SeqA: A Negative Modulator of Replication Initiation in E. coli

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Summary

In E. coli, replication initiates at a genetically unique origin, oriC. Rapidly growing cells contain multiple oriC copies. Initiation occurs synchronously, once and only once per cell cycle at all origins present. Secondary initiations are prevented by a sequestration process that acts uniquely on newly replicated origins, which are marked because they are hemimethylated at GATC sites. We report the identification of a gene required for sequestration and demonstrate that this gene, seqA, also serves as a negative modulator of the primary initiation process. All previously identified in vivo initiation factors play positive roles. Thus, precise control of replication initiation may involve a balance between positive and negative elements. We suggest that SeqA might be a cooperativity factor, acting to make the replication initiation process dependent upon cooperative interactions among components.

Introduction

The Escherichia coli genome is a single circular chromosome whose replication initiates from a unique site oriC. Replication initiation is very precisely controlled. The interval between initiation events varies with growth rate. The basis for growth rate regulation is unknown. The timing of initiation can be varied somewhat by changing the level of DnaA initiator protein, but additional factors remain undiscovered (reviewed by Skarstad and Boye, 1994). Additional complexities exist because rapidly growing cells contain multiple copies of oriC. A complete round of DNA replication and subsequent cell division requires more than 60 min, but bacteria have the capacity to divide as frequently as once every 20 min. Thus, new rounds of initiation occur while previous round(s) are still in progress.

At the appropriate time in the cell cycle, all origins within a given cell initiate replication synchronously (Skarstad et al., 1986). Thus, the initiation process responds sensitively and dramatically to a small incremental change in internal cell physiology. Nonetheless, each copy of oriC only undergoes one round of initiation per cell cycle. This outcome may be achieved by the combined effects of two processes (Campbell and Kleckner, 1990; Hansen et al., 1991; Mahaffy and Zyskind, 1989). Initiation potential increases to a high level and triggers efficient and synchronous initiation at all available origins by virtue of one or more highly cooperative interactions. Then, unwanted additional rounds of initiation are prevented by a secondary block that we call sequestration.

The existence of a mechanism that blocks secondary initiation events was proposed originally by Russell and Zinder (1987) who found that oriC minichromosome plasmids efficiently transform a strain lacking DNA adenine methylase (dam) only if the plasmid DNA is unmethylated; for fully methylated DNAs, transformation efficiency was dramatically reduced. The 245 bp minimal oriC contains 11 GATC sites (Zyskind and Smith, 1986), substrates for Dam methylation. Fully methylated plasmids introduced into a Dam− host replicate once and are blocked from further replication by their (permanent) hemimethylated state. In normal chromosomal replication, freshly replicated origins will be hemimethylated until newly synthesized strands become methylated, so a process that specifically blocks replication initiation at hemimethylated oriC could serve to block additional rounds of initiation at a time when initiation potential is high.

The existence of such a process has been demonstrated further. First, GATC sites in oriC remain hemimethylated for a long time following replication initiation (Ogden et al., 1988; Campbell and Kleckner, 1990). So too does a GATC site in the promoter region of the dnaA gene, which encodes the primary oriC recognition protein; here, sequestration blocks transcription (Campbell and Kleckner, 1990). Since this gene is located close to oriC, sequestration at the dnaA locus could facilitate a drop in initiation potential immediately after replication initiation (see Theisen et al., 1993). Second, certain subcellular fraction(s) containing outer membrane components are highly enriched in hemimethylated oriC sequences (Ogden et al., 1988), specifically bind exogenously added hemimethylated oriC DNA (Hendickson et al., 1982; Kusano et al., 1984; Ogden et al., 1988; Chakraborti et al., 1992), and block replication initiation (Landoulsi et al., 1990). Third, in Dam− cells, initiations are asynchronous and interinitiation times are variable, as expected from a sequestration defect (Boyé et al., 1988; Boye and Løbner-Olesen, 1990; Bakker and Smith, 1989).

Sequestration does not determine the timing of replication initiation in the cell cycle as the origin is released from sequestration after about one-third of a generation time (Ogden et al., 1988; Campbell and Kleckner, 1990). oriC may be released from sequestration passively, by action of dam methylase (Landoulsi et al., 1990; A. Løbner-Olesen, personal communication).

We report here the identification of a gene required for sequestration, seqA. The phenotypic effects of both a seqA− null mutation and of increased seqA expression, alone and in combination with other mutations, suggest...
that seqA also negatively modulates the primary replication initiation process.

Results

The seqA Gene and Its Role in Sequestration
Isolation of a Sequestration-Defective Mutant

Because of sequestration, a fully methylated oriC plasmid gives transformants in a Dam" strain at about a 200-fold reduction of the frequency of an unmethylated version of the same plasmid (Table 1, line 2). An E. coli mutant defective in sequestration was obtained as a derivative of a Dam" strain that now efficiently accepts oriC minichromosomes (Campbell, 1991). A dam::Tn9 muth3 strain was mutagenized and subjected to two rounds of selection with differentially marked, fully methylated oriC plasmids. Independent plasmids were constructed from oriC-kan. KanR transformants from each culture were pooled and transformed with oriC-tet. One pool gave a very high level of transformants, indicating that at least one original KanR transformant was the desired mutant. Dam" and Seq" phenotypes of several such TetR transformants were confirmed. The mutation in one transformant was mapped by P1 transduction to 16 min on the E. coli genetic map. This mutation, seqA1, abolishes sequestration in an unmutagenized strain background (Table 1).

Molecular Analysis

The seqA gene was localized by complementation analysis to two Kohara library phages (Experimental Procedures). Subcloning yielded a 930 bp BssHII fragment that fully complements seqA1 (Table 1). Sequence analysis revealed two linked open reading frames preceded by a promoter consensus sequence (Figures 1A and 1R). The first open reading frame is seqA; a plasmid encoding only this gene and the putative promoter complements segA1 (Table 1). Sequence analysis revealed two linked open reading frames preceded by a promoter consensus sequence (Figures 1A and 1B). The second open reading frame is seqA; a plasmid encoding only this gene and the putative promoter complements seqA1 (Table 1).

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Table 1. Effect of seqA Mutations on the Ability of Dam" Strains To Be Transformed by Methylated and Unmethylated Minichromosomes

<table>
<thead>
<tr>
<th>Strain Genotype (chromosome with plasmid)</th>
<th>Relative Transformation Efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unmethylated (A)</td>
</tr>
<tr>
<td>dam&quot;</td>
<td>0.85</td>
</tr>
<tr>
<td>dam::Tn9</td>
<td>1.0</td>
</tr>
<tr>
<td>dam::Tn9 seqA1</td>
<td>0.70</td>
</tr>
<tr>
<td>dam::Tn9 seqA1 with pseqA*</td>
<td>0.85</td>
</tr>
<tr>
<td>dam::Tn9 seqA::tet</td>
<td>0.76</td>
</tr>
<tr>
<td>dam::Tn9 seqA::tet with pseqA*</td>
<td>0.96</td>
</tr>
</tbody>
</table>

poriC-kan is pJC35, and pseqA* is pML5. All strains are derivatives of MM294 and contain plasmid pML14 to the Pgm- defect conferred by the seqA::tet allele. For unmethylated poriC-kan in the dam::Tn9 strain, the absolute efficiency of transformation was 4.6 x 10^6 transformants/µg. Essentially identical B/A ratios were obtained in three other strain backgrounds (NK7254, CM735, and W1485).

* Average of three separate experiments.

The protein contains an unusually high percentage of positively and negatively charged residues, and the region preceding and overlapping the beginning of seqA includes putative binding sites for several factors implicated in growth rate regulation, replication initiation, or both: factor for inversion stimulation (FIS), integration host factor (IHF), leucine-responsive protein, and DnaA (Figure 1B). GATC sites occur only at the distal end of the gene. Codon composition and analysis of extracts with SeqA antibodies suggest that the in vivo level of SeqA protein is very low.

A seqA Null Mutant Is Viable and Sequestration Defective

A strain carrying a seqA null allele marked by tet genes (Figure 1A) is viable. The null allele was first integrated as part of a nontandem direct repeat in which mutant and wild-type alleles are separated by the sacB gene (Slater and Maurer, 1993). Recombinational segregants retaining only one allele were obtained by selection against sacB. Wild-type and mutant versions arose at similar frequencies, (55% and 45%, respectively) corresponding to the nearly equal lengths of homology available for the two requisite recombination events. The seqA deletion also eliminates sequestration (Table 1).

seqA Is Required for Maximally Delayed Remethylation at Sequestered GATC Sites

Two individual GATC sites in oriC and one GATC site in the dnaA promoter normally persist in hemimethylated form for about 13 min following replication, after which remethylation rapidly ensues; at a control site in uncB not subject to sequestration, the hemimethylated state persists for 1.5 min (Campbell and Kleckner, 1990). The seqA null mutation reduces the lifetime of hemimethylation at sequestration-sensitive sites to 5 min and has no effect at the control site (Figure 2).

Remethylation kinetics are determined by analysis of DNA extracted at appropriate intervals from a culture in which replication has initiated simultaneously at all origins in all cells. A culture of an initiation-defective dnaC" mutant (seqA" or seqA::tet) is held at nonpermissive temperature (42°C) for 1 hr and then returned to permissive temperature (30°C). During 42°C incubation, ongoing rounds of replication are completed, and replication initiation arrests at the DNA step. Upon return to 30°C, initiation occurs synchronously at all origins; secondary initiations...
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(A) Restriction map drawn to scale in kb. seqA::tet contains a 2.8 kb BglII fragment of Tn10 substituted for a 0.36 kb Apol-Eagl FIS (Finkel and Johnson, 1992), leucine-responsive protein (Rex et al., 1991) are indicated by solid underlining, dashed underlining, and wavy underlining, respectively. GATC sites are prevented by returning cells to 42°C after 6 min at 30°C. In DNA extracted from such cells, the methylation status of individual GATC sites is determined at positions where half of the GATC sequence overlaps the recognition site for a restriction enzyme whose cleavage is sensitive to methylation at the shared A residue (Campbell and Kleckner, 1990; Figure 2A). When the chromosomal region containing such a site is fully methylated, the diagnostic restriction enzyme fails to cleave at the site of interest but cleaves successfully at flanking sites that do not overlap GATCs. After replication, 1 of 2 hemimethylated species can be cleaved, and the level of a corresponding fragment represents half of the hemimethylation level at this site.

In seqA+ dnaC0, the level of hemimethylated DNA at the GATCs in oriC and dnaA rises to 100% and remains there for several min. In seqA+ dnaC0, at both sites analyzed, hemimethylated DNA rises but never reaches 100% and then rapidly falls. The durations of the hemimethylated state at these sites in seqA+ are 13.9 and 13.3 min and in seqA- are 5.5 and 5.3 min. The kinetics of remethylation at the unsequestered uncB site is the same for both strains: the level of hemimethylated DNA rapidly increases to 15% and then rapidly decreases with a corresponding duration of 1.5 min.

Two additional findings are of interest. First, even in seqA-, both sequestration-sensitive sites remain hemimethylated much longer than the uncB control site and longer than most other GATCs in the genome (Campbell and Kleckner, 1990). These two sites might simply be poor substrates for Dam methylase. Alternatively, sequestration may involve components in addition to SeqA protein that still have effects by themselves. Second, the seqA+::tet mutation delays replication initiation by about 3 min, as appearance of hemimethylated DNA is delayed by this amount at all sites examined.

Genes Not Required for Sequestration
Dam- strains carrying each of the following mutations are sequestration proficient by the oriC transformation assay: himA::Tn10, hip::cat, fic::kan, mutH::Tn5, hupB::kan, lrp::Tn10, and iciA::kan (data not shown). Also, dnaA+ (all alleles, below), dnaA46+, and dnaC-PC2 mutants are Seq’ strains, both sequestration-sensitive sites remain hemimethylated much longer than the onc6 control site and longer than most other GATCs in the genome (Campbell and Kleckner, 1990). These two sites might simply be poor substrates for Dam methylase. Alternatively, sequestration may involve components in addition to SeqA protein that still have effects by themselves. Second, the seqA+::tet mutation delays replication initiation by about 3 min, as appearance of hemimethylated DNA is delayed by this amount at all sites examined.

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Aberrant Physiology of a seqA Mutant
Replication Initiation Asynchrony
Since replication initiation is normally synchronous at all origins within each given cell, wild-type cells always contain a number of origins that is an integral power of 2; the exact distribution depends on growth rate (Skarstad et al.,

Bank accession number is U07651.

Figure 1. The seqA Locus
(A) Restriction map drawn to scale ± 0.1 kb. seqA::tet contains a 2.8 kb BglII fragment of Tn10 substituted for a 0.36 kb Apol-Eagl segment encoding residues 34-154 of SeqA protein. (B) seqA coding region (161 codons) and flanking sequences. Consensus sequences for transcription and translation initiation are boxed; a circled methionine is a second in-frame ATG. Sequences that exhibit a reasonable match to suggested consensus binding sequences for FIS (Finkel and Johnson, 1992), leucine-responsive protein (Rex et al., 1991), IHF (Goodrich et al., 1990), and DnaA proteins (Schaefer and Messer, 1991) are indicated by solid underlining, dashed underlining, dotted overlining, and wavy overlining, respectively. GATC sites...
Origin content is determined by blocking replication initiation and cell division, permitting ongoing rounds of replication to be completed, and counting the genome equivalents of DNA present in individual cells after run-out is complete. The distribution of DNA contents in a large number of such cells is determined by fluorescence labeling and flow cytometry (Boye and Løbner-Olesen, 1991).

In M9 minimal glucose medium, wild-type cells mostly contain two origins (Figure 3a). In contrast, seqAd::tet mutant cells contain all possible numbers of origins between one and five (Figure 3b). The presence of cells containing inappropriate numbers of origins indicates that replication initiation is aberrant (Boye and Løbner-Olesen, 1991). Cells with two or four origins are more frequent than other types, however, suggesting that some degree of synchrony is retained. As for Dam- cells (Boye et al., 1988), asynchrony in seqAd::tet is expected from its sequestration defect, but inefficiency or aberrant control of the primary initiation process could also contribute.

**Overinitiation of DNA Replication**

The seqAd::tet mutant grows aberrantly. In Luria–Bertani (LB) medium, colonies are very small (Figures 4a and 4b), doubling time is 1.6 x that of wild type (Table 2), and long septate snakes are abundant (Figures 5a and 5f). From several observations, these defects can be explained as follows. Overinitiation leads to the presence of excess replication forks that are often more closely spaced than normal and cannot be processed as rapidly as they are generated. Accumulation of stalled forks causes induction of SOS functions, delayed cell division, and cell growth defects. The first observation is as follows. All growth aberrancies decrease in severity as growth rate decreases (see Figures 4c and 4d; Table 2; Figures 5a–5d). A slower growth rate means a longer interval between initiations and thus a longer time for clearance of one pair of forks prior to arrival of the next.

The second observation is as follows. Colony size of seqAd::tet on LB is dramatically improved by mutations that compromise replication initiation: dnaA alleles at intermediate but permissive temperatures (Figure 6A, compare panels b and d with panels f, h, i, and n; Table 3), hip::cat, a mutation affecting IHF (Figure 7C, compare panels a–d; Table 4, line 3), and dam::Tn9 (Figure 7C, panels a, b, g, and h; Table 4, line 9).
The third observation is as follows. Viability of seqAΔ::tet on LB requires expression of the SOS-induced cell division inhibitor, sulA: a seqAΔ::tet sulA::kan double mutant is inviable but is rescued by the presence of a Plac-sulA gene (Table 5). In fact, seqA cells are induced for SOS functions (data not shown). Also, the seqA mutant is dependent upon SOS induction for viability, and this requirement is due solely to the need for sulA expression: a block to SOS induction, due to overexpression of a dominant lexA allele, is lethal to a seqA mutant on LB. And this lethality is alleviated by expression of sulA from the heterologous promoter Plac (Table 5).

The fourth observation is as follows. seqA mutant cells growing in LB medium fail to give prominent peaks of fluorescence intensity in flow cytometry assays (see Figures 3e and 3f), suggesting that most ongoing rounds of replication cannot be completed. A similar phenotype occurs in cells overexpressing DnaA protein (Atlung and Hansen, 1993).

The fifth observation is as follows. The third and fourth phenotypes are all reduced or absent in seqA mutant cells growing more slowly (see Table 5; see Figures 3a–3d).

**seqA Negatively Modulates the Primary Replication Initiation Process**

**A seqA Null Mutant Suppresses dnaAΔ Lethality**

DnaA protein recognizes oriC during replication initiation, and temperature-sensitive dnaA alleles have been characterized (reviewed by Skarstad and Boye, 1994). The seqAΔ::tet mutation substantially suppresses dnaAΔ growth defects (see Table 3; see Figure 6A, compare panels g, h, m, and n). For every dnaAΔ allele at nonpermissive temperature (40°C), semipermissive temperature (42°C), or both, the seqAΔ::tet mutation restores growth, increases colony size irrespective of growth medium (see Table 3), or both. seqAΔ::tet suppresses dnaAΔ inviability even in the presence of mutations that strongly exacerbate the dnaAΔ defect. IHF and FIS proteins are suspected to
Table 2. Growth and Chromosome Segregation in seqAΔ::tet and seqA+ Strains

<table>
<thead>
<tr>
<th>Strain Genotype</th>
<th>Growth Medium</th>
<th>Doubling Time (minutes)</th>
<th>Anucleate Cells/1000 (Three cultures)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>LB</td>
<td>28</td>
<td>26 (±1)</td>
</tr>
<tr>
<td>seqAΔ::tet</td>
<td>LB</td>
<td>44</td>
<td>39 (1.6)</td>
</tr>
<tr>
<td>wild type</td>
<td>M9 glucose</td>
<td>54</td>
<td>55 (±1)</td>
</tr>
<tr>
<td>seqAΔ::tet</td>
<td>M9 glucose</td>
<td>70</td>
<td>69 (1.3)</td>
</tr>
<tr>
<td>wild type</td>
<td>M9 glycerol</td>
<td>144</td>
<td>138 (±1)</td>
</tr>
<tr>
<td>seqAΔ::tet</td>
<td>M9 glycerol</td>
<td>160</td>
<td>156 (1.1)</td>
</tr>
</tbody>
</table>

Cultures of a wild-type strain NK7254 and a seqAΔ::tet derivative, both containing pML14, were grown in exponential phase in the indicated medium at 37°C. Absolute times required for doubling of colony forming units (CFU/ml) and cell mass (OD600) and doubling times of seqAΔ::tet relative to wild type in the same medium are shown. Cells were examined microscopically for the presence or absence of DNA as described in Figure 5, and they were examined without regard to whether they were of normal or aberrant length. Of the 17 anucleate cells observed in seqAΔ::tet in LB, 12 were snakes and 5 were single cells.

The dnaA46 hip::cat fis::kan seqAΔ::tet quadruple mutant is viable at all temperatures up through 39°C. The seqAΔ::tet mutation suppresses both the dnaA46 defect and the enhancement of that defect by hip::cat, but not the enhancement of that defect by fis::kan. Thus, a fis dnaA46 seqA triple mutant behaves essentially identically to a hip fis dnaA46 seqA quadruple mutant (see Table 4, lines 6 and 8, right side), while these triple and quadruple mutants both grow less well than their fis+ counterparts (see Table 4, lines 6 and 2, lines 8 and 4, right side).

Suppression of dnaA46 mutations by seqAΔ::tet does not appear to result from the presence in seqA- cells of an

![Figure 5](image-url)

**Figure 5.** Cells of the seqA+ and seqAΔ::tet Strains

NK7254 seqA+ and seqAΔ::tet strains containing the pgm+ complementing plasmid pML14 were grown exponentially at 37°C in the indicated media, fixed in ethanol, air dried on microscope slides, overlaid with a DAPI solution (1 μg/ml), and examined and photographed by combined fluorescence and phase contrast microscopy (with the Zeiss Axioplan fluorescence microscope).
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Figure 6. Genetic Interactions between seqA and dnaA

Single colonies on LB plates of strains mutations, plasmids, or both as indicated. All strains are isogenic derivatives of CM735, except for dnaA46cos, which is KA441.

(A) Mutually beneficial interactions between seqAΔ:tet and dnaA* mutations. Exponentially growing cultures were diluted and plated, and plates were incubated for 20 hr. All strains carry pML14(ori+). WT, wild type.

(B) Effect of seqA overexpression on dnaA* mutants. pML6 is a multicopy seqA plasmid; pNB8 is an isogenic seqAΔ control. Each plasmid was transformed into the indicated strain; transformation mixture was plated on selective plates that were scored after incubation at 30°C for 30 hr or at 34°C for 20 hr, except for dnaA4tF, for which plates were incubated at 30°C for 46 hr or 42°C for 16 hr. Other dnaA* alleles (Table 3) behaved similarly to dnaA46 and dnaA203.

Overexpression of seqA is Lethal To Strains Compromised for Replication Initiation

Overexpression of the seqA has the opposite effect of the seqAΔ::tet mutation; it exacerbates the defects conferred by dnaA*, hip::cat, himA::Tnl0, and fis::kan single mutations. The presence of a multicopy plasmid encoding the seqA gene and its upstream region, but lacking pgl, has little or no effect on a wild-type strain but impairs growth of all dnaA* mutants at the normally permissive temperature of 30°C and is lethal at 34°C or 37°C, depending upon the particular dnaA* allele (see Figure 6B, panels a–j). Furthermore, the seqA plasmid is lethal or nearly so for hip::cat, himA::Tnl0, and fis::kan single mutants (Figure 7B). These effects are particularly remarkable since these three mutations by themselves have little or no effect...
Table 3. Interactions between dnaA* and seqA::tet Mutations

<table>
<thead>
<tr>
<th>dnaA* Allele</th>
<th>seqA*</th>
<th>seqA::tet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>34°C</td>
<td>37°C</td>
</tr>
<tr>
<td>seqA+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>dnaAD Allele</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asynchrony Index</td>
<td></td>
<td></td>
</tr>
<tr>
<td>203</td>
<td>0.25</td>
<td>L</td>
</tr>
<tr>
<td>204</td>
<td>0.20</td>
<td>L</td>
</tr>
<tr>
<td>508</td>
<td>0.27</td>
<td>L</td>
</tr>
<tr>
<td>167</td>
<td>0.38</td>
<td>L</td>
</tr>
<tr>
<td>205</td>
<td>0.71</td>
<td>M&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>602</td>
<td>1.29</td>
<td>L</td>
</tr>
<tr>
<td>46</td>
<td>1.32</td>
<td>L</td>
</tr>
</tbody>
</table>

The seqA::tet mutation was introduced by F1 transduction into isogenic wild type and dnaA* derivatives of CM735 containing pML14. Duplicate cultures grown to exponential phase in LB at 30°C were diluted and titered on LB medium at the indicated temperatures. The minus sign indicates an efficiency of plating (<0.01) relative to the same cultures at 30°C; in all other cases, the efficiency of plating was 1 ± 0.1. Colony sizes (L, large; M, medium; S, small) are normalized to the colony size of dnaA+ CM735 at the same temperature.

The asynchrony index (Skarstad et al., 1966) is defined as the ratio of the frequencies of three- and five-chromosome cells (f<sub>T</sub> + f<sub>5</sub>) to four-chromosome cells (f<sub>4</sub>) in rifampicin-treated cultures after completion of ongoing rounds of replication assayed at 29°C in LB medium.

The seqA+ allele was introduced by F1 transduction into isogenic wild type and dnaA* derivatives of CM735 containing pML14. Duplicate cultures grown to exponential phase in LB at 30°C were diluted and titered on LB medium at the indicated temperatures. The minus sign indicates an efficiency of plating (<0.01) relative to the same cultures at 30°C; in all other cases, the efficiency of plating was 1 ± 0.1. Colony sizes (L, large; M, medium; S, small) are normalized to the colony size of dnaA+ CM735 at the same temperature.

Overexpression of seqA suppresses the hyperinitiation phenotype of dnaA<sup>add</sup>.

dnaA<sup>add</sup> is an intragenic suppressor of the ts allele dnaA46 (Kellenberger-Gujer et al., 1978). It retains the two codon substitutions of dnaA46 and has acquired two additional codon changes (reviewed by Skarstad and Boye, 1994). The dnaA<sup>add</sup> mutant is viable at 42°C but exhibits strong overinitiation at this temperature. In addition, dnaA<sup>add</sup> is now cold sensitive and fails to grow at or below 40°C (Kellenberger-Gujer et al., 1978), presumably owing to accentuation of the overinitiation phenotype at temperatures where the mutant protein is more active. The presence of the multicopy seqA plasmid or the Plac-seqA plasmid reverses the effects of the cos suppressor mutation: cold sensitivity is eliminated and temperature sensitivity is restored. Growth occurs only at temperatures <34°C, where the parent dnaA<sup>add</sup> strain carrying a control plasmid is inviable (see Figure 6B, panels k–n).

Dam Methylase Plays an Important Positive Role in Replication Initiation

GATC sites (nine) in oriC are spatially conserved among different bacteria (Zyskind and Smith, 1986). However, it has not been clear whether methylation at these sites plays any role in replication initiation beyond a role in sequestration. Neither absence nor moderate overexpression of Dam methylase dramatically inhibits replication initiation or cell growth (Boye and LaBnber-Olesen, 1990), and methylation of oriC has only minor effects on replication initiation in vitro (Hughes et al., 1984; Messer et al., 1985; Smith et al., 1985; Boye, 1991).

Table 4. Genetic Interactions among dnaA46, seqA::tet, hip::kan, and dam::Tn9 mutHLS::TnS Mutations As Reflected in Colony Sizes

<table>
<thead>
<tr>
<th>Strain Genotype</th>
<th>seqA&lt;sup&gt;*&lt;/sup&gt;</th>
<th>seqA::tet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30°C</td>
<td>34°C</td>
</tr>
<tr>
<td>wild type</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>dnaA46</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>hip::cat</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>hip::cat dnaA46</td>
<td>L</td>
<td>L</td>
</tr>
<tr>
<td>fis::kan</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>fis::kan dnaA46</td>
<td>L</td>
<td>M</td>
</tr>
<tr>
<td>hip::cat fis::kan dnaA46</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>dam::Tn9 mutHLS::TnS</td>
<td>M&lt;sup&gt;+&lt;/sup&gt;</td>
<td>M&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td>dam::Tn9 dnaA46 mutHLS::TnS</td>
<td>M</td>
<td>S</td>
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</tbody>
</table>

Isogenic derivatives of wild-type strain CM735 and its dnaA46 derivative containing pML14 were constructed, and duplicate cultures of each strain were analyzed on LB medium exactly as in Table 3. Essentially identical interactions were observed on M9 glucose medium and for three other dnaA<sup>*</sup> alleles (dnaA167, dnaA205, dnaA602).

a Colony sizes are abbreviated as follows: L, large; M, medium; and S, small.

b Plus sign represents a colony size slightly larger than that indicated by the letter code.

c Minus sign represents a colony size slightly smaller than that indicated by the letter code.
A significant role for dam methylase in the primary initiation process is revealed here in three different situations. First, a dam::Tn9 mutation is strongly deleterious to dnaA" mutants. A dnaA46 mutant makes normal size colonies at 37°C, while a dam::Tn9 dnaA46 double mutant is inviable at this temperature (Figure 7A, panels g and h; see Table 4, lines 2, 9 and 10, left side). Potential complications from uncontrolled mismatch correction (Radman et al., 1980) were eliminated by mutS, mutL, or mutH insertion mutations. Corresponding effects are observed at other temperatures and with other alleles (data not shown). Second, overexpression of seqA via either of the plasmids discussed above is lethal to a dam::Tn9 mutant (Figure 7B, panels g and h). Third, growth of a seqA mutant is greatly improved by a dam mutation, as by other mutations that compromise initiation (Figure 7C, panels b and h; see Table 4, lines 1 and 9, right side). Dam methylase presumably exerts these effects indirectly via GATC methylation at oriC, though a direct role for methylase protein per se is not excluded.

GATC Methylation and seqA Have At Least Some Independent Functions

GATC methylation and SeqA function can each influence cell growth (and, thus presumptively, replication initiation) in the absence of the other determinant. In the absence of GATC methylation or hemimethylation, i.e., in a dam- strain, overproduction of SeqA protein is lethal (above). Conversely, in a seqA::tet strain, a dam- mutation improves growth (above); and in a dnaA46 mutHLS seqAΔ strain, a dam- mutation is strongly deleterious (see Table 4, compare lines 2 and 10, right side).

In a dnaA" Strain, a Dam Mutation Is Epistatic to a seqA Mutation

A dam mutation is equally deleterious to dnaA" in the presence or absence of seqA; that is, the dam defect is epistatic to the seqA" defect in dnaA". The simplest interpretation of this result is that the effect of dam methylation on dnaA" function precedes the effect of seqA function. If methylation and seqA function acted independently but at the same step, elimination of seqA should still improve dnaA" growth. An alternative but more complicated possibility would be that dam methylation cooperatively affects both the dnaA" defect and the seqA" defect. If so, the seqA"-dependent step could be at the dam-dependent step or later.

None of the Genetic Interactions Observed Extend to dnaC-PC2

DnaC is required at a stage in the initiation process subsequent to the major UnaA-promoted events defined thus far, i.e., subsequent to formation of an open complex at oriC.

Figure 7. Genetic Interactions among dnaA46, seqAΔ, seqA Overexpression, and Mutations in the Structural Genes for IHF, FIS, and Dam Methylase

(A and C) The effects in these derivatives of either the dnaA46 mutation (A) or the seqAΔ::tet mutation plus the pgm complementing plasmid pML6 (C) were determined by the plating of exponentially growing cultures at appropriate dilutions. (B) Effects in these derivatives of either a plasmid overexpressing seqA (pML6) or a control plasmid lacking seqA (pNB8) were examined by transforming each strain with each of the two plasmids and plating an appropriate dilution of the transformation mixture. Effects observed in (A) were confirmed in CM735; effects observed in (B) and (C) were confirmed in MM294, NK725, and CM735 backgrounds.
the prominent delay in remethylation following replication at sequestration-sensitive GATC sites.

to establish themselves in a Dam- host, and it eliminates seqA Function Plays a Negative Role in the assays: it permits fully methylated oriC minichromosomes dnaA, hip, him, fis, or dam genes (data not shown).

Primary Replication Initiation Process Genetic interactions between seqA alleles and mutations described above could all act at the earlier DnaA step(s) of replication initiation. If a DnaC-dependent step is strongly rate limiting for replication initiation in the dnaC-PC2 mutant, minor defects at earlier steps should have little effect on growth rate.

iciA Mutants Are Unaffected by Genetic Alteration of Replication Initiation The iciA gene encodes a protein that binds oriC and can inhibit replication initiation in vitro (see Hwang et al., 1992). However, iciA mutants have not yet been found to have any defect in replication initiation in vivo (see Boye et al., 1992). In this analysis, we have found that an iciA null mutation does not affect sequestration (above), does not confer sensitivity to overproduction of seqA, and has no effect on the growth of cells carrying mutations in the seqA, dnaA*, hip, him, fis, or dam genes (data not shown).

Discussion

The seqA Gene Is Required for Sequestration A seqA mutation confers a defect in sequestration by two assays: it permits fully methylated oriC minichromosomes to establish themselves in a Dam- host, and it eliminates the prominent delay in remethylation following replication at sequestration-sensitive GATC sites.

seqA Function Plays a Negative Role in the Primary Replication Initiation Process Genetic interactions between seqA alleles and mutations in other genes further define seqA as a negative factor for replication initiation. Elimination of seqA function ameliorates initiation defects conferred by dnaA* mutations even when exaggerated by additional mutations in fis, himA, hip, or both. There is no reason to suppose that a defect in the efficiency with which first rounds of replication are initiated should be suppressed by permitting an additional round of replication to occur on templates that have already initiated once. Conversely, overexpression of the seqA gene exacerbates defects conferred by dnaA*, hip, himA, fis, and dam mutations and suppresses the overinitiation phenotype of dnaA*.

Does seqA Act Directly? seqA might act directly on sequestration and replication initiation or indirectly, for example, by affecting expression of other genes such as dnaA. We favor the view that SeqA acts directly at oriC in both cases. With respect to sequestration, seqA is the only gene other than dam* known to be involved and is likely to be the only nonessential gene required (above). If SeqA acts directly during sequestration, it is then simplest to invoke a related role during initiation. The finding that SeqA overproduction qualitatively alters the effect of the dnaA* mutation also seems suggestive of a direct interaction.

The ability of seqA to suppress dnaA* is not so easily attributable to increased expression of dnaA (or any other gene inhibited by hemimethylation-dependent sequestration). dnaA is normally turned off by SeqA during a small fraction of the cell cycle only, so relief of sequestration should result only in a small increase in the overall level of expression. Since the dnaA gene is autoregulated, a seqA mutation could increase dnaA expression independent of sequestration. If SeqA affects DnaA activity per se (below). But in this case, initiation should also be affected directly as well.

Coordinate Evolution of Synchrony, Growth Rate Control, and Sequestration Is Biologically Sensible Why should the same protein be involved, directly or indi-
Regulation of Replication Initiation Involves a Balance of Positive and Negative Factors

Identification of a negative factor for replication is particularly interesting because all previously identified components known to affect initiation in vivo act positively. We suggest that the role of seqA in replication initiation is primarily regulatory and that replication initiation, like many other biological processes, is controlled by changes in the balance between positive and negative factors.

How might a negative factor participate in regulation of replication initiation? Early considerations of negative control mechanisms suggested that initiation might be triggered by dilution of an inhibitor (see Samitt et al., 1989). We would like to formulate the possible role of a negative regulator somewhat differently and to suggest that SeqA might function as a cooperativity factor.

Recent models for replication control have favored triggering of replication by accumulation of positive factor(s), usually DnaA protein (e.g., Hansen et al., 1991; Atlung and Hansen, 1993; Mahaffy and Zyskind, 1989). In such positive models, both the sensitivity of initiation to small differences in cell physiology and the synchronous initiation of all origins present in any individual cell can be attributed to the dependence of replication initiation on one or more highly cooperative interactions that would in turn be sensitive to the appropriate physiological signal(s). In the context of such models, the role of a negative factor (e.g., SeqA) might be to confer requisite cooperativity upon the initiation process. If such a factor were to impose or strengthen kinetic barriers, energetic barrier(s), or both to an appropriate critical transition(s) in the replication initiation pathway, the occurrence of initiation could thereby be made dependent upon the appropriate cooperative interactions. As a specific example, SeqA might allosterically impede the transition of DnaA from a conformation that functions early in the replication initiation process to another that is required for later steps, thereby making the required transition sharply dependent upon either the concentration of DnaA itself or the concentration of some other critical factor(s), as yet unidentified.

An interesting potential paradigm for replication initiation is provided by bacteriophage Mu transposition, where the biologically critical event is the DNA-promoted assembly of an active transposase tetramer at the ends of Mu. Cooperative collaborations involving weak interactions among many different components (including supercoiling, IHF, histone factor U, multiple transposase monomers, and an intricate array of protein-binding sites) ultimately provoke an important divalent cation-dependent conformational change in Mu transposase as well as assembly of selected monomers into the active ends-bound tetramer (Baker and Mizuuchi, 1992).

Specific Roles of SeqA, DnaA, and GATC Methylation during Replication Initiation

If the genetic interactions observed here reflect direct physical interactions during replication initiation, they imply that DnaA activity directly senses the methylation status of GATC sites, while SeqA interacts negatively with DnaA. A strong interaction is observed between dnaA* and dam* mutations: mutations in the two genes are synergistically lethal. Strong genetic interactions are also observed between dnaA and seqA (above).

Furthermore, the DnaA-GATC interaction may precede the DnaA-SeqA interaction because augmentation of the dnaA* defect by a dam mutation is epistatic to amelioration of the dnaA* defect by a seqA mutation. Finally, these interactions all appear to precede at least some DnaC-dependent step(s).

If the steps in replication initiation at oriC in vivo correspond to those defined in vitro, a reasonable biochemical representation of the observed genetic relationships would be that DnaA protein is sensitive to GATC methylation at early steps (e.g., binding of DnaA to DNA and initial interactions among DnaA monomers), while SeqA protein is inhibitory to DnaA action at latter stages in the process (e.g., during formation of an open complex).

An effect of SeqA on DnaA activity during open complex formation is suggested by both genetic and biochemical observations. First, the dnaA* mutant defect is suppressible by alterations that should facilitate open complex formation, e.g., increased superhelical tension (in a topA mutant; Louarn et al., 1984). Second, the dnaA* mutant defect is enhanced by himA and hip mutations. IFH often facilitates formation of complex protein-DNA structures and is stimulatory for open complex formation at oriC under appropriate in vitro conditions (Skarstad et al., 1990). Furthermore, a seqA mutation appears to suppress the IFH enhancement of the dnaA* defect as well as the dnaA* defect per se; this is the situation expected if the two factors affect the same step in the reaction. Third, DnaA mutant proteins have recently been shown to be specifically defective in open complex formation but not in DnaA binding (see Hupp and Kaguni, 1993).

A simple specific mechanism for SeqA action consistent with existing biochemical observations would be that SeqA blocks the allosteric transition of DnaA from an inactive form (potentially the ADP form) to an active (ATP) form.
The ATP form of DnaA is required for open complex formation in vitro (Sekimizu et al., 1987), and purified DnaA**R** protein is defective in ADP binding (T. Katayama and A. Kornberg, personal communication).

That GATC methylation acts at an earlier stage(s) is inferred from epistasis results. Dramatic effects of GATC methylation on DnaA binding or initiation have not been observed in vitro (above), but conditions, substrates, reaction components, or both substrates and reaction components may have been inappropriate. We note that fis function probably affects the same step as GATC methylation because there is no evidence of genetic interactions between seqA and fis in either dnaA or dnaA**R** strains (above) and because fis**-** dam**-** double mutants are essentially inviable even in combination with a mutSL mutation with or without a seqA mutation, while hip**-** dam**-** double mutants grow essentially normally (unpublished data).

Variations in seqA and dam activity can each have important effects in the absence of the other determinant. Thus, SeqA protein may not directly sense GATC methylation status. None of the observations presented excludes such a possibility, however.

Specific Roles of SeqA, DnaA, and GATC Methylation during Sequestration

It is attractive to suppose that the interactions between SeqA and DnaA and between DnaA and GATC methylation suggested above for replication initiation might also apply during sequestration.

The strong genetic interactions between seqA and dnaA have led us to consider the possibility that the primary effector of sequestration is in fact DnaA protein with its activity suitably modified by SeqA. A primordial form of sequestration might have existed or have evolved quite naturally as a consequence of a failure of used DnaA protein to become dissociated from oriC following replication initiation. Such an intrinsic tendency might then be further sharpened by the presence of SeqA (and methylation). This notion is supported by the fact that two seqA null mutant phenotypes are suggestive of residual sequestration capacity: an intermediate rate of remethylation at sequestration-sensitive GATC sites after replication initiation and residual initiation synchrony.

Similarly, if DnaA protein senses GATC methylation status during replication initiation, it could be responsible for recognition of hemimethylated DNA during sequestration as well. A general model that can explain the hemimethylation dependence of sequestration is presented in Figure 8. The effective determinant for sequestration is proposed to be a pair of specificity units present as inverted repeats, with each unit comprising a GATC methylation site in appropriate juxtaposition to a second, asymmetric sequence. Involvement of a second sequence in the specificity determinant has the specific advantage of explaining why only a small subset of GATC sites are subject to sequestration; clustering of GATC sites is clearly not sufficient (Campbell and Kleckner, 1990). In the present context, the asymmetric sequence should be a DnaA-binding site, and appropriate juxtapositions of DnaA-binding sites and GATC sites do occur at both oriC and the dnaA promoter (diagram not shown).

Both in vivo and in vitro studies demonstrate that a hemimethylated oriC is blocked from replication initiation even if introduced de novo rather than emerging from a replication initiation event (Russell and Zinder, 1987; Landoulsi et al., 1990). Furthermore, partially purified outer membrane fractions exhibit specific binding of hemimethylated linear fragments encoding part or all of oriC (Landoulsi et al., 1990; Ogden et al., 1988; Chakraborti et al., 1992). These observations can be reconciled with the above suggestions if used DnaA protein with appropriate substrate specificity resides by itself, even in the absence of oriC, in the appropriate subcellular location(s). Several lines of evidence argue for important interactions between DnaA and the membrane (reviewed by Kornberg and Baker, 1991; Skarstad and Boye, 1994). Furthermore, the block to replication of hemimethylated substrates may well be tighter upon emergence from active replication initiation: hemimethylated oriC minichromosomes are established in a Dam**-** strain after transformation several times more efficiently than are fully methylated minichromosomes, despite the fact that the latter molecules undergo a round of Ura replication prior to becoming sensitive to sequestration (Russell and Zinder, 1987).

Experimental Procedures

E. coli K12 Strains

MM294 is F+ supE44 hsdS endA1 pro thiA. NK7254 is leuD trp31 metB1
Replication Initiation in Escherichia coli

his1 argG trp140 tonA tpv supE44 k- (Hansen et al., 1984). RV and dnaAQ derivatives is meIE46 trp3 his4 thil galK2 lacy7 or lacZ4 mtll425

Replication Initiation in Escherichia coli

is dnac" lea thy stf' (Carl, 1970). KA449 and its dnaA- derivative

motL706:Tn5 and mutS704c:Tn5 (RH302 and 303, gifts of R. Hoess).

oriC-kan (pJC35) and oriC-tet (pJC50) are pCM959 (Meijer et al.,

Plasmids

(a gift of A. Wright). Sources of marked deletion or disruption alleles:

TnlO (NS373, a gift of A. Wright); su/A::TnC pyrD (GC4540; a gifi of

et al., 1988); iciA::kan (WBTl3, a gift of B. Thony); dnaA46 tnaA:c

Iran (DSCQO, Starts and Markovitz, 1988); fis::767 (RJ1617, Johnson

seqAA::tet (NK9050, this work); daml3::TnQ (GM2159, a gifl of M.

containing the pgm gene plus its own promoter. pMLl5 (pPlac-seqA

pNB8 (Bishop et al., 1992), respectively. pML14 is pACYCl77 con-

pNN388 (Elledge and Davis, 1989) and the multicopy Bluescript vector

chloramphenicol, 25 pg/ml; kanamycin, 25 vglml; tetracycline, 15 kg/

Media, Enzymes, and Chemicals

pACYCl84 (a gift of S. Gottesman).

p transduction was performed according to the procedure of Miller

Morrison (1977). Transformation mixtures contained 2 x 108 cells and

nant DNA techniques were based on those of Sambrook et al. (1989).

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