

# Reduced initiation frequency from *oriC* restores viability of a temperature-sensitive *Escherichia coli* replisome mutant

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The *dnaX* gene of *Escherichia coli* encodes  $\tau$  and  $\gamma$  clamp loader subunits of the replisome. Cells carrying the temperature-sensitive *dnaX2016* mutation were induced for the SOS response at non-permissive temperature. The SOS induction most likely resulted from extensive replication fork collapse that exceeded the cells' capacity for restart. Seven mutations in the *dnaA* gene that partly suppressed the *dnaX2016* temperature sensitivity were isolated and characterized. Each of the mutations caused a single amino acid change in domains III and IV of the DnaA protein, where nucleotide binding and DNA binding, respectively, reside. The diversity of *dnaA(Sx)* mutants obtained indicated that a direct interaction between the DnaA protein and  $\tau$  or  $\gamma$  is unlikely and that the mechanism behind suppression is related to DnaA function. All *dnaA(Sx)* mutant cells were compromised for initiation of DNA replication, and contained fewer active replication forks than their wild-type counterparts. Conceivably, this led to a reduced number of replication fork collapses within each *dnaX2016 dnaA(Sx)* cell and prevented the SOS response. Lowered availability of wild-type DnaA protein also led to partial suppression of the *dnaX2016* mutation, confirming that the *dnaA(Sx)* mode of suppression is indirect and results from a reduced initiation frequency at *oriC*.

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## INTRODUCTION

The DnaA protein is the initiator of chromosomal DNA replication from the origin of replication, *oriC*, in *Escherichia coli*. Analysis of sequence conservation, mutational analysis (Messer, 2002) and structural analysis (Erzberger *et al.*, 2002) suggest that the DnaA protein is composed of four domains. The conserved N-terminal domain I is involved in protein oligomerization and interaction with the DnaB helicase. Domain II lacks conservation, has a flexible structure and varies in length between different bacteria. Domains IIIa and IIIb are the ADP/ATP-binding and the sensor II domains, respectively, of the AAA+ family of ATPase-type proteins. Domain IIIa has a second interaction with the DnaB helicase and also has another oligomerization determinant as a part of the AAA+ motif. The start of domain IV is involved in membrane binding, and the remaining part of domain IV recognizes and binds to the 9-mer recognition sequences (DnaA-box) sequence motif. The crystal structure of domain IV in complex with a DnaA-box was recently determined, revealing the interactions between individual amino acids and nucleotides in the DnaA-box (Fujikawa *et al.*, 2003).

Initiation of *E. coli* chromosome replication is coupled to cell growth in such a way that it occurs when a certain cell mass per origin, the initiation mass, has accumulated

(Donachie, 1968). The initiation mass is fairly constant over a wide range of growth rates (reviewed by Bipatnath *et al.*, 1998) and is mainly set by accumulation of the DnaA protein in wild-type cells (Løbner-Olesen *et al.*, 1989). In different *dnaA* mutants the initiation mass is increased, indicating that the reduced activity of the DnaA protein is compensated for by a decreased concentration of origins in the cell (Boye *et al.*, 1996).

In the initiation process, the DnaA protein bound to either ATP or ADP recognizes and binds to its five DnaA-boxes in *oriC* (Fuller *et al.*, 1984). Subsequently, ATP-bound DnaA recognizes an additional set of 9-mer sequences (I-boxes; McGarry *et al.*, 2004). This triggers duplex opening in the AT-rich region, and DnaA-ATP stabilizes this 'open complex' by binding 6-mer motifs in the single-stranded AT-rich region (Speck *et al.*, 1999). In the final stages of initiation, DnaA interacts with the DnaB helicase and recruits it to the open complex (Marszalek & Kaguni, 1994; Seitz *et al.*, 2000) to form the 'pre-priming complex' that facilitates further strand separation and allows for entry of the replication machinery.

Fast-growing *E. coli* cells contain multiple origins of replication, which are synchronously initiated, once and only once per cell cycle. Several mechanisms contribute to this stringent control of chromosome replication. Immediate

reinitiation is prevented by sequestration of hemimethylated origins (Campbell & Kleckner, 1990). During sequestration at least three mechanisms operate to lower the activity of the DnaA initiator protein sufficiently for initiation only to occur one mass doubling later. First, the *dnaA* promoter is also sequestered (Lu *et al.*, 1994). This prevents *de novo* DnaA synthesis and accumulation during the sequestration period (Campbell & Kleckner, 1990). Second, new DnaA-binding sites outside the origin are generated by replication. These sites serve to lower the amount of free DnaA protein available for initiation. The most prominent of these DnaA binding sites, *datA*, is located about 0.7 Mb away from *oriC*. The *datA* region consists of two active DnaA-boxes that titrate large amounts of DnaA protein (Kitagawa *et al.*, 1998; Ogawa *et al.*, 2002) presumably in either ATP- or ADP-bound form. Third, DnaA-ATP is converted to DnaA-ADP by a process called RIDA involving the sliding clamp of active replication forks as well as the Hda protein (Suetsugu *et al.*, 2004).

*E. coli* cells with reduced DnaA protein activity, due either to certain mutations in domain III or IV of the protein, or to the introduction of additional *datA* sites, initiate replication at an increased cell mass per origin. Initiations are often asynchronous, indicating that not all origins are initiated each cell cycle. In such cells the time required to replicate the chromosome is reduced and cells contain only few active replication forks (Morigen *et al.*, 2003).

The *dnaX* gene encodes both the  $\gamma$  and the  $\tau$  subunits of DNA polymerase III holoenzyme by a frame-shifting mechanism and consequently these proteins are identical for the first 430 amino acids (domains I–III) (Blinkova & Walker, 1990; Flower & McHenry, 1990). The intensive research on the role of  $\tau$  and  $\gamma$  in formation of the DnaX complex was recently reviewed by McHenry (2003). The DnaX complex has a dual role in organizing the asymmetric dimeric replicase and loading the  $\beta_2$  clamp onto DNA. Domain I of  $\tau/\gamma$  has similarity to the AAA+ class ATPases, and both  $\tau$  and  $\gamma$  possess ATPase activity. Domains IV and V are only present in the longer  $\tau$  and are necessary for interaction with DnaB helicase and the core of DNA polymerase III. Two  $\tau$  subunits organize the replisome by connecting two cores with DnaB helicase, imparting coordinated leading and lagging strand synthesis and rapid fork movement. The  $\gamma$  subunit appears to organize the other subunits of the DnaX complex and connects this complex to the two  $\tau$  subunits. The entire DnaX complex has the stoichiometry  $\tau_2\gamma_1\delta_1\delta'_1\chi_1\psi_1$  in the mature *in vivo* DNA polymerase III. The *dnaX2016* temperature-sensitive mutation changes glycine 118 to aspartate in both  $\tau$  and  $\gamma$  (Blinkova *et al.*, 1993). This glycine is located between the Box IV' and the Walker B motif in the AAA+ domain I (Neuwald *et al.*, 1999), and the *dnaX2016* mutation potentially affects the ATPase activity of  $\tau/\gamma$ .

Extragenic mutations that partially suppress the temperature-sensitive phenotype of *dnaX2016* (Sx phenotype) are easily obtained. Some of these mutations reside in the *dnaA* gene

and consequently a direct interaction between the DnaA and the DnaX proteins was suggested (Walker *et al.*, 1982; Ginés-Candelaria *et al.*, 1995). Because these mutants were isolated for being concomitantly cold-sensitive, they are termed *dnaA*(Cs,Sx) mutants.

In this work seven new *dnaX*(Ts)-suppressing mutations in the *dnaA* gene are characterized. The amino acids changed in these mutants are widely distributed over domains III and IV of the DnaA protein. The initiation of replication is compromised in all the *dnaA*(Sx) mutants, implying that the mutant DnaA proteins are partly deficient in sustaining initiation of chromosomal DNA replication from *oriC*. Furthermore, all the *dnaA*(Sx) mutants alleviated the strong SOS response induced in the *dnaX2016* mutant at non-permissive temperature.

## METHODS

**Strains, plasmids and growth conditions.** Relevant markers of principal *E. coli* K-12 strains are AX727 (*dnaX2016*; Filip *et al.*, 1974), RUC839 (AX727, *tna2123::Tn10*), KM22 [ $\Delta$ (*recC ptr recB recD*)::*Plac-bet exo kan*; Murphy, 1998], RUC1227 (KM22, *dnaA46 tna2123::Tn10*), RUC1000 [ $\Delta$ (*lac*)X74; Berenstein *et al.*, 2002], RUC1024 (RUC1000, *dnaAp''lacZ*; Berenstein *et al.*, 2002), RUC1282 (RUC1000,  $\lambda$ *sulA''lacZ cl ind att''*; Lin & Little, 1988), RUC1291 [RUC1282, *dnaX2016*, Tn5(CamR) inserted near *dnaX*].

Strains for measuring SOS induction were derived from RUC1282 or RUC1291 and strains for all other growth experiments were derived from RUC1024 by P1 transductions with selection for resistance to tetracycline [*dnaA*(Sx) alleles] or chloramphenicol (*dnaX2016* mutation) and screening with restriction site polymorphism or with RG-PCR (Gasparini *et al.*, 1992).

Plasmids pACYC177, pMW119 (a pSC101-based vector), pMOR6 (pACYC177-*datA*) and pMOR8 (pMW119-*datA*) were all obtained from Morigen (Morigen *et al.*, 2001).

Bacterial cultures were grown in AB minimal medium supplemented with 0.1  $\mu$ g thiamine ml<sup>-1</sup>, 0.2 % glucose, 1 % Casamino acids (Difco) and 0.05 % serine (ABTG-*caa* medium).

**Chromosomal gene replacement.** A part of the *dnaA* gene containing the *dnaA*(Sx) mutation was PCR-amplified with primers *dnaA3* (GGCGGATAACCCTGGCG) and *dnaA4* (CCACCTAACGGACCGCTCAC). The DNA fragment was used to replace the temperature-sensitive *dnaA46* allele on the chromosome of RUC1227 (KM22, *dnaA46*, *tna2123::Tn10*) with  $\lambda$  Red-mediated recombination (Murphy, 1998) by selection for growth at 42 °C. The replaced DNA fragment was amplified by PCR in candidates, screened for restriction site polymorphism or with RG-PCR (Gasparini *et al.*, 1992) and finally sequenced to ensure perfect gene replacement.

**DNA sequencing.** Sequences were obtained with the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 310 Genetic Analyser (Applied Biosystems).

**Autorepression.** Strains derived from RUC1024 were grown exponentially at 34 °C in ABGT-*caa* medium for five doubling times and triplicate samples were taken at OD<sub>450</sub> 0.5 for measuring specific  $\beta$ -galactosidase activity (Miller, 1992) from the *dnaAp''lacZ* fusion in the *attB* site.

**SOS induction.** Strains derived from RUC1282 or RUC1291 were grown exponentially at 34 °C in ABGT-*caa* medium for five

doubling times. At OD<sub>450</sub> 0.5, cultures were diluted 10-fold into prewarmed medium at 38 °C for continuous growth at 38 °C. Duplicate samples for specific β-galactosidase activity from the *sulA''lacZ* fusion were taken every 30 min. Cultures were diluted further into prewarmed medium when OD<sub>450</sub> reached 0.5.

**Quantity and stability of DnaA proteins.** Strains derived from RUC1024 were grown exponentially at 34 °C in ABTG-*caa* medium. At OD<sub>450</sub> 0.5, a *t*=0 sample was taken and chloramphenicol was added to 200 μg ml<sup>-1</sup> to two aliquots followed by incubation at either 34 °C or 42 °C. Samples from the chloramphenicol-treated aliquots were taken at 30, 60 and 120 min. Proteins were separated by SDS-PAGE and immunoblotted with DnaA antibody obtained from K. Skarstad (Institute for Cancer Research, Oslo, Norway). Alkaline phosphatase activity of the secondary antibody was visualized with ECF substrate (Amersham Biosciences), scanned with a STORM840 PhosphorImager (Amersham Biosciences), and quantified with TotalLab software (Nonlinear Dynamics/Amersham Biosciences).

**Flow cytometry.** This was performed as described by Løbner-Olesen *et al.* (1989) on a Bryte-HS flowcytometer (Bio-Rad) equipped with a 100 W mercury lamp. Strains derived from RUC1024 were grown exponentially at 34 °C in ABTG-*caa* medium. At OD<sub>450</sub> 0.5, samples were incubated with rifampicin (300 μg ml<sup>-1</sup>) and cephalixin (36 μg ml<sup>-1</sup>) to prevent new initiations of DNA replication and cell division.

**Determination of the replication time, C, and the number of replication forks per cell, F<sub>c</sub>.** Chromosomal DNA was prepared, and the origin to terminus ratio (*O/T*) was determined by marker frequency analysis exactly as described by Atlung & Hansen (1999). When a culture has a doubling time  $\tau$  and a chromosomal replication time *C*, the stoichiometry between origins, *O*, and termini, *T*, is given by the formula  $O/T=2^{C/\tau}$  (Bremer & Churchward, 1977), from which *C* is extracted as  $C=\ln(O/T)\cdot\tau\cdot[\ln(2)^{-1}]$ . The number of active replication forks, *F<sub>c</sub>*, is given by the formula  $F_c=2(I_c-T_c)$  (Bremer & Churchward, 1977), where *I<sub>c</sub>* is the mean number of origins per cell and *T<sub>c</sub>* is the mean number of termini per cell. *I<sub>c</sub>* was determined by flow cytometry and *T<sub>c</sub>* was determined by *I<sub>c</sub>* divided by *O/T*.

## RESULTS

### Isolation of new *dnaA* mutations suppressing the *dnaX2016(Ts)* phenotype

*E. coli* strain RUC839 carries the *dnaX2016* mutation and a *tna2123::Tn10* insertion. The *dnaX2016* allele confers temperature-sensitive growth upon the cells, and suppressor mutations were readily isolated by selecting cells able to form colonies after 24 h incubation at 40 °C, conditions non-permissive to strain RUC839. Groups of approximately 50 suppressor colonies were pooled; a P1 lysate was grown on each pool and used to transduce strain AX727 (*dnaX2016*) to tetracycline resistance at 39 °C. Then one or two colonies from each transduction were tested for having an Sx [suppression of *dnaX2016(Ts)*] phenotype linked to *Tn10* and thus having the suppressor mutation located in the vicinity of the *tna* gene. Some of the suppressor mutations were suspected to be in the *dnaA* gene located 5 kb away from *tna* on the *E. coli* chromosome. The *dnaA* genes from 17 suppressor clones were sequenced and nine of these had changes in the *dnaA* gene. A total of seven different single-nucleotide mutations were found and all mutations changed a single amino acid in the DnaA protein (Table 1). The changed amino acids all resided in the C-terminal half of the DnaA protein where the previously isolated *dnaA(Cs,Sx)* mutations *dnaA71*, *dnaA73* and *dnaA721* (Ginés-Candelaria *et al.*, 1995) also resided (Fig. 1). The remaining eight clones are puzzling. The suppressor mutations must be near *tna*, and it was suspected that they could reside in *oriC* and/or *dnaN*. The *dnaN* gene and the *oriC* regions were therefore sequenced in these eight clones, but no mutations were found (not shown).

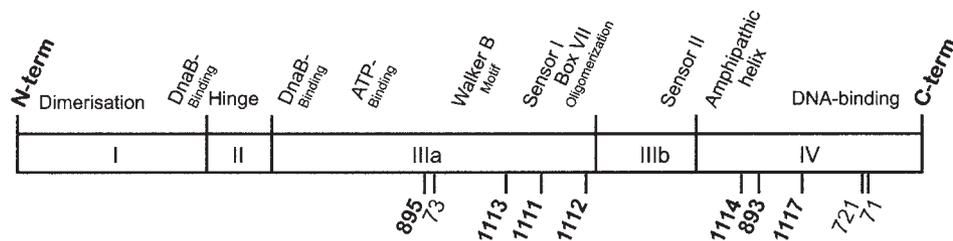
**Table 1.** The new *dnaA* mutations and their effect on autorepression and DnaA protein concentration

Allele	Nucleotide substitution		Amino acid changed	Verified by chromosomal gene replacement	<i>dnaA</i> p* (normalized)	Quantification of DnaA protein†
	Wild-type codon	Mutant codon				
<i>dnaA</i> <sup>+</sup>					1.00 ± 0.14	1.00 ± 0.06
<i>dnaA893</i>	ATT	ATG	I379M	No	1.58 ± 0.27	0.45‡
<i>dnaA895</i>	CAG	AAG	Q208K	Yes	1.94 ± 0.20	1.12 ± 0.04
<i>dnaA1111</i>	TCG	ACG	S268T	Yes	1.57 ± 0.24	1.26 ± 0.05
<i>dnaA1112</i>	ACT	AAT	T291N	Yes	1.59 ± 0.14	0.88 ± 0.06
<i>dnaA1113</i>	TTT	TGT	F250C	No	1.90 ± 0.18	0.93 ± 0.10
<i>dnaA1114</i>	CAG	AAG	Q370K	Yes	1.89 ± 0.13	0.81 ± 0.03
<i>dnaA1117</i>	CGC	AGC	R401S	Yes	1.90 ± 0.17	0.87 ± 0.06

\*The promoter activity of the *dnaA* promoter was measured as specific β-galactosidase activity expressed by the *dnaA''lacZ* fusion and normalized to the *dnaA*<sup>+</sup> strain (9.6 Miller units). Mean values and standard deviations were calculated from triplicates.

†Total DnaA protein concentration was quantified from immunoblots; see legend to Fig. 3. Mean values and standard deviations were calculated from the 0, 30, 60 and 120 min samples at 34 °C and are normalized to the DnaA protein concentration in RUC1024 (*dnaA*<sup>+</sup>). Values for samples taken at 40 °C were similar to those for the 34 °C samples.

‡This protein is unstable (Fig. 3) and the value is from the 0 min sample.



**Fig. 1.** Localization of *dnaA*(Sx) mutations relative to known functional regions. The new mutations are indicated in bold; the previously described *dnaA*(Cs,Sx) mutations (Ginés-Candelaria *et al.*, 1995) are in lighter type.

To ensure that the Sx phenotype resulted from the mutations in the *dnaA* gene only and not from additional mutations in nearby genes, five of the *dnaA*(Sx) mutations were transferred into a new host by gene replacement (Methods, Table 1). The Sx phenotype followed the *dnaA* allele in all cases, demonstrating that this alone was sufficient for suppression of the *dnaX2016* mutation. Strains with gene-replaced alleles were used in all subsequent experiments.

Previously isolated *dnaX2016*(Ts) suppressors residing in the *dnaA* gene were all selected by sensitivity to growth at 20 °C (Walker *et al.*, 1982; Ginés-Candelaria *et al.*, 1995). None of the new mutants isolated as described above were cold-sensitive, demonstrating that cold-sensitivity and Sx phenotype are independent.

### The *dnaA*(Sx) mutations reduce the SOS induction in a *dnaX2016* strain

The role of the  $\tau$  and  $\gamma$  proteins in organizing the DNA polymerase III holoenzyme and loading of the  $\beta$ -clamp suggests that cells carrying the *dnaX2016* mutation might suffer replication fork collapse at non-permissive temperatures and consequently induce the SOS response (Seigneur *et al.*, 1998). The SOS response was measured as  $\beta$ -galactosidase activity from a *sulA''lacZ* fusion carried by a  $\lambda$  phage (Lin & Little, 1988).

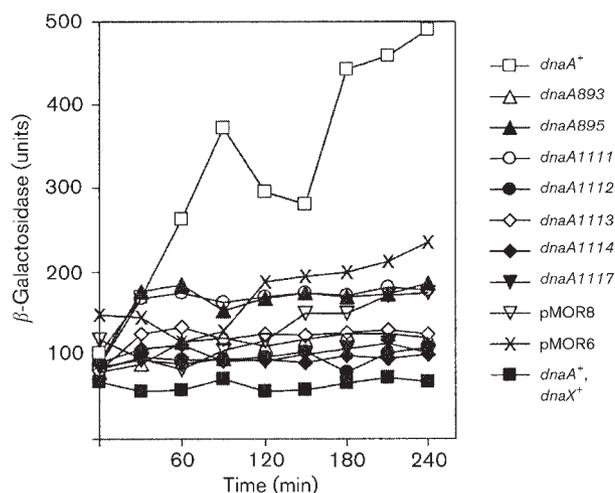
During steady-state growth at 34 °C, the amount of  $\beta$ -galactosidase expressed from this fusion in wild-type cells was 62 units. The  $\beta$ -galactosidase activity of wild-type cells did not change upon a shift to 38 °C (Fig. 2, filled squares). In cells carrying the *dnaX2016* mutation the amount of  $\beta$ -galactosidase was 102 units, an indication of slight SOS induction even at permissive temperature. After shifting the *dnaX2016* strain to 38 °C the  $\beta$ -galactosidase activity increased immediately; over time a fivefold increase was observed (Fig. 2, open squares). When the *dnaX2016* mutation was combined with either of the *dnaA*(Sx) mutations, the activity of the *sulA''lacZ* fusion was reduced to levels between 80 and 92 units, indicating a partial relief of the signal for SOS induction at permissive temperature. No or little increase in  $\beta$ -galactosidase activity upon a shift to

38 °C was observed for any of the *dnaX2016 dnaA*(Sx) double mutants (Fig. 2).

It can therefore be concluded that *dnaX2016* cells are somewhat SOS-induced even at permissive temperature, and that this induction increases dramatically upon a shift to nonpermissive temperature. The *dnaA*(Sx) mutations isolated here were able to suppress this SOS induction. This may in turn suggest that the SOS-inducing signal is no longer generated in the *dnaA*(Sx) cells.

### The *dnaA*(Sx) mutants have reduced autorepression

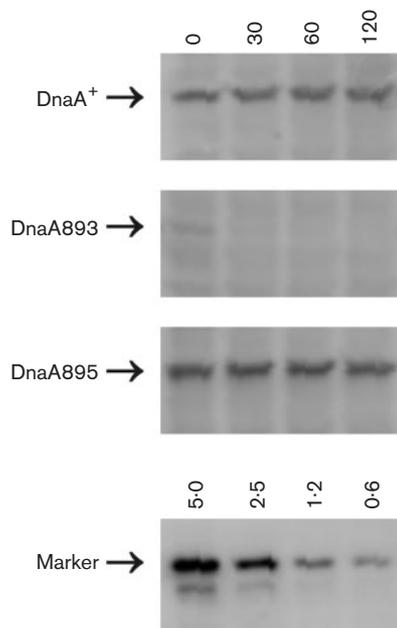
The *E. coli dnaA* gene is autoregulated by a mechanism in which the DnaA protein binds to a region between promoters p1 and p2 of the *dnaA* gene, thereby repressing



**Fig. 2.** SOS induction in *dnaA*(Sx) *dnaX2016* strains after temperature upshift. Cultures were grown exponentially at 34 °C in ABTG-*caa* medium with doubling times between 36 and 41 min. At time zero, cultures were diluted 10-fold into the same pre-warmed medium at 38 °C and samples for  $\beta$ -galactosidase activity from the *sulA''lacZ* fusion were taken every 30 min. Data presented are from one representative experiment.

transcription from both promoters (Braun *et al.*, 1985). The expression of a *dnaA* gene transcriptionally fused to *lacZ* is therefore a convenient way of measuring DnaA protein–DnaA-box interaction *in vivo* (Braun *et al.*, 1985). A *dnaA''lacZ* gene fusion integrated in the  $\lambda$  attachment site *attB* of the chromosome (Berenstein *et al.*, 2002) was used. This was convenient because the gene dosage of *attB* changes little with changing growth rates and/or replication times (C-periods). Transcription from *dnaAp* was increased for all of the new *dnaA*(Sx) mutants. The degree of derepression was in the range 1.5–2.0 fold (Table 1).

The observed derepression of *dnaA* gene transcription could be due either to instability of the DnaA(Sx) proteins resulting in lowered DnaA protein concentration, or to a poor interaction with DnaA-boxes. The concentration and the stability of each DnaA(Sx) protein was therefore determined (Fig. 3, Table 1). Most of the *dnaA*(Sx) mutations led to little or no change in DnaA protein concentration in the cells relative to wild-type. There was, however, one exception. The *dnaA893* mutation had less than 50% of wild-type DnaA protein concentration. The reduced DnaA concentration resulting from the *dnaA893* allele was due to a greatly decreased stability of this protein because this protein was already absent 30 min after *de novo* protein synthesis was stopped with chloramphenicol



**Fig. 3.** Concentration and stability of DnaA proteins. Samples from exponentially growing cultures of the indicated *dnaA* strains were withdrawn and *de novo* protein synthesis blocked with chloramphenicol at time zero. Samples were taken at 0, 30, 60 and 120 min as indicated. Proteins were separated by SDS-PAGE and immunoblotted with DnaA antibody. The marker contains 5.0, 2.5, 1.2 and 0.6 ng purified DnaA protein.

(Fig. 3). Wild-type DnaA protein as well as the remaining DnaA(Sx) proteins were stable over a 120 min period (Fig. 3).

It can be concluded that all of the isolated *dnaA*(Sx) suppressor mutations led to a derepression of the *dnaA* promoter. For one of the mutations (*dnaA893*) the derepression resulted from instability of the DnaA protein itself causing a reduction in intracellular concentration. For the remaining mutants the DnaA protein content was around normal or elevated, indicating that these mutations resulted in DnaA proteins with a lower than normal affinity for the DnaA-boxes contained in the *dnaA* promoter region.

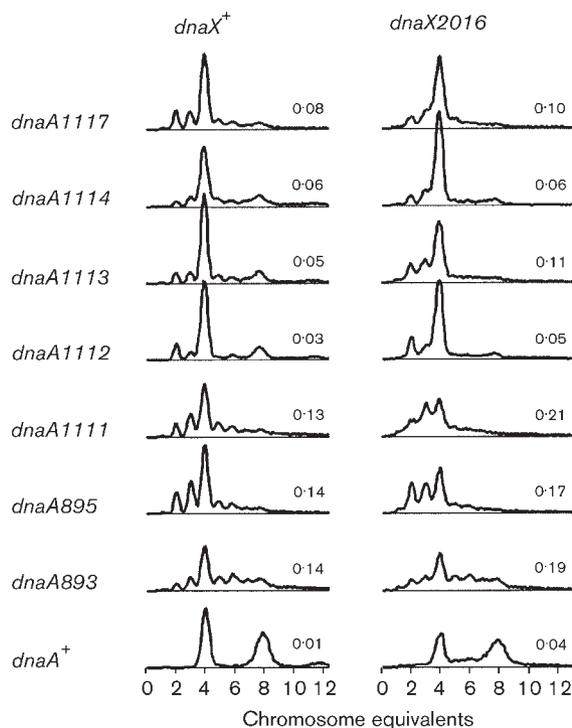
### The *dnaA*(Sx) mutants have increased initiation mass

The reduced ability to repress *dnaA* gene transcription suggests that the mutant DnaA(Sx) proteins may also interact poorly with the DnaA-boxes located in *oriC*, leading to feeble initiation of chromosome replication.

A flow cytometric analysis of cells treated with rifampicin and cephalixin (for details see Methods) revealed that the number of origins per cell was 5.9 for both *dnaX*<sup>+</sup> and *dnaX2016*(Sx) mutant cells at permissive temperature (Table 2). All *dnaA*(Sx) mutations reduced this number of origins per cell by 15–30% in combination with the *dnaX*<sup>+</sup> allele and by 20–40% in combination with the *dnaX2016* allele (Table 2). The mean cell mass was also determined for each culture (Table 2) and this value was used to calculate the mean cell mass per origin. The mean cell mass per origin is equal to the initiation mass of the cells multiplied by ln 2 (Bremer *et al.*, 1979) and is therefore a reliable measure of the relative initiation mass. All *dnaA*(Sx) mutants had an increased initiation mass (Table 2). The increase in initiation mass was around 50% for most mutants but smaller (8–23%) in the *dnaA893* mutant. This clearly demonstrates that all *dnaA*(Sx) mutations resulted in reduced initiation efficiency that led to delayed initiation in the cell cycle.

### The synchrony of initiation is reduced in *dnaA*(Sx) mutants

In *E. coli* wild-type cells all origins of replication are initiated simultaneously. Cells consequently contain  $2^n$  ( $n=0, 1, 2, 3$  or  $4$ ) origins (Skarstad *et al.*, 1986). In agreement with this, *dnaA*<sup>+</sup> cells mainly contained 4 or 8 fully replicated chromosomes after treatment with rifampicin and cephalixin. The synchrony of *dnaX2016* cells was slightly reduced relative to *dnaX*<sup>+</sup> cells (Fig. 4, lower panels). Asynchrony indices,  $A_i$ , were calculated according to Olsson *et al.* (2003) and are indicated on each panel of Fig. 4. The presence of either *dnaA*(Sx) mutation led to a greater fraction of cells containing numbers of origins different from  $2^n$ , indicating that origins were no longer initiated in synchrony (Fig. 4). However, the degree of asynchrony conferred by



**Fig. 4.** Distributions of origin numbers in individual cells. Cultures of strains with the indicated combinations of *dnaA(Sx)* and *dnaX2016* alleles were grown exponentially and samples were subjected to flow cytometry after rifampicin/cephalexin treatment. The number of fully replicated chromosomes after rifampicin/cephalexin treatment reflects the number of origins present in the cell at the time of drug treatment. Asynchrony indices were calculated as  $A_i = (f_3 + f_5 + f_6 + f_7) / (f_2 + f_4 + f_8) / (\text{origins per cell})$  (Olsson *et al.*, 2003), and are indicated in each panel.

**Table 2.** Effect on initiation of DNA replication

Cells were grown in ABTG-*caa* medium at 34 °C, subjected to rifampicin plus cephalexin run-out and analysed by flow cytometry. Data presented are from one representative experiment. The origin distributions are shown in Fig. 4.

<i>dnaA</i> allele	Cell mass*		Origins per cell†		Initiation mass‡	
	<i>dnaX</i> <sup>+</sup>	<i>dnaX2016</i>	<i>dnaX</i> <sup>+</sup>	<i>dnaX2016</i>	<i>dnaX</i> <sup>+</sup>	<i>dnaX2016</i>
<i>dnaA</i> <sup>+</sup>	73	71	5.9	5.9	1.00	0.97
<i>dnaA893</i>	78	64	5.1	4.8	1.23	1.08
<i>dnaA895</i>	93	72	4.2	3.8	1.78	1.54
<i>dnaA1111</i>	80	64	4.6	3.9	1.42	1.33
<i>dnaA1112</i>	81	76	4.6	4.0	1.43	1.52
<i>dnaA1113</i>	81	71	4.6	4.0	1.43	1.44
<i>dnaA1114</i>	84	82	4.8	4.2	1.41	1.58
<i>dnaA1117</i>	87	77	4.3	4.1	1.63	1.51

\*Cell mass is the mean light scatter per cell after replication run-out.

†Origins per cell is the sum of chromosome equivalents divided by the sum of cells after replication run-out.

‡Initiation mass is the mean of light scatter per cell divided by mean chromosome equivalents per cell after replication run-out. Values were normalized to the *dnaA*<sup>+</sup> *dnaX*<sup>+</sup> strain.

the individual *dnaA(Sx)* mutations varied greatly. The *dnaA1112*, *dnaA1113*, *dnaA1114* and *dnaA1117* alleles led to only slight asynchrony of initiation in otherwise wild-type cells ( $A_i$  0.03–0.08), whereas *dnaA893*, *dnaA895* and *dnaA1111* alleles led to a higher degree of asynchrony ( $A_i$  0.13–0.14). Similar asynchrony indices were found in combination with the *dnaX2016* mutation (Fig. 4). For comparison, the asynchrony index of the well-characterized *dnaA46* mutant is 0.21 (Olsson *et al.*, 2003).

### The *dnaA(Sx)* mutants have a decreased replication time and reduced number of replication forks per cell

Cells carrying *dnaA(Sx)* mutations grew with doubling times that were only slightly increased compared to wild-type cells at permissive temperature (Table 3). The low origin content of these cells (Table 2) therefore indicated that *dnaA(Sx)* cells replicated their chromosome faster than wild-type cells, i.e. had a reduced C-period. The ratio of *oriC* to *terC* for each *dnaA(Sx)* mutant alone or in combination with the *dnaX2016* mutation was determined by hybridization with specific probes. This ratio is solely dependent on the duration of the replication time and the culture doubling time (Methods).

The C-period was 49 min in wild-type cells growing with a doubling time of 34 min (Table 3). Introduction of *dnaX2016* increased the C-period slightly to 53 min without any effect on the doubling time. This may indicate a slight defect of the DnaX2016 protein even at permissive temperature. The *dnaA893* allele only reduced the C-period slightly, from 49 to 43 min in wild-type cells and from 53 to 52 min in *dnaX2016* cells. All other *dnaA(Sx)* mutations reduced the C-period significantly either alone

**Table 3.** Effect on DNA-replication time (C)

The doubling time ( $\tau$ ) and the *oriC* to *terC* ratio (*O/T*) was determined in cells growing exponentially in ABTG-*caa* medium at 34 °C. The replication time (C) of one round of DNA replication and the number of replication forks ( $F_c$ ) were calculated; see Methods. Each *O/T* value is the mean of three determinations.

<i>dnaA</i> allele	<i>dnaX</i> <sup>+</sup>				<i>dnaX2016</i>			
	$\tau$ (min)	<i>O/T</i>	C (min)	$F_c$	$\tau$ (min)	<i>O/T</i>	C (min)	$F_c$
<i>dnaA</i> <sup>+</sup>	34	2.73 ± 0.22	49	7.5	35	2.84 ± 0.13	53	7.6
<i>dnaA893</i>	37	2.22 ± 0.35	43	5.6	37	2.63 ± 0.16	52	5.9
<i>dnaA895</i>	39	1.63 ± 0.15	27	3.2	39	1.97 ± 0.12	38	3.7
<i>dnaA1111</i>	38	1.96 ± 0.12	37	4.5	37	1.88 ± 0.19	34	3.7
<i>dnaA1112</i>	37	1.80 ± 0.12	31	4.1	39	1.95 ± 0.20	38	3.9
<i>dnaA1113</i>	35	1.92 ± 0.07	33	4.4	38	2.09 ± 0.34	40	4.2
<i>dnaA1114</i>	35	2.00 ± 0.08	35	4.8	38	2.01 ± 0.18	38	4.2
<i>dnaA1117</i>	36	1.99 ± 0.10	36	4.3	37	2.28 ± 0.15	44	4.6

(27–37 min) or in combination with the *dnaX2016* mutation (34–44 min) (Table 3).

The low origin content of the *dnaA*(Sx) cells also indicated that these cells contained fewer replication forks than their wild-type counterparts. The mean number of replication forks per cell in exponentially growing cells,  $F_c$ , is a function of the replication time, C, the time from termination of replication to cell division, D, and the doubling time,  $\tau$ , of the culture (Bremer & Churchward, 1977). We calculated the mean number of replication forks per cell,  $F_c$ , from the number of origins per cell (Table 2) and the origin to terminus (*O/T*) ratio (Table 3). All the *dnaA*(Sx) mutations reduced  $F_c$  significantly and irrespectively of the *dnaX* allele carried (Table 3). For most mutations  $F_c$  was reduced to approximately 50–60 % of the wild-type, but the *dnaA893* mutation only reduced  $F_c$  to 75 % of the wild-type.

It can be concluded that the increased initiation mass observed for *dnaA*(Sx) cells was accompanied by a decrease in replication time. This indicates that replication forks in wild-type cells either do not travel with maximal velocity or make frequent pauses during replication of the chromosome. The decreased replication time subsequently resulted in a reduction of active replication forks per cell.

### Increased gene dosage of *datA* suppresses the *dnaX2016* Ts phenotype

The *datA* locus titrates large amounts of DnaA protein and an increased gene dosage of *datA* therefore delays initiation of DNA replication from *oriC* (Ogawa *et al.*, 2002; Morigen *et al.*, 2001), induces asynchrony and decreases the replication time (C) (Morigen *et al.*, 2003). These phenotypes are similar to those observed for the *dnaA*(Sx) mutations isolated here. It was therefore tempting to speculate that the mechanism of suppression of the *dnaX2016* mutation is related to one of these phenotypes.

Transformation of a *dnaX2016* strain with either of the

*datA*-containing plasmids pMOR8 (~5 copies per cell; Morigen *et al.*, 2001) or pMOR6 (~10 copies per cell; Morigen *et al.*, 2001) increased the permissive temperature by 2 °C, whereas transformation with the parent plasmids pMW119 or pACYC177 did not change the permissive temperature (data not shown). The pMOR6 and pMOR8 plasmids also suppressed the SOS induction in a *dnaX2016* mutant at 38 °C (Fig. 2) and restored the plating efficiency (EOP) at 39 °C (Table 4).

It can therefore be concluded that a reduction in free DnaA protein in the cell by introduction of *datA*-containing plasmids suppresses the temperature sensitivity of the *dnaX2016* allele partly and to the same extent as the *dnaA*(Sx) mutations.

**Table 4.** Efficiency of plating at 39 °C

Overnight cultures of strains with the indicated genotypes were diluted in steps of 10-fold; 100  $\mu$ l samples of relevant dilutions were spread on pairs of LB plates that were incubated at 34 °C and 39 °C respectively. EOP is the fraction of colonies formed at 39 °C compared to colonies at 34 °C.

<i>dnaA</i> allele	<i>dnaX</i> allele	Plasmid	EOP at 39 °C
<i>dnaA</i> <sup>+</sup>	<i>dnaX</i> <sup>+</sup>	–	0.9
<i>dnaA</i> <sup>+</sup>	<i>dnaX2016</i>	–	< 10 <sup>-5</sup>
<i>dnaA893</i>	<i>dnaX2016</i>	–	1.3
<i>dnaA895</i>	<i>dnaX2016</i>	–	0.6
<i>dnaA1111</i>	<i>dnaX2016</i>	–	1.0
<i>dnaA1112</i>	<i>dnaX2016</i>	–	0.8
<i>dnaA1113</i>	<i>dnaX2016</i>	–	1.4
<i>dnaA1114</i>	<i>dnaX2016</i>	–	0.9
<i>dnaA1117</i>	<i>dnaX2016</i>	–	1.0
<i>dnaA</i> <sup>+</sup>	<i>dnaX2016</i>	pACYC177	< 10 <sup>-5</sup>
<i>dnaA</i> <sup>+</sup>	<i>dnaX2016</i>	pMOR6	0.3
<i>dnaA</i> <sup>+</sup>	<i>dnaX2016</i>	pMOR8	0.9

## DISCUSSION

Seven *dnaA* mutations that partially suppressed temperature sensitivity and SOS induction of the *dnaX2016* mutant were isolated and characterized. Each of the seven *dnaA*(Sx) mutations gave rise to a single amino acid change in domain III or IV of the DnaA protein. Flow cytometric studies revealed that cells carrying any one of the *dnaA*(Sx) mutations initiated DNA replication later in the cell cycle and at an increased initiation mass and somewhat asynchronously, relative to wild-type cells. The time required to replicate the chromosome (C-period) was reduced for all *dnaA*(Sx) mutants. Plasmids containing the *datA* region, known to lower the availability of DnaA for initiation of replication, also partially suppressed the *dnaX2016* mutation.

### SOS induction in *dnaX2016* cells

The *dnaX2016* mutation causes an immediate stop of DNA synthesis and gradual inhibition of cell division after temperature shift from 30 °C to 42 °C, whereas protein synthesis continues for at least 30 min (Chu *et al.*, 1977). The SOS response was found to be somewhat induced even at permissive temperature in *dnaX2016* cells. Because the *dnaX2016* mutation affects the  $\tau$  and  $\gamma$  clamp loader subunits of the replisome, the most likely explanation for this is that replication forks collapse with low frequency at permissive temperature, generating an SOS-inducing signal, but the collapsed forks can be efficiently restarted, and cells are fully viable (Seigneur *et al.*, 1998). At higher temperatures a strong SOS response was elicited in *dnaX2016* cells, indicating that the SOS-inducing signal persisted or was continuously generated. An increased frequency of replication fork collapse that exceeds the cells' capacity for replication fork restart is the most likely explanation for the increased SOS induction. At high temperature, cell division is consequently inhibited and viability lost. The presence of any *dnaA*(Sx) allele or one of the *datA* plasmids suppressed the SOS induction of *dnaX2016* mutant cells. This suggests that the *dnaA*(Sx) mutations reduce replication fork collapse in *dnaX2016* cells.

### Chromosome replication in the *dnaA*(Sx) mutants

Transcription of the *dnaA* gene was derepressed in all *dnaA*(Sx) mutants compared to wild-type, indicating that the DnaA(Sx) proteins were somewhat deficient in auto-repression. Efficient repression of *dnaA* transcription is dependent on binding of DnaA associated with either ATP or ADP to a single DnaA-box and subsequent cooperative DnaA-ATP binding to adjacent 6-mer regions, located between the *dnaAp1* and *p2* promoters. The DnaA(Sx) mutant proteins were therefore expected to fall into two groups: those with altered binding to the DnaA-box and those that affected nucleotide binding. Both of these deficiencies were also expected to affect replication initiation

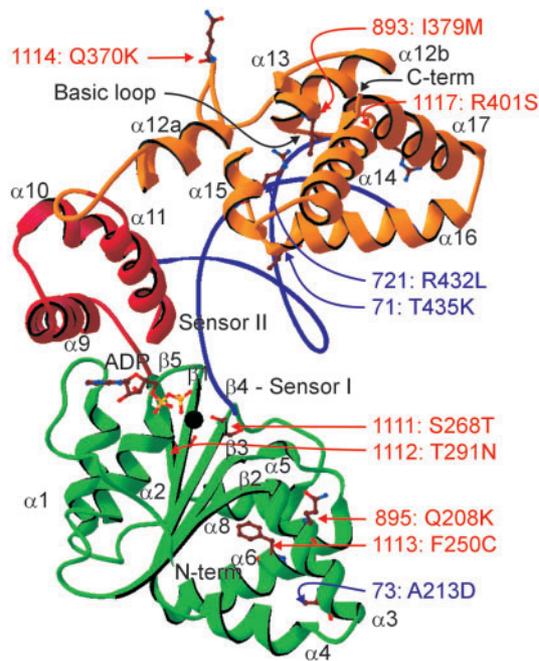
from *oriC*, where binding of DnaA protein to DnaA-boxes and subsequently to DnaA-ATP-specific sites ensures open complex formation. In agreement with this it was found that all *dnaA*(Sx) mutations led to initiation at increased cell mass per origin (initiation mass; Table 2). In all cases, the increased initiation mass was accompanied by a decrease in the chromosome replication time (C-period). This is in agreement with previous observations where a decrease in availability of wild-type DnaA protein led to increased initiation mass and decreased C-period (Morigen *et al.*, 2003). A decreased C-period has also been observed in cells carrying *dnaA* mutations (Boye *et al.*, 1996; Morigen *et al.*, 2003) or a mutation in the *hns* gene (Atlung & Hansen, 2002). An increased replication rate might therefore be a general consequence of reduced initiation frequency that in turn leads to fewer replication forks per cell mass. In wild-type cells activity of DNA polymerase III may therefore normally be limited by availability of nucleotides or other factors. Alternatively, frequent pausing of polymerase III could occur in wild-type cells, and a reduced concentration of active replication forks alleviates this pausing.

The synchrony of initiation with the single cell was also reduced by the *dnaA*(Sx) mutations, albeit to different extents. This reduced affinity of DnaA(Sx) proteins for the origin DnaA-boxes most likely led to an extended initiation interval in these cells. The extended initiation interval causes asynchrony because timely once-per-cell-cycle initiation (synchrony) is dependent on a period of initiation significantly shorter than the sequestration period (Skarstad & Løbner-Olesen, 2003) and the latter is not expected to be affected by *dnaA*(Sx) mutations. One group of mutants (*dnaA893*, *dnaA895* and *dnaA1111*) initiate DNA replication very asynchronously and another group (*dnaA1112*, *dnaA1113*, *dnaA1114* and *dnaA1117*) are only weakly asynchronous (Fig. 4). The amino acid changes in the two groups of synchrony mutants are not clustered to a particular region of the DnaA protein (Fig. 1), but connections to a particular property of the DnaA protein can be made (discussed below).

### The highly asynchronous *dnaA*(Sx) mutants

Domains IIIa and IIIb of the DnaA protein comprise an AAA + nucleotide-binding fold (Neuwald *et al.*, 1999). The structure of domains III and IV was solved for the *Aquifex aeolicus* DnaA protein complexed with ADP (Erzberger *et al.*, 2002) and the structure of domain IV complexed with a DnaA-box was solved for *E. coli* DnaA (Fujikawa *et al.*, 2003). Fig. 5 shows the predicted structure of domains III + IV of the *E. coli* DnaA protein complexed with a DnaA-box. The positions of individual mutations are indicated.

Three *dnaA*(Sx) mutations resulted in a very asynchronous phenotype (Fig. 4). The Q208, changed to K in *dnaA895*, is in contact with E204, important for the intrinsic ATPase activity of DnaA (Mizushima *et al.*, 1997; Nishida *et al.*, 2002). The A213 changed to D in the previously described



**Fig. 5.** Positions of mutated amino acids in the DnaA protein. The structure of domains III and IV of *E. coli* DnaA complexed with a DnaA-box and ADP was predicted by threading the primary sequence of the *E. coli* DnaA protein on the structures of the *Aquifex aeolicus* DnaA protein complexed with ADP (Erzberger *et al.*, 2002) and domain IV of *E. coli* complexed with a DnaA-box (Fujikawa *et al.*, 2003) using the ExPASy server (Gasteiger *et al.*, 2003). Green, domain IIIa; red, domain IIIb; orange, domain IV; black sphere,  $Mg^{2+}$  ion; blue string, the ribose-phosphate backbone of the complexed DnaA-box.  $\alpha$ -Helices and  $\beta$ -strands are numbered according to Erzberger *et al.* (2002).  $\alpha 7$  did not form and  $\alpha 12$  was broken into two helices, here marked as  $\alpha 12a$  and  $\alpha 12b$ , during the modelling. The locations of the *dnaA*(Cs,Sx) (Ginés-Candelaria *et al.*, 1995) mutations are indicated in blue and the new *dnaA*(Sx) mutations are indicated in red.

*dnaA73* (Ginés-Candelaria *et al.*, 1995) is also located in this  $\alpha 3$ -helix (Erzberger *et al.*, 2002) and also confers asynchrony (Blinkova *et al.*, 2003). Altered ATPase activity is therefore a likely reason for the defect of the DnaA895 and DnaA73 proteins.

The *dnaA1111* (S268T) mutation is located in the sensor I motif in  $\beta 4$ , very close to where the  $\gamma$ -phosphate would be if ATP was bound (Erzberger *et al.*, 2002). A changed sensing of the bound nucleotide is therefore the suggested cause of the reduced initiation capacity.

The *dnaA893* (I379M) mutation is located in  $\alpha$ -helix  $\alpha 12$  (Erzberger *et al.*, 2002), but outside the amphipathic part of this helix. This mutant deviated from the other *dnaA*(Sx) mutants by having only slightly increased initiation mass and replication rate. The present data show that the DnaA893 protein is very unstable (Fig. 3), and the DnaA

protein concentration is much lower than in wild-type cells. The DnaA893 protein therefore must be more active than the wild-type DnaA protein in DNA replication and autorepression. The increased activity of the DnaA893 protein suggests that this mutation eliminates a negative element; perhaps it escapes the RIDA-dependent inactivation.

The asynchrony of *dnaA73*, *dnaA895* and *dnaA1111* together with the classical *dnaA* mutants in the ATP-binding site (*dnaA5*, *dnaA46*, *dnaA601* and *dnaA604*) (Skarstad *et al.*, 1988) could indicate that asynchrony is a common property of mutations affecting binding, hydrolysis or sensing of the ATP molecule.

### The slightly asynchronous *dnaA*(Sx) mutants

Four *dnaA*(Sx) mutations were only slightly affected in initiation synchrony. Two of these, *dnaA1112* (T291N) located in the  $\beta 5$  strand (Erzberger *et al.*, 2002), and *dnaA1113* (F250C) located in the  $\alpha 6$  helix (Erzberger *et al.*, 2002), are in regions for which no specific function is known (Fig. 5). F250 is however a very conserved amino acid and close to H252, which is changed to Y in *dnaA46*.

The *dnaA1114* (Q370K) mutation is located in the amphipathic  $\alpha$ -helix 12, which in the model in Fig. 5 is broken by a random structure. This region is associated with phospholipids involved in rejuvenation of DnaA-ATP from DnaA-ADP (Garner *et al.*, 1998) and consequently the *dnaA1114* mutation may alter the balance between these two species of DnaA protein to favour the DnaA-ADP form, inactive for both autorepression and initiation.

The *dnaA1117* (R401S) mutation is located in the basic loop (Fig. 5; Erzberger *et al.*, 2002) and is in contact with the backbone phosphate of DNA via a water molecule during DnaA-box binding (Fujikawa *et al.*, 2003). This suggests that the *dnaA1117* mutation leads to a weakening of DnaA-box binding, but maintains the specificity for DnaA-boxes. This is different from the base-specific binding of T435, changed to K in the asynchronous *dnaA71* mutant (data not shown), which leads to loss of specificity in DNA binding (Blaesing *et al.*, 2000). This suggests an explanation for the clear differences in their effect on DNA replication *in vivo*.

### The suppression mechanism is indirect

At least two different scenarios by which mutations in the *dnaA* gene suppress the *dnaX2016* temperature sensitivity can be imagined. The DnaA [and DnaA(Sx)] proteins may contact the  $\tau$  and/or  $\gamma$  components of the DNA polymerase III holoenzyme either directly or indirectly. The suppressors obtained could be considered allele-specific suppressors of the *dnaX2016* mutation. Because the affected amino acids in the mutant DnaA(Sx) proteins are located in both domains III and IV of DnaA (Fig. 1), and affect different functions of the protein, we consider the DnaA and  $\tau$  and/or  $\gamma$  interaction unlikely and therefore we investigated indirect suppression mechanisms.

All the *dnaA*(Sx) mutants analysed shared three characteristics: first, the SOS induction in *dnaX2016* was suppressed at elevated temperature; second, initiation of replication was delayed in the cell cycle and occurred at an increased initiation mass; and third, replication of the chromosome was faster than in wild-type cells (the C-period was shorter). The presence of plasmids carrying the *datA* locus also delayed initiation of DNA replication in the cell cycle, and increased the replication rate and suppressed the *dnaX2016* mutation. Delayed initiation in the cell cycle and an increased chromosomal replication rate both contribute to a reduced number of replication forks in cells. It is conceivable that the number of replication forks that collapse in the *dnaX2016* cells also carrying a *dnaA*(Sx) suppressor is reduced at nonpermissive temperature as well. We speculate that the frequency of replication fork collapse in suppressed cells is such that they can be efficiently repaired by the host. This explains why the *dnaA*(Sx) mutations suppress both temperature sensitivity and SOS induction in cells carrying *dnaX2016*. Recently, support for an initiation deficiency model was obtained by Blinkova *et al.* (2003), who analysed the classical *dnaA*(Cs,Sx) mutants, primarily *dnaA721*. Like the *dnaA*(Sx) mutants described here, *dnaA721* cells had increased length and reduced number of origins per cell. Suppression of the *dnaX2016* temperature-sensitive phenotype by multicopy plasmids carrying the *datA* locus (this work) or by certain *oriC* mutations (Blinkova *et al.*, 2003) also supports an initiation deficiency model; in both cases binding of DnaA<sup>+</sup> protein to *oriC* is reduced, leading to delayed initiation and an increase in the initiation mass.

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