coli* replication initiates at a unique site, *oriC*, located at min 84.5 on the genetic map. The minimal sequence that promotes replication was found to be 258 bp long (25). The left side of *oriC* is an AT-rich region consisting of an AT cluster and three similar sequences, each 13 nucleotides long and each starting with GATC (1, 5). This AT-rich region provides the helical instability of *oriC*. Apparently, it is the AT richness that is important in this region, and except for the R-13-mer, the sequence can be replaced by different AT-rich sequences with similar properties (5, 6, 21). Within *oriC* there are eight 9-mer DnaA binding sites; five of these sites can bind either DnaA-ADP or DnaA-ATP with equal affinity (R boxes) (27), but the remaining three sites preferentially bind DnaA-ATP (I boxes) (23, 26). The minimal *oriC* sequence also contains 11 GATC sites specific for Dam methyltransferase that must be methylated for optimal origin function. In addition, there are binding sites for the DNA bending protein IHF, which assists in *oriC* unwinding, and the DNA bending protein Fis. There are also binding sites for IciA, ROB, SeqA, and Cnu/Hha among other proteins (18, 25).

Initiation of replication begins with the binding to *oriC* and cooperative interaction of 20 to 30 monomers of DnaA-ATP and bending of the DNA that promotes the unwinding of the AT-rich region (31). This strand opening also requires transcriptional activation (2, 29, 34) and is enhanced by a certain DNA topology (9, 30). DnaA-ATP then binds to the single-stranded DNA, providing the initial stabilization of the single-stranded state. The stable oligomeric DnaA-*oriC* complex is required for the interaction of DnaA with the two replicative helicase DnaB-DnaC hexamers and their loading into the unwound DNA (7, 28, 33). The binding of DnaB to the single-stranded DNA seems to facilitate dissociation of DnaC from the complexes. Subsequent loading of primase permits the recruitment of the rest of the proteins for replisome assembly.

In addition to cyclic replication during exponential growth, there are stress conditions that can induce extra chromosome replication. An increase in the environmental temperature of 10°C or more induces the initiation of extra rounds of replication (4, 13). We named this phenomenon heat-induced replication (HIR). This stress replication initiates at *oriC*, is not a heat shock response, and, in contrast to cyclic replication, requires neither RNA polymerase activity nor protein synthesis (4). Other physiological changes, such as DNA damage and a change in the growth medium, can induce replication by one of two ways, termed inducible stable DNA replication and constitutive stable DNA replication (19). HIR exhibits some homology with both types of stable DNA replication. The three replication modes do not require protein synthesis. In contrast to constitutive stable DNA replication, HIR requires active RNase H; in contrast to inducible stable DNA replication, HIR is independent of SOS induction; and, as we showed in this work, HIR initiates only in *oriC* and requires DnaA.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All of the strains used are derivatives of *E. coli* K-12 and are listed in Table 1. Bacteria were incubated at 30°C in M9 minimal medium containing 0.4% glucose, 20 μg/ml required amino acids, 2 μg/ml thiamine, and 0.1% Casamino Acids. Antibiotics, including 20 μg/ml ampicillin, 50 μg/ml kanamycin, and 15 μg/ml tetracycline, were added when appropriate.

DNA synthesis and number of origins. DNA synthesis was determined by growing cells in minimal medium containing 1 μg/ml thymidine and 1 μCi/ml [*methyl*-^{3}H]thymidine (20 Ci/mmol; ICN) in the presence of 1.5 mM uridine. The isotope was added to both the overnight and exponentially growing cultures. Trichloroacetic acid-insoluble material from 0.2-ml aliquots was assayed using a Beckman LS3801 scintillation counter. Rifampin (150 μg/ml) was added to

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Other characteristics	Reference or source
CM1565	*ilv192 asnB32 relA1 spoT1 thi-1 lys*		35
CM1588	CM1565 *zif*::pCM197	F′ ilv^+	35
CM1671	CM1588 Δ*oriC1071*	Deletion from *gidB* to *asnA*	35
CM1793	CM1565 *zij*::pKN500	R1 derivative, $asnA^+$	35
CM1843	CM1793 Δ*oriC1071*	Deletion from *gidB* to *asnA*	35
CM735	*metE46 trp3 his4 thi1*		8
CM735-*intFs-B*	CM735 Δ*oriC*::pAE15	Nucleotides 23 to 38 deleted (L-13-mer), integratively suppressed by mini-F	8
ER	*asnA asnB thi1*		20
LK211	ER Δ*oriC*::pKN1562	Nucleotides 23 to 38 deleted (L-13-mer), integratively suppressed by R1 derivative	20
WM2482	*ilvG*	MG1655	36
WM2759	WM2482 *oriC160*	Δ275-352	36
WM2762	WM2482 *oriC13*	R2 scrambled	36
WM2763	WM2482 *oriC14*	R3 scrambled	36
WM2764	WM2482 *oriC15*	R4 scrambled	36
WM2765	WM2482 *oriC21*	R1 at R3 position	36
WM2766	WM2482 *oriC131*	Mutant FIS site	36
WM2767	WM2482 *oriC132*	Mutant IHF site	36
WM2768	WM2482 *oriC136*	R4 inverted	36
WM2844	WM2482 *oriC17*	M scrambled	36
WM2845	WM2482 *oriC162*	+14 bp between R3 and R4	36
JK607	*thyA arg his thi*		Our lab
JK876	JK607 *dnaA*46	*dnaA46*	Our lab

exponentially growing cultures to inhibit initiation of replication, and replication runout (ΔG at 30°C) was determined by measuring the [^{3}H]thymidine that was incorporated over 4 h. All measurements were made relative to the radioactive count at the time that rifampin was added, which was always around 5,000 dpm. The accumulated DNA synthesis was proportional to the number of active replication forks, as in these growth conditions bacteria had overlapping replication cycles. The number of overlapping replication cycles (n) was determined from the formula $\Delta G = 2^n \times n \times \ln 2/(2^n - 1) - 1$, as described by Sueoka and Yoshikawa (32).

Measurement of HIR. HIR was determined by shifting a culture from 30 to 41°C and adding 150 μg/ml rifampin. Replication runout (ΔG at 41°C) was determined by measuring the [^{3}H]thymidine that accumulated over 4 h. The number of induced origins was determined as described by Jiménez-Sánchez and Guzmán (17). Briefly, if i is the frequency of reinitiated origins after any inductive treatment resulting in complete chromosome replication and n is the number of replication cycles per chromosome in the exponentially growing culture at 30°C, the final amount of chromosomes is $2^n(i + 1)$ and the replication runout is $\Delta G = 2^n \times (i + 1) \times n \times \ln 2/(2^n - 1) - 1$.

SIDD. The probability that superhelically stressed DNA underwent strand separation events was analyzed by introducing DNA sequences into Benham's stress-induced DNA duplex destabilization (SIDD) program (http://genomics.ucdavis.edu/benham/sidd/index.php) and determining the probability profile using near-neighbor computing (3).

RESULTS

***oriC* is the only initiation site for HIR.** To study the strict requirement of *oriC* for HIR, we used strain CM1671, in which the complete *oriC* sequence from *gidB* to *asnA* was deleted. Inhibition of replication was suppressed by F plasmid inte-

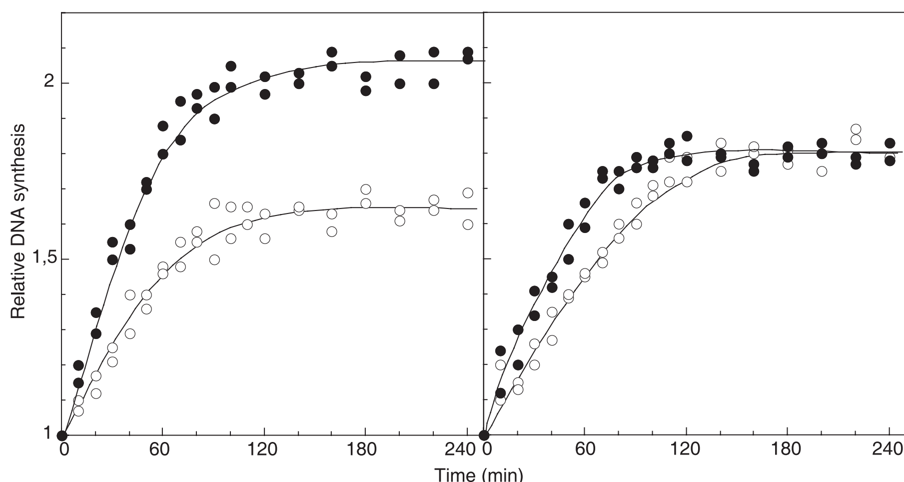

FIG. 1. Replication runout of strains CM1588 (left panel) and CM1671 (right panel) after addition of rifampin at 30°C (○) or together with a shift to 41°C (●).

TABLE 2. HIR of a strain with *oriC* deleted

Strain	Characteristics	ΔG at 30°C	ΔG at 41°C	HIR (%)
CM1565	*oriC*$^+$	64	103	23.8
CM1588	*oriC*$^+$ *zif*::F	65	105	24.2
CM1671	Δ*oriC1071 zif*::F	80	80	0

grated at min 85. Replication runout after inhibition of initiation with rifampin was used to determine the relative numbers of origins before and after the temperature shift from 30°C to 41°C in the control strain and the strain with *oriC* deleted (Fig. 1). We found HIR in about 24% of the origins present in parental strain CM1565 and in strain CM1588 with F integrated but not in strain CM1671 with *oriC* deleted (Table 2). Thus, HIR is initiated neither outside *oriC* nor in the F replication origins.

Functional *oriC* mutations allow HIR. In order to detect the specific *oriC* sequence required for HIR, a number of *oriC* mutant strains were used. These strains included strains with scrambled, shifted, and inverted DnaA boxes, altered IHF and FIS binding sites, deletion of nucleotides 275 to 352, and insertion of 14 nucleotides between R3 and R4. All the mutations tested induced HIR after the temperature upshift (Table 3). Some of the mutations tested affected the synchrony of cyclic initiation to different extents, but others did not affect the synchrony of cyclic initiation (36). All mutant strains induced about the same amount of HIR, and there was no detectable correlation with the asynchrony phenotype.

Induction of HIR requires the L-13-mer of *oriC*. Deletion of the L-13-mer made *oriC* inactive and could be rescued only by integrative suppression. To test the induction of HIR in a mutant bacterium, we used strain CM735 *intFs-B*, in which the L-13-mer was replaced by a mini-F plasmid (8). No HIR was detected in this strain (Table 3). This shows that L-13-mer is a sequence that is required for HIR.

HIR can be induced in other replicons. Above we show that HIR is specific for *oriC* and is not induced from the F plasmid. It might seem that this stress replication is specific for the *E. coli oriC*. To check for this specificity, we studied HIR in a strain with all of *oriC* deleted, CM1843, and in a strain with L-13-mer deleted, LK211, both suppressed by the integration of an R1 derivative plasmid. When these R1-suppressed strains were shifted from 30°C to 41°C, they exhibited high levels of HIR induction (Table 4). This indicates that a replicon other than *oriC* can perform HIR.

TABLE 3. HIR of *oriC* mutant strains

Strain	Characteristics	Asynchrony phenotype[a]	HIR (%)
WM2482	MG1655	None	19.5
WM2759	Δ275-352	None	22.8
WM2762	R2 scrambled	Low	19.2
WM2763	R3 scrambled	None	20.0
WM2764	R4 scrambled	High	20.0
WM2765	R1 at R3 position	None	19.0
WM2766	Mutant FIS site	None	22.0
WM2767	Mutant IHF site	Low	19.5
WM2768	R4 inverted	None	23.4
WM2844	M scrambled	Low	20.4
WM2845	+14 bp between R3 and R4	High	10.1
CM735 *intFs-B*	L-13-mer deleted, integratively suppressed by mini-F	High	0

[a] Data from references 8 and 36.

TABLE 4. HIR at R1 replicon

Strain	Characteristics	ΔG at 30°C	ΔG at 41°C	HIR (%)
CM1793	*oriC*$^+$ *zij*::R1	60	95	21.8
CM1843	Δ*oriC1071 zij*::R1	60	100	25.0
ER	*oriC*$^+$	75	115	22.8
LK211	L-13-mer deleted, integratively suppressed by R1	81	120	21.5

Expected SIDD sites in *oriC* suggest that there is local DNA denaturation of L-13-mer for HIR initiation. Local denaturation is a fundamental process required for initiation of replication, as well as other biological processes, such as transcription. We studied the SIDD of the wild-type *oriC* sequence and of mutants with mutations that did or did not affect HIR induction. The analysis of wild-type *oriC* showed that there was a high local probability of stress-inducible denaturation for a site located between positions 25 and 36 containing the L-13-mer (Fig. 2). This SIDD analysis performed with the mutant sequences, which conferred an HIR-inducible origin, revealed the same denaturation profile with the same local stress-destabilized site as the wild type. Deletion of the 16 bases from positions 23 to 38 eliminated the high probability of a stress-destabilized site, as expected. These results indicate that the heat-induced destabilization of the L-13-mer is an essential determinant for HIR initiation.

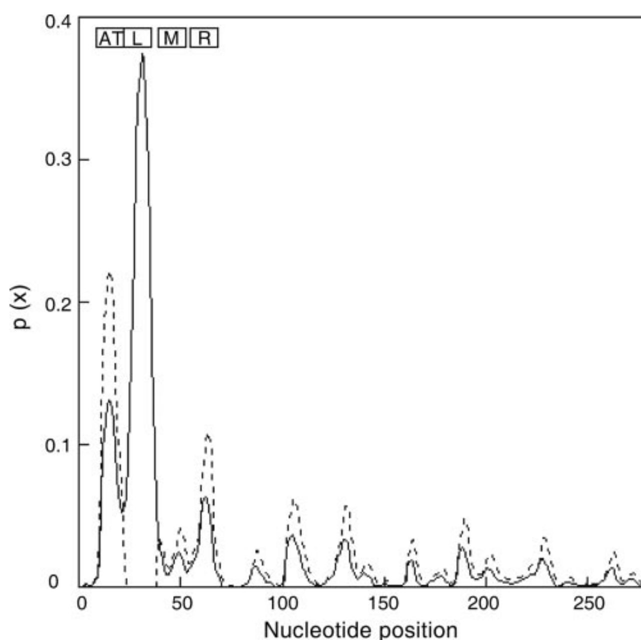

FIG. 2. Benham's probability of stress-induced DNA duplex destabilization in wild-type *oriC* (solid line) or in the sequence with L-13-mer deleted (dashed line). The nucleotide positions are the positions described by Gille and Messer (11). The boxes show the positions of the AT cluster and the L-, M-, and R-13-mers.

TABLE 5. DnaA requirement for HIR

Strain	Characteristics	ΔG at 30°C	ΔG at 41°C	HIR (%)
JK607	Wild type	42	70	19.7
JK876	*dnaA*46	45	35	0

HIR requires DnaA protein. RNA polymerase activity is required at the initiation step of chromosome replication for local DNA destabilization, facilitating opening of the chromosome by DnaA (2, 5, 29). In previous work we showed that HIR does not require RNA polymerase activity (4). In this study we found that mutations in single DnaA binding sequences that allow *oriC* function also allow HIR. Consequently, we tested whether the DnaA protein was also required for HIR initiation. We used a *dnaA46* mutant strain, which was thermosensitive at the initiation of chromosome replication due to the failure of DnaA46 in ATP binding and its thermolabile interaction with other proteins (14, 15, 16, 24). Table 5 shows that the presence of the mutant DnaA protein prevented HIR induction. This dependence of HIR on DnaA must be *oriC* specific, since R1 suppressed strain LK211, which did not require DnaA for initiation of replication, exhibited similar HIR values in a *dnaA* wild-type background and in a *dnaA* null mutant strain (data not shown).

DISCUSSION

Thermal stress induces in all kinds of cells a number of new functions that counteract the adverse consequences of a sudden increase in the environmental temperature. The main physiological switch in *E. coli* is induction of the synthesis of σ^{32}, which promotes expression of the heat shock genes (12). We reported previously that another consequence of thermal stress is induction of unscheduled replication, which we named HIR (4, 13). This stress replication was not a heat shock response, as it was not affected either by an *rpoH* deletion or by RNA or protein synthesis inhibition, and by using marker frequency analysis we determined that it initiates in *oriC*.

Here we found that HIR initiates in *oriC* and only in this site, as indicated by the following findings. First, a bacterial strain in which *oriC* was completely deleted and which replicated from an integrated F plasmid did not induce HIR. Second, deletion of the L-13-mer of *oriC* and insertion of a mini-F plasmid at this site, which made the chromosome replicate from the integrated plasmid replicon, did not induce HIR. These results show that the L-13-mer sequence is required for HIR and that no other sequence outside *oriC* is used for this stress-induced initiation of replication.

To look for other specific sequence requirements in *oriC* for HIR, we tested a number of mutants with altered DnaA, Fis, or IHF binding sites or with deletions and insertions that altered *oriC* functionality, such as the synchrony of the initiation step, but did not inhibit the initiation activity. All these mutant strains induced HIR, and no effect of the asynchrony phenotype on the number of stress-induced origins was observed.

DNA strand opening is the first step required for initiation of replication. The required *oriC* opening for the initiation of cyclic replication is accomplished by binding of the DnaA protein to the eight DnaA boxes in *oriC* with the aid of RNA polymerase and other proteins. Structural distortion of DNA is a prerequisite for the second function of DnaA in the initiation process, loading of the replicative helicase DnaB. Using chemically modified single-stranded DNA, Gille and Messer (11) obtained the first in vivo evidence that the region containing the three 13-mers in the left part of *oriC* is the first sequence that destabilizes during unwinding of *oriC*, and they supported the proposal that unwinding is initiated at the R-13-mer (5, 6). Our results obtained with deletion mutants show that HIR requires the L-13-mer, and in the absence of this sequence no other site is used to initiate this stress replication. The study of the SIDD of *oriC* by computer simulation as described by Fye and Benham (10) showed that there is a highly local stress-inducible denaturation site consisting of 12 nucleotides in the L-13-mer. In the absence of this sequence, no other SIDD site appears. This analysis, although not probative, correlates with our results for inhibition of HIR in the absence of L-13-mer. Consequently, we propose that HIR initiates by denaturation of the L-13-mer. This local denaturation could promote the unwinding of the nearby AT-rich region, assisted by the transient changes in DNA topology due to the thermal stress (22). We also found similar SIDD sequences in the *oriF* sequences of mini-F and in *oriR1* of mini-R1 (data not shown), although an integrated F plasmid showed no detectable HIR. RepE initiator protein-mediated *incC-oriF* DNA looping negatively regulates F plasmid replication by inhibiting the formation of an open complex at *oriF* sequences in vitro (37). We suggest that *the oriF* SIDD sequence is not available for duplex destabilization as a result of steric hindrance, due to RepE-*oriF* complex formation, and therefore is not available for HIR.

Thermal DNA unwinding might supersede the requirement for the melting action of DnaA assisted by RNA polymerase. In previous work we showed that this stress replication does not require RNA polymerase for initiation (4). In the present work we found that DnaA activity is required for HIR in *oriC*. Consequently, we suggest that the initial local denaturation induced by the heat stress is located at the L-13-mer and that a functional *oriC* is required for HIR initiation. In this initiation step the DnaA protein might not be required for strand opening, as strand opening does not require the RNA polymerase destabilization function, but DnaA might be required for other initiation functionality, such as DnaB loading.

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