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Mutant DnaAs of *Escherichia coli* that are refractory to negative control

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ABSTRACT

DnaA is the initiator of DNA replication in bacteria. A mutant DnaA named DnaAcos is unusual because it is refractory to negative regulation. We developed a genetic method to isolate other mutant DnaAs that circumvent regulation to extend our understanding of mechanisms that control replication initiation. Like DnaAcos, one mutant bearing a tyrosine substitution for histidine 202 (H202Y) withstands the regulation exerted by *datA*, *hda* and *dnaN* (β clamp), and both DnaAcos and H202Y resist inhibition by the Hda-β clamp complex *in vitro*. Other mutant DnaAs carrying G79D, E244K, V303M or E445K substitutions are either only partially sensitive or refractory to inhibition by the Hda-β clamp complex *in vitro* but are responsive to *hda* expression *in vivo*. All mutant DnaAs remain able to interact directly with Hda. Of interest, both DnaAcos and DnaAE244K bind more avidly to Hda. These mutants, by sequestrating Hda, may limit its availability to regulate other DnaA molecules, which remain active to induce extra rounds of DNA replication. Other evidence suggests that a mutant bearing a V292M substitution hyperinitiates by escaping the effect of an unknown regulatory factor. Together, our results provide new insight into the mechanisms that regulate replication initiation in *Escherichia coli*.

INTRODUCTION

In all free-living organisms, DNA replication is regulated to occur only once per cell cycle. In *Escherichia coli*, DnaA protein bound to the *E. coli* replication origin (*oriC*) regulates the frequency of DNA replication by periodically assembling the enzymes destined to act at the replication fork for duplication of the genome. Structure-function studies have identified several domains of DnaA [reviewed in (1,2)]. To summarize, domain I (amino acid residues 1–90) is involved in the self-oligomerization of DnaA at *oriC* and in binding to other proteins (Figure 1) (5–12). Domain II (residues 90–130) appears to act as a flexible linker joining domains I and III (13). Depending on the region in this domain, a minimum of 21–27 amino acids is required for its function. Domain III (residues 130–347) binds ATP, and domain IV (residues 347–467) binds to the DnaA box, which is present in a number of chromosomal locations including several copies at *oriC* [reviewed in (1,2)]. As shown by the genetic characterization of many *dnaA* alleles, and the biochemical characterization of mutant DnaAs, these domains are essential for DnaA function at *oriC*.

Among scores of *dnaA* alleles, one named *dnaAcos* is unusual because it induces excessive initiation (14). It was originally isolated as an intragenic suppressor of the *dnaA46*(TS) allele. At 42°C, *dnaAcos* strains are viable, but they are unable to grow at 30°C because DnaAcos hyperinitiates (15,16). To explain how the uncontrolled initiation causes inviability, we suggested that the new replication forks collide from behind with stalled forks, leading to fork collapse (17). The accumulated double-strand breaks (DSBs) then overwhelm the cell's capacity to repair them, resulting in cell death.

The *dnaAcos* allele encodes four substitutions (Q156L, A184V, H252Y and Y271H; Figure 1) (18). The A184V and H252Y substitutions derive from the *dnaA46* allele. Previous work investigated the effect of each substitution of DnaAcos individually and in the different possible combinations (16). These and other studies showed that the A184V substitution not only leads to overinitiation at 30°C but also causes a phenotype of temperature-sensitive growth (16,19,20). The Y271H substitution appears to stabilize the activity of DnaAcos at an elevated temperature

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as Joint First Authors.

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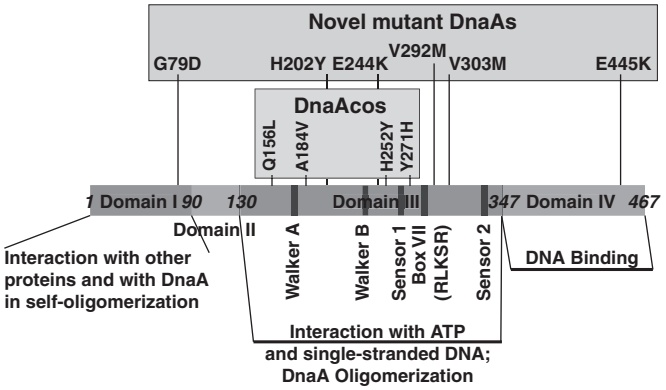


Figure 1. DnaA and its functional domains. The amino acid substitutions of the novel mutant DnaAs and DnaAcos are shown within the gray boxes. Relative to coordinates for *E. coli* DnaA, domain 1 near the N-terminus is involved in the interactions between DnaA and DnaB, HU, Dps, DiaA or ribosomal protein L2, and is also required for DnaA oligomerization [reviewed in (2)]. Domain II apparently functions as a linker to join domain I and III. Domain III is involved in ATP binding and hydrolysis. It carries the amino acid sequence motifs shared among AAA+ family members and named Walker A box, Walker B box, Sensor 1, Box VII and Sensor 2. Domain IV functions in binding to the DnaA box. A region that interacts with acidic phospholipids is not shown [reviewed in (3)]. The boundaries separating the domains are derived from functional analyses [reviewed in (2)], comparative amino acid sequence alignment of DnaA homologues (http://www.molgen.mpg.de/~messer/) and from a homology model based on the X-ray crystal structure of domain 3 and 4 of *Aquifex aeolicus* DnaA (4).

(16). *In vitro*, the A184V substitution substantially reduces the affinity of DnaA for ATP (21). As the ATP-bound form of DnaA is much more active than DnaA-ADP in initiation (22), the residual ATP binding by DnaAcos is apparently enough to support its activity in initiation. However, we do not know if the substitution also impairs the already weak ATPase activity of DnaA to result in DnaA remaining in a persistent ATP-bound state, or interferes with the interaction between DnaA and the Hda-β clamp complex (see later in the text).

Current evidence suggests that several independent mechanisms control the frequency of initiation in *E. coli* [reviewed in (23)]. One involves SeqA, which may block unscheduled initiations by its ability to bind specifically to hemi-methylated *oriC* that is produced from a new round of DNA replication (24). The second requires Hda complexed with the β clamp bound to DNA (25). This complex stimulates the hydrolysis of ATP bound to DnaA in a process named the regulatory inactivation of DnaA. As DnaA-ADP is less active in *oriC* plasmid replication *in vitro*, this mechanism is thought to block initiation from occurring at improper times in the cell cycle (26,27). The third pathway involves *datA*, a site in the bacterial chromosome to which estimates of several hundred DnaA molecules can bind (28–30). This site is speculated to take up excess DnaA that would otherwise induce extra initiation events.

In an earlier study, we described a genetic method to select for genes that regulate initiation (31). We showed that *hda*, *dnaN*, *datA* or *seqA* in a multicopy plasmid (pACYC184) suppressed the toxic effect caused by elevated $dnaA^+$ expression in DSB repair-defective *recA* or *recB* mutants. As an increased level of DnaA in an isogenic $recA^+$ strain is not lethal, these results suggest that the toxicity originates from the cell's inability to repair DSBs formed when replication forks assembled from new rounds of initiation run into stalled forks. The effect of the respective multicopy plasmids support the model that the increased abundance of Hda or the β clamp encoded by *dnaN* reduces initiation by stimulating the hydrolysis of ATP bound to DnaA. These results also suggest that the respective chromosomally encoded levels are limiting, and that an increased level either stimulates the formation of the Hda-β clamp complex and/or allows for the complex to efficiently interact with and down-regulate DnaA. The current model explaining *datA*-mediated regulation of replication initiation is that extra copies of this locus titrates excess DnaA to reduce initiation frequency. The multicopy *seqA* plasmid may suppress hyperinitiation by two mechanisms. In one, an increased steady-state level of SeqA bound to hemi-methylated *oriC* may prolong its sequestered state to block the binding of DnaA to *oriC* (32,33). Alternatively, as *in vitro* replication of an *oriC*-containing plasmid requires that this DNA is supercoiled, SeqA's ability to inhibit topoisomerase IV and its increased level may lower the superhelix density of the chromosomal domain carrying *oriC* to reduce initiation frequency (34).

This study also showed that DnaAcos hyperinitiates by apparently circumventing the regulatory pathway dependent on Hda and the β clamp (31). As the *datA* plasmid fails to suppress the toxicity caused by an increased DnaAcos level, those molecules of DnaAcos not bound to *datA* can apparently induce extra initiations, thus overcoming the effect of this locus.

Recent evidence indicates that Hda complexed with the β clamp bound to DNA interacts directly with DnaA to stimulate the hydrolysis of the bound ATP (35,36). Hda apparently interacts with both the ATP binding domain of DnaA, and with specific amino acids in domain 1 (N44), and in domain 4 (L422 and P423) that bind to the DnaA box (37). In addition, a mutant DnaA lacking domain 1 and 2 retained its ability to bind ATP and its intrinsic ATPase activity, but was almost inert in responding to the Hda-β clamp complex (27), suggesting that Hda complexed to the β clamp may interact with domain 2. However, it is not known whether DnaA also interacts directly with the β clamp complexed with Hda. To extend our understanding of the mechanisms that control initiation, we developed a genetic method based on properties conveyed by DnaAcos. Our goal was to isolate other mutant DnaAs that overinitiate on the basis that their characterization may lead to new insight into how initiation is regulated. A second objective was to seek evidence for novel regulatory pathways. Herein, we describe the study of six novel mutant DnaAs. Their characterization suggests that some mutants are defective in ATP hydrolysis, which is normally stimulated by Hda complexed to the β clamp. One mutant DnaA (V292M) may initiate excessively by resisting a novel regulatory factor. We also present direct biochemical evidence that, except for V292M, these mutants and DnaAcos are more resistant than wild-type DnaA (wtDnaA) to the inhibitory

effects of Hda and the β clamp; yet, all retain the ability to interact physically with Hda complexed with the β clamp. Of interest, DnaAcos and another mutant (E244K) bind more avidly to Hda than wtDnaA, suggesting that these particular mutants complexed to Hda restrict its availability so that free DnaA molecules are able to initiate DNA replication at improper times in the cell cycle.

MATERIALS AND METHODS

Proteins and DNAs

Where noted, highly purified wtDnaA, and DnaA or mutant DnaAs fused at their N-termini to polyhistidine, were used. The concentration of these protein fractions was determined by the dye-binding method (38), and by quantitative densitometry of Coomassie Blue-stained SDS polyacrylamide gels relative to bovine serum albumin. Other purified replication proteins have been described (39,40). Creatine kinase and phosphocreatine were purchased from Sigma-Aldrich. Plasmid DNAs are described in Supplementary Table S1.

Isolation of novel *dnaA* alleles

An hydroxylamine-treated DNA restriction fragment carrying the *dnaA* coding region prepared from plasmid pDS596 as described (41) was used to replace this same 1.5 kb fragment in the untreated plasmid. The resultant ligation mixture was then transformed into LS1073 [relevant genotype: *dnaA46*(Ts)] followed by selection at 42°C on Luria–Bertani (LB) plates containing ampicillin (100 μg/ml) to obtain *dnaA* alleles that can complement the *dnaA46* mutant. To identify *dnaA* mutations like *dnaAcos*, the isolates were then screened for their inability to grow on LB medium supplemented with 0.5% arabinose and ampicillin (100 μg/ml) at 30°C while remaining able to grow at 42°C in the absence of the inducer. Thirty-four isolates were originally obtained of 1289 transformants screened. After isolation of plasmid DNA and retransformation into LS1073, six conferred growth interference, confirming that the phenotype is plasmid-dependent. DNA sequence analysis was performed to map the respective mutations.

Real-time PCR analysis

Escherichia coli MC1061 [*araD139* Δ(*ara*, *leu*)*7697* Δ*lacX74 galU galK rpsL hsdR2* (r_K- m_{K+}) *mcrB1*] bearing plasmids encoding the respective *dnaA* alleles under *araBAD* promoter control (Table 3) was grown with aeration in LB medium supplemented with 1% (w/v) glucose and ampicillin (100 μg/ml) at 30, 37 and 42°C. At an optical density (OD; 595 nm) of ~0.15, cultures were centrifuged at 14 000 rpm for 2 min in a Beckman F0650 rotor at 20°C and immediately resuspended in the above pre-warmed media, but lacking glucose. Portions were then adjusted to contain 0.5% L-arabinose or 1% glucose, and samples were removed at various times during growth with aeration at the respective temperatures. For real-time PCR analysis, reactions (25 μl) contained 5 ng of genomic DNA isolated from these samples, SYBR Green PCR master mix (Applied Biosystems) and primers that amplify 100 base pair fragments of *oriC* or *relE* as described (10,31). As interplate variation is a major source of inaccuracy, we used one PCR plate to determine the abundance of *oriC* in the experimental samples relative to a standard curve prepared with increasing amounts of bacterial DNA from a stationary phase culture. We also measured the abundance of *relE* in the same DNA samples on a separate plate and then calculated the ratio of *oriC* to *relE*. For each of three identical DNA samples for a specific time point, we quantified the abundance of these loci in quadruplicate.

Purification of mutant DnaAs, DnaAcos and Hda

wtDnaA fused at its N-terminus to a polyhistidine sequence encoded by pET16b (Novagen) was overproduced and purified from *E. coli* BL21 [(λDE3) (pLysS) *ompT dcm gal lon hsdS*$_B$ (r_B-, m_B-)] as described (42). Because polyhistidine joined to the N-terminus of DnaA has no measurable effect on its activity in any assay (8,12,42,43), corresponding derivatives of pET16b encoding *dnaAcos* or other *dnaA* alleles were constructed. The strain described here or a derivative encoding Δ*oriC* and Δ*dnaA* mutations was used for the purification of the novel mutant DnaAs, or DnaAcos, respectively. Cultures bearing the respective *dnaA* plasmids were grown at 37°C in LB media supplemented with ampicillin (100 μg/ml), and chloramphenicol (35 μg/ml) to a turbidity (595 nm) of ~0.6. For the strain encoding Δ*oriC* and Δ*dnaA* mutations and also carrying the plasmid bearing *dnaAcos*, cultures additionally contained kanamycin (25 μg/ml), and tetracycline (15 μg/ml). To induce expression of the plasmid-borne alleles, 0.4 mM IPTG was added followed by incubation for 3 h. Purification of DnaAcos or the mutant DnaAs was performed essentially as described (42).

For the purification of Hda, we inserted the *hda* coding region between the NdeI and BamHI cleavage sites of pET16b (Novagen). The insertion joins an open reading frame for polyhistidine encoded by the parent plasmid to the *hda* gene, which was amplified with primers that replaced both the originally annotated GUG initiation codon (27) with AUG, and the natural Shine–Dalgarno sequence with the corresponding DNA sequence for gene 10 of T7 bacteriophage. This plasmid named pET16b*hda* is essentially identical to one encoding Hda joined to polyhistidine that was described to be comparable in activity with a partially purified fraction of wild type Hda (27), suggesting that the polyhistidine tag does not affect Hda function *in vitro*. In support, we observed that pET16b*hda* but not the parental plasmid carried in a Δ*hda* strain [JC125 F^- λ^- *rph-1* Δ*hda*::*tet*^{R}; (44)] conferred a colony size in the absence of inducer comparable to the isogenic *hda*$^+$ strain (MG1655 F^- λ^- *rph-1*).

At a turbidity of ~0.6 (595 nm) at 37°C, *E. coli* BL21 (λDE3) (pLysS) carrying pET16b*hda* in LB medium containing ampicillin (100 μg/ml) was supplemented with IPTG to a final concentration of 0.4 mM, followed by continued growth for 2 h. After centrifugation, the cell pellets were frozen and stored at −70°C. The thawed

cells were resuspended in 20 mM Tris–HCl (pH 7.5) and 10% sucrose, and then adjusted to contain 0.5 M NaCl, 20 mM spermidine and 5 mM imidazole. After centrifugation in a Beckman Ti 45 rotor at 40 000 rpm for 30 min at 4°C, the pellet was resuspended in buffer [20 mM Tris–HCl (pH 7.5), 0.5 M NaCl and 15% glycerol] containing 5 mM imidazole, and 6 M urea, and the solubilized material was then chromatographed onto a HiTrap Chelating HP column (4 ml; GE Healthcare) equilibrated in the above buffer plus 5 mM imidazole, followed by elution with a 20 ml of linear gradient from 5 to 400 mM imidazole in this buffer essentially as described (42). Based on the analysis of Coomassie blue-stained SDS-polyacrylamide gels, fractions containing Hda were pooled and chromatographed on a Superose 12 column (HR 10/30, GE Healthcare) equilibrated in 50 mM Tris–HCl (pH 7.6), 10% (w/v) glycerol, 120 mM potassium glutamate, 0.01% Brij58, 8 mM dithiothreitol and 0.5 mM EDTA. SDS-polyacrylamide gel electrophoresis revealed that Hda eluted at a volume expected for the homodimer and was greater than 95% pure.

Assays to measure DNA replication

Reactions containing a supercoiled plasmid bearing *oriC* (M13*oriC*2LB5, 200 ng or 46 fmol), purified replication proteins and other required components were incubated at 30°C for 30 min with various levels of the different forms of DnaA as described (12). Where indicated, a crude protein fraction lacking DnaA protein activity, which was prepared from *E. coli* WM433 [*leu-19 pro-19 trp-25 his-47 thyA59 arg-28 met-55 deoB23 lac-11 gal-11 strA56 sul1 hsdS* ^{K12} *dnaA204* as described (45,46), was used in place of the purified replication proteins. These reactions also contained 8% (w/v) polyvinyl alcohol (Mr 30 000–70 000; Sigma-Aldrich].

To measure the inactivation of DnaA by Hda and the β clamp, incubations were separated into two stages using a similar approach to that described (26). In reactions with DnaAcos in which DNA replication during the second stage relied on a crude protein fraction, the first stage of incubation in 5 μl contained 40 mM HEPES–KOH (pH 7.6), 20 mM Tris–HCl (pH 7.5), 10 mM magnesium acetate, 4 mM dithiothreitol, 0.08 mg/ml bovine serum albumin, 4% (w/v) sucrose, 5 mM ATP (which we estimate naturally contains ~50 μM ADP), 40 mM phosphocreatine, creatine kinase (200 μg/ml), M13*oriC*2LB5 (200 ng; 46 fmol) and unless otherwise stated, DNA polymerase III* (30 ng; 0.06 pmol) as the source of the clamp loader, the β clamp (25 ng; 0.3 pmol), Hda as indicated, and either wtDnaA (64 ng; 1.2 pmol) or DnaAcos (160 ng; 3 pmol). After the first incubation at 30°C for 20 min, each reaction was supplemented with 20 μl of a mixture containing 40 mM HEPES–KOH (pH 7.6), 40 mM phosphocreatine, creatine kinase (75 μg/ml), 1.25 mM ATP, 0.5 mM each of CTP, UTP and GTP, 100 μM each of dATP, dCTP, dGTP and [methyl-^{3}H] dTTP (25–30 cpm/pmol), 10 mM magnesium acetate, 8% (w/v) polyvinyl alcohol, and a crude enzyme fraction (350 ng) lacking DnaA activity that was prepared from *E. coli* WM433 (46), followed by incubation at 30°C for 20 min for wtDnaA or for 30 min for DnaAcos. Alternatively in assays with purified components in both stages, the first incubation was performed as described earlier in the text, but the reactions (23 μl) differed in that they also contained 0.5 mM each of CTP, GTP and UTP, 100 μM each of dATP, dCTP, dGTP and [methyl-^{3}H] dTTP (25–30 cpm/pmol), and instead included 2 mM ATP, 6 mM phosphocreatine, creatine kinase (108 μg/ml), DNA polymerase III* (88 ng; 0.2 pmol), the β clamp (49 ng; 0.6 pmol), wtDnaA or mutant DnaA (100 ng; 2 pmol), and the indicated amounts of Hda. After the first incubation, a mixture (2 μl) that contained SSB (310 ng; 4 pmol), HU (α dimer; 5 ng; 0.3 pmol), DNA gyrase A subunit (370 ng; 3.8 pmol), DNA gyrase B subunit (520 ng; 5.8 pmol), DnaB (100 ng; 0.3 pmol), DnaC (58 ng; 2 pmol) and primase (11 ng; 0.2 pmol) was added followed by incubation for 20 min at 30°C. After trichloroacetic acid precipitation of acid-insoluble radioactivity onto glass fiber filters (Whatman GF/C), DNA synthesis (in pmol) was measured by liquid scintillation spectrometry.

ATP hydrolysis

Following established methods (26,47), wtDnaA or the mutants (2 pmol) were incubated with 1.5 μM [α^{32}P]-ATP for 15 min at 0°C in 0.5 mM magnesium acetate, 15% glycerol, 0.01% Triton X-100 and 50 mM Tris–HCl (pH 8.0). A mixture containing the β-clamp (100 ng), DNA polymerase III* (140 ng; a subassembly of this DNA polymerase that contains the clamp-loader and the core complex) and M13*oriC*2LB5 supercoiled DNA (200 ng of this *oriC* plasmid) in 20 mM Tris–HCl (pH 7.5), 10% (v/v) glycerol, 8 mM DTT, 0.05% Brij 58, 8 mM magnesium acetate, 120 mM potassium glutamate, 0.1 mg/ml bovine serum albumin, 2 mM ATP and 30 μM ADP was added. After adding the indicated amounts of Hda, reactions (25 μl) were incubated for 20 min at 30°C. DnaA was then immunoprecipitated with Protein A agarose beads that were pre-incubated with rabbit antiserum that specifically recognizes the DNA binding domain of DnaA, and the bound nucleotide was analyzed by polyethyleneimine-cellulose thin-layer chromatography.

RESULTS

Isolation of *dnaA* alleles that hyperinitiate

Relying on a *dnaA46*(Ts) strain and plasmids carrying various *dnaA* alleles downstream from an inducible promoter, we developed a genetic method to isolate *dnaA* mutations that initiate DNA replication excessively. This method is based on an earlier observation that an elevated level of DnaAcos expressed from the *araBAD* promoter interferes with viability (31). In confirmation, Table 1 shows that induced expression of *dnaAcos* caused a reduction in the frequency of colony formation at 30°C and 42°C. In contrast, the *dnaA*$^+$ gene had no effect on colony formation at 30°C, regardless of whether its plasmid-borne expression was induced, and was able to complement the temperature sensitivity of the *dnaA46* mutant at 42°C. In comparison, the plasmid carrying the *dnaA204*(Ts) allele in the absence of inducer,

Table 1. Elevated expression of *dnaAcos* but not *dnaA*$^+$ interferes with viability of a *dnaA46*(Ts) strain

Plasmid and *dnaA* allele	Colony forming units+inducer[a] / Colony forming units−inducer	
	30°C	42°C
pDS596 (*dnaA*$^+$)	1.0	0.9
pLS120 (*dnaAcos*)	2.2×10^{-3}	4.2×10^{-3}
pDS319 (*dnaA204*)	1.0	⋙1
pBR322	1.0	None detected

[a]*E. coli* LS1073 (relevant genotype: *dnaA46*) was transformed by electroporation with the indicated plasmids that encode the respective *dnaA* alleles under *araBAD* promoter control, or with pBR322 (Supplementary Table S1). Colony formation was measured on LB medium supplemented with ampicillin (100 μg/ml) that either contained or lacked arabinose, and then the ratio of colony forming units obtained on ampicillin-supplemented LB plates containing 0.5% arabinose relative to plates lacking the inducer was calculated. In the absence of inducer, transformants were obtained at a frequency of 10^6-10^7 per μg of plasmid DNA at both temperatures with the following exceptions. For pLS120, the frequency of colony formation was two-fold less at 42°C compared with 30°C, indicating that the plasmid-borne *dnaAcos* allele partially complements the *dnaA46* mutant at 42°C. For pDS319, the ratio of colonies obtained on antibiotic-supplemented LB medium lacking 0.5% arabinose was 1.3×10^6-fold less relative to this media containing arabinose, indicating that the *dnaA204* allele under uninduced conditions is not able to complement the *dnaA46* mutant. For pBR322, no colonies were detected at 42°C in the presence or absence of inducer.

like pBR322, led to a similar low frequency of colony formation at 42°C, showing that these plasmids fail to complement the *dnaA46* defect. However, elevated *dnaA204* expression conferred temperature-resistant growth for the host strain, yielding a ratio much greater than 1 for the frequency of colony formation in the presence of arabinose compared with its absence. Hence, this mutant DnaA may be partially active at the higher temperature, or forms active, mixed complexes with DnaA46. To summarize, this genetic assay shows that *dnaAcos* is unique in that it complements the *dnaA46* strain at 42°C in the absence of inducer and interferes with colony formation when its expression is elevated at either temperature.

To obtain hyperactive *dnaA* alleles, we mutagenized the *dnaA* gene and placed its expression under control of the *araBAD* promoter. With the objective of obtaining functional mutations, we selected for *dnaA* alleles that, like *dnaAcos*, were able to complement the *dnaA46*(Ts) defect at 42°C in the absence of inducer. To identify *dnaA* alleles that interfered with viability, we then screened the transformants at 30°C for growth interference on media with arabinose compared with media lacking the inducer. After immunoblot analysis with a monoclonal antibody (M43) that specifically recognizes DnaA (48), we chose six mutant DnaAs for further study whose abundance and proteolytic stability in whole-cell lysates were comparable with wtDnaA (data not shown).

The *dnaA* alleles support DNA replication of an *oriC*-containing plasmid

Earlier work showed that a pair of *dnaA* alleles that are each defective in a separate function can complement the other, apparently interacting to form a DnaA oligomer that is active in initiation (6). Hence, a mutant DnaA may be inactive by itself but cooperate with DnaA46 of the host strain to form an active DnaA oligomer. To separate such *dnaA* alleles from those that are active without assistance, we used a strain lacking the chromosomal *dnaA* gene. This strain (LS1062, relevant genotype: *dnaA850*::Tn*10* Δ*oriC*::pKN1562) bears the replication origin of plasmid R1 integrated within *oriC*, which lacks 16 base pairs that renders *oriC* non-functional (49). Because R1 does not require DnaA for its maintenance, this plasmid replicon in the absence of DnaA serves as an alternate replication origin to preserve viability (50). This host strain was transformed with a plasmid bearing each *dnaA* allele with expression controlled by the *dnaA* promoters. Their function was measured by maintenance of a second plasmid that carries *oriC* and depends on this locus for DNA replication. Compared with the inactivity of pBR322 at 30 and 42°C, we found that most *dnaA* alleles, each encoding a unique amino acid substitution, were able to maintain the *oriC*-containing plasmid at either temperature (Table 2A). However, the reduced activity of V303M and E445K at 42°C compared with their ability to complement the temperature sensitivity of the *dnaA46* mutant described earlier in the text suggests that they are able to initiate DNA replication at the elevated temperature by oligomerizing with DnaA46. Nevertheless, these mutant DnaAs are active in replication from *oriC* at 30°C (Table 2).

The *dnaA* alleles induce excessive initiation from *oriC*

The results described earlier in the text assume that initiation from *oriC* is required to observe toxicity when the *dnaA* alleles are induced. To support this assumption, we showed that their induced expression compared with *dnaAcos* is lethal when the host strain carries *oriC* but not when the isogenic (integratively suppressed) strain carries a Δ*oriC* mutation (Table 2B). These results with the *oriC*-containing strain obtained at 42°C (and at lower temperatures in Table 3 later in the text) suggest that overinitiation from *oriC* leads to growth interference. For V303M and E445K, it appears that increased expression overcomes the temperature sensitivity observed in Table 2A.

To demonstrate that these *dnaA* alleles induce more frequent initiation than *dnaA*$^+$, we analyzed chromosomal DNA isolated at various times from cultures that either were induced to express each *dnaA* allele or were untreated. With these DNAs, we measured the ratio of *oriC* to *relE* (a locus in the terminus region) by quantitative PCR analysis. This ratio reflects the frequency of initiation. For simplicity, Figure 2 summarizes the *oriC*-to-*relE* ratios obtained only at 30°C and 60 min post-induction, demonstrating that these novel *dnaA* alleles like *dnaAcos* promote excessive initiation. The entire time course for cultures grown at 30, 37 and 42°C shows that the ratios increased with time after expression was induced (Supplementary Figure S1). In contrast, the ratios from the uninduced cultures bearing these alleles were similar to that for the *dnaA*$^+$ gene without induction (data not shown; Supplementary Figure S1).

Table 2. The *dnaA* alleles are active in maintenance of an *oriC*-containing plasmid, and are lethal when overexpressed in an *oriC*$^+$ strain but not in a Δ*oriC* strain

(A) Plasmid	Mutation (nucleotide position and substitution)[a]	Allele or amino acid substitution	Relative plating efficiency[b]
pRB100	None	*dnaA*$^+$	1
pBR322	not applicable	none	none detected
pLS*dnaAcos*	See ref. (18)	*dnaAcos*	1
pLS132	236, G→A	G79D	1.4
pLS134	604, C→T	H202Y	0.7
pLS135	730, G→A	E244K	1
pLS136	874, G→A	V292M	1
pLS137	907, G→A	V303M	9×10^{-4}[c]
pLS138	1333, G→A	E445K	2×10^{-3}

(B) Plasmid	Amino acid substitution	Relative plating efficiency[d]	
		LS1073 (*oriC*$^+$)	LS1062 (Δ*oriC*)
pDS596	*dnaA*$^+$	1	1
pLS120	*dnaAcos*	5×10^{-3}	1
pLS125	G79D	7×10^{-4}	1
pLS127	H202Y	5×10^{-4}	0.9
pLS128	E244K	5×10^{-3}	0.9
pLS129	V292M	1×10^{-4}	1
pLS130	V303M	7×10^{-4}	1
pLS131	E445K	2×10^{-4}	1

[a]The positions in the coding strand of each mutation and the respective nucleotide substitution are indicated relative to the first nucleotide of the *dnaA* coding sequence (51). The *dnaAcos* allele encodes Q156L, A184V, H252Y and Y271H substitutions (18).
[b]The indicated plasmids (see Supplementary Table S1) were transformed by electroporation into *E. coli* LS1062 (relevant genotype: Δ*oriC*::pKN1562, *dnaA850*::Tn*10*) followed by growth on LB plates supplemented with ampicillin (100 μg/ml). Transformants were obtained at 10^5–10^7 colony forming units per μg of plasmid DNA. After confirming the presence of the properly sized plasmid isolated from randomly chosen isolates by agarose gel electrophoresis, an *oriC* plasmid (pCM959-Cm^r) was introduced into each plasmid-bearing strain followed by selection on LB media containing ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml) at 30°C and 42°C. The relative plating efficiency is the ratio of transformants harboring pCM959-Cm^r at 42°C compared with 30°C.
[c]At 30°C, colonies that bear the *V303M* allele were very small compared with colonies expressing other *dnaA* alleles.
[d]The relative plating efficiency is the ratio of the number of colonies obtained at 42°C on LB medium supplemented with 100 μg/ml ampicillin and 0.5% arabinose relative to this medium lacking the inducer. On plates lacking arabinose, transformants were obtained at 10^5–10^7 colony forming units per μg of plasmid DNA. Colonies of LS1062 (relevant genotype: Δ*oriC*::pKN1562, *dnaA850*::Tn*10*) carrying the plasmid-borne *dnaA* alleles were comparable in size but smaller on media supplemented with arabinose compared with this media lacking it. LS1062 is an isogenic derivative of LS1073 (relevant genotype: *oriC*$^+$).

Interestingly, elevated *dnaA*$^+$ expression at 30°C only caused a modest increase in initiation frequency (Figure 2), which we observed previously (31). Although not shown, quantitative immunoblot analysis of portions from the same cultures used for PCR analysis revealed comparable levels of the mutants and wtDnaA protein relative with HU protein used as an internal control. Hence, the modest increase in initiation with wtDnaA at 30°C is not due to its poor expression.

Like DnaAcos, elevated levels of H202Y and V292M are refractory to regulation by *datA*, *hda* and *dnaN*

Results from an earlier study support the conclusion that DnaAcos initiates excessively because it fails to respond to several pathways that regulate the frequency of initiation (31). To summarize, we showed that DnaAcos and not wtDnaA resists regulation by *datA*, *hda* and *dnaN* when their gene dosage is either at the chromosomally encoded level, or elevated via a multicopy plasmid. As Hda complexed to the β clamp stimulates the hydrolysis of ATP bound to DnaA (26), one explanation is that DnaAcos hyperinitiates, despite the increased copies of *hda* or *dnaN* by remaining in the initiation-competent state. A possible mechanism is that one or more of its amino acid substitutions interferes with its interaction with the Hda-β clamp complex. Alternatively, this complex remains able to interact with DnaAcos but fails to stimulate ATP hydrolysis. Regarding *datA*, DnaAcos should remain able to bind to the DnaA boxes within this DNA because the amino acid substitutions of DnaAcos are not in its DNA-binding domain. We suggest that the extra copies of *datA* may titrate only a portion of the excess DnaAcos so that the remainder causes overinitiation.

If the novel mutants behave like DnaAcos, the regulatory genes described earlier in the text may fail to suppress their toxicity. Table 3 summarizes the results of an experiment testing this idea. Confirming previous results (31), we observed that the induced expression of *dnaA*$^+$ did not reduce viability of a repair-proficient (*recA*$^+$) strain at various temperatures (Table 3). We also confirmed that induced *dnaAcos* expression is toxic in this strain carrying the empty vector (pACYC184) or plasmids encoding *hda*, *dnaN* or *datA* (Table 3). Like DnaAcos, H202Y and V292M resisted the effect of *hda*, *dnaN* and *datA*, suggesting that these mutants withstand these regulatory pathways.

Table 3. Like DnaAcos, H202Y or V292M fail to respond to multicopy plasmids encoding *hda*, *dnaN*, or *datA*[a]

Allele	Temperature	Plasmid-borne gene[a]				
		None (pACYC184)	*seqA*	*hda*	*dnaN*	*datA*
A						
dnaA	30°C	1.1	1.1	1	1.4	1.4
	37°C	1.0	0.9	1.1	1.0	1.1
	42°C	1.0	1.0	1.2	1.1	1.0
dnaAcos	30°C	6×10^{-4}	0.5	4×10^{-4}	3×10^{-4}	4×10^{-4}
	37°C	6×10^{-4}	0.5	4×10^{-4}	3×10^{-4}	3×10^{-4}
	42°C	1×10^{-3}	0.6	4×10^{-4}	6×10^{-4}	6×10^{-4}
G79D	30°C	1×10^{-2}	1.1[b]	1.0	0.7[b]	0.9[b]
	37°C	4×10^{-3}	0.3[b]	0.9	0.6[b]	0.7[b]
	42°C	3×10^{-3}	0.3[b]	0.9	0.2[b]	0.7[b]
H202Y	30°C	1×10^{-3}	0.4	5×10^{-3}	2×10^{-3}	3×10^{-3}
	37°C	4×10^{-4}	0.9	2×10^{-3}	2×10^{-3}	4×10^{-3}
	42°C	7×10^{-4}	0.5	8×10^{-4}	2×10^{-3}	3×10^{-3}
V292M	30°C	6×10^{-4}	0.6	3×10^{-3}	1×10^{-3}	9×10^{-4}
	37°C	6×10^{-4}	0.2	1×10^{-3}	6×10^{-4}	4×10^{-4}
	42°C	3×10^{-4}	0.3	1×10^{-3}	1×10^{-3}	5×10^{-4}
B						
dnaA	30°C	1.0	0.9	0.8	1.3	0.9
	37°C	1.0[a]	0.8	1.0	0.9	1.1
	42°C	1.0[a]	0.8	0.8	1.3	1.1
E244K	30°C	$<3 \times 10^{-3}$	0.9	1.1	0.9[b]	$<1 \times 10^{-2}$
	37°C	$<4 \times 10^{-3}$	0.7	0.8	$<9 \times 10^{-3}$	$<8 \times 10^{-3}$
	42°C	$<3 \times 10^{-4}$	1.1[b]	0.9	$<8 \times 10^{-3}$	$<8 \times 10^{-3}$
V303M	30°C	$<4 \times 10^{-3}$	1.0	1.0	0.9[b]	1.0[b]
	37°C	$<4 \times 10^{-3}$	0.9	0.9	8×10^{-3}	1.1[b]
	42°C	$<2 \times 10^{-4}$	0.7	0.9	$<4 \times 10^{-3}$	0.9[b]
E445K	30°C	$<3 \times 10^{-3}$	1.4[b]	1	0.8	$<1 \times 10^{-2}$
	37°C	$<6 \times 10^{-3}$	0.4[b]	0.8	0.3[b]	$<5 \times 10^{-3}$
	42°C	$<6 \times 10^{-4}$	0.9[b]	0.9	$<1 \times 10^{-2}$	$<6 \times 10^{-3}$

[a]*E. coli* MC1061 was co-transformed with the respective *dnaA* plasmid and either pACYC184 or its derivatives. These plasmids are described in Table S1. The relative plating efficiency is the ratio of colonies obtained on antibiotic-supplemented LB medium containing 0.5% arabinose or lacking the inducer.
[b]Colony size ranged from small to very tiny colonies.

Only the *seqA* plasmid was able to suppress the toxicity caused by increased levels of these and the other mutant DnaAs (Table 3). However compared with media lacking the inducer, the smaller size of colonies on arabinose-supplemented media varied with the respective plasmid. Because the known regulatory pathways are thought to work independently, our interpretation is that colony formation reflects the relative sensitivity of a mutant to an elevated level of SeqA as well as to the other pathways operating at their normal levels.

Suppression by the plasmid encoding *dnaN* was effective at all temperatures for G79D but was ineffective at the higher temperatures for E244K, V303M and E445K (Table 3). Although the temperature dependence suggests that a conformational change reduces the ability of the latter mutants to interact with the Hda-β clamp complex, the conundrum is that suppression by the *hda* plasmid was not affected by temperature. We do not understand the reason for this discrepancy.

We found that the plasmid bearing *datA* varied in its suppressing activity (Table 3). For G79D and V303M, we observed a reduction in colony size instead of a lower frequency of colony formation. The other mutants were not suppressed by the *datA* plasmid. Although one explanation is that *datA* is only partially effective because the mutants bind poorly to this site, the dilemma is that most amino acid substitutions do not reside in the DNA-binding domain of DnaA. For E445K, glutamate 445 does not make contact with DNA in the crystal structure of the DNA-binding domain (domain IV) complexed with a DnaA box (52). Hence, these mutants should be able to bind to the DnaA boxes in *datA*. An alternative interpretation is that G79D and V303M not bound to *datA* may still respond to other regulators present at their normal levels, whereas E244K and E445K can withstand them. An alternate possibility is based on the suggestion that the *datA* site does not titrate DnaA to prevent reinitiation but acts to ensure that initiation occurs at the proper time in the cell cycle (53). Although the mechanism of this timekeeper role for *datA* is not understood, our observation that the *datA* plasmid does not uniformly suppress hyperinitiation by these *dnaA* mutations suggests that the mutant DnaAs vary in their reactivity to this regulatory process.

In summary, one major conclusion of Table 3 is that H202Y and V292M are genetically like DnaAcos in that they remain toxic in the presence of multicopy plasmids carrying *datA*, *hda* and *dnaN*. The remaining alleles may still respond but with less efficacy to regulation by the Hda-β clamp complex.

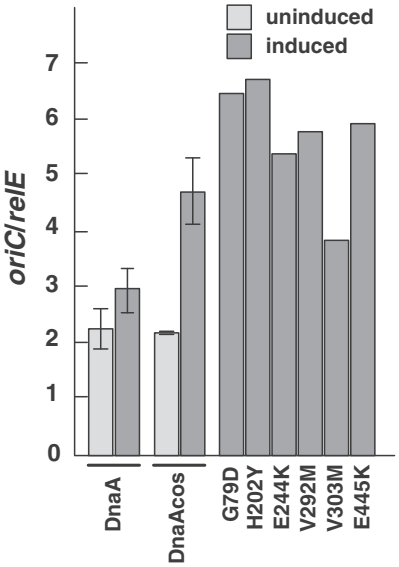


Figure 2. Increased levels of the mutant DnaAs lead to excessive initiation. *Escherichia coli* MC1061 carrying the various plasmid-borne *dnaA* alleles under *araBAD* promoter control was grown at 30°C as described in 'Materials and Methods' section and then transferred into pre-warmed media supplemented with either glucose (1% w/v) as a control or arabinose (0.5% w/v) to induce expression. After 60 min, chromosomal DNA was isolated for real-time PCR analysis to quantify the amounts of *oriC* and *relE*. The ratio of these loci reflects the frequency of initiation. For simplicity, the ratios for the respective mutants under uninduced conditions are not shown as they are comparable with those for wtDnaA and DnaAcos. The brackets for wtDnaA and DnaAcos denote the standard deviation from several determinations. These results were part of a data set described in a previous study (31). The ratios for the respective mutants were determined from a representative experiment. To confirm that expression of the various forms of DnaA was comparable, quantitative immunoblot analysis of DnaA from whole-cell lysates was performed using 0.1 OD (595 nm) of cells from the 60 min samples [(31), data not shown]. As an internal standard, the membranes were then stripped and reprobed with affinity-purified antibody that recognizes HU (α dimer). Among the samples, the normalized amounts of DnaA varied by less than 2-fold.

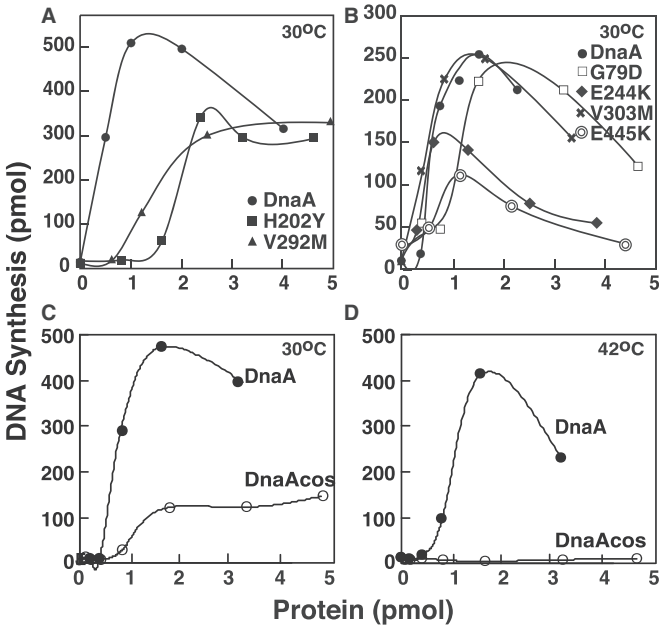


Figure 3. At 30°C, all mutant DnaAs including DnaAcos are active in DNA replication of an *oriC*-containing plasmid. In (**A**) and (**B**), reactions (25 μl) were assembled with a supercoiled plasmid carrying *oriC* (200 ng or 46 fmol of M13*oriC*2LB5), purified replication proteins and the indicated amounts of wild-type or mutant DnaAs as described in 'Materials and Methods' section. Incubation was for 20 min at 30°C. In (**C**) and (**D**), a crude protein fraction that lacks DnaA protein activity replaced the purified replication proteins (see 'Materials and Methods' section), and incubations with wtDnaA or DnaAcos were performed for 30 min at the indicated temperatures. DNA synthesis (as pmol of acid-insoluble radioactivity) was measured by liquid scintillation spectrometry.

The mutant DnaAs are active in DNA replication *in vitro*

To characterize the defects of the mutants *in vitro*, and to address the notion that H202Y, V292M and perhaps the other mutants are less responsive than wtDnaA to Hda and the β clamp, we purified each protein (see 'Materials and Methods' section). We first examined the mutant DnaAs in a replication system reconstituted with purified proteins and a supercoiled plasmid carrying *oriC* (Figure 3). At subsaturating levels, E244K and V303M were comparable with wtDnaA in DNA replication of the *oriC*-containing plasmid, but DNA synthesis was reduced at higher levels of E244K (Figure 3B). In contrast, G79D, H202Y, V292M and E445K were less active than wtDnaA (Figure 3A and B), suggesting that the respective amino acid substitution impairs their DNA replication activity. Although the affinity of H202Y for ATP (K_d of 0.36 μM) is almost 10-fold weaker than wtDnaA, the affinities of the other mutant DnaAs are comparable with wtDnaA (Supplementary Table S2). As the assay of DNA replication contains 2 mM ATP, the mutants and a substantial portion of H202Y should be bound to ATP. Hence, impaired ATP binding does not apparently explain their reduced activity.

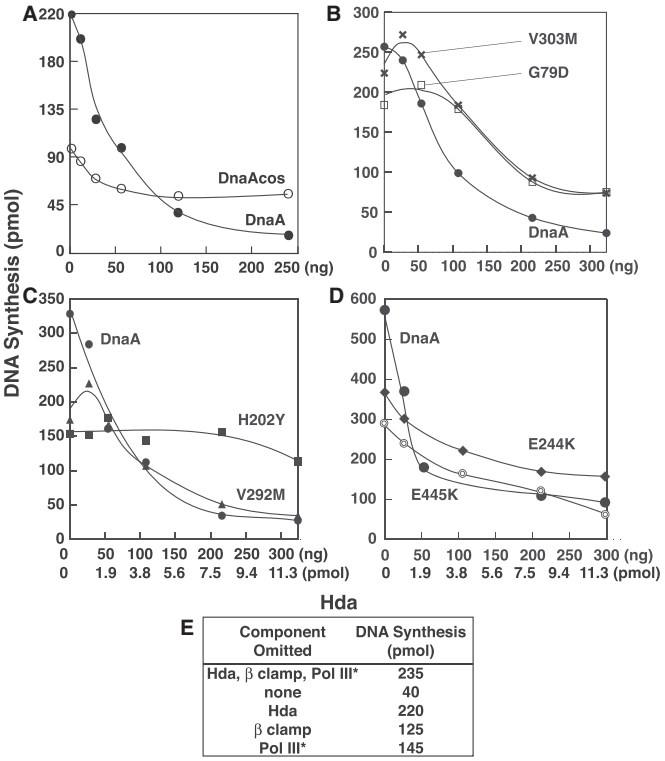
Because of our plan to measure the effect of Hda on the activity of these mutants in comparison with DnaAcos, we also examined DnaAcos in this reconstituted system. Its inactivity (data not shown) contrasts with its function at 30°C when proteins in the reconstituted system were replaced with a crude enzyme fraction (Figure 3C), which confirms earlier work (54). On the basis that a mutant DnaA bearing the A184V substitution requires DnaK and GrpE for activity (21), we presume that the crude enzyme fraction provides these proteins. The inactivity of DnaAcos at 42°C (Figure 3D) conflicts with its ability to induce hyperinitiation *in vivo* at this temperature. Experiments that explore this apparent discrepancy show that DnaAcos is active at both temperatures in an independent replication system (Supplementary Figure S2A and B); therefore, its inertness in Figure 3D is not due to gross unfolding. Other supplementary experiments show that DnaAcos supports *in vitro* DNA replication of an *oriC*-containing plasmid at and below 37°C, but not at higher temperatures, which inversely correlates with impaired growth of a *dnaAcos* mutant at and below 37°C (Supplementary Figure S2C and D) (14,17). Nevertheless, its *in vitro* activity at 30°C provides the opportunity to test whether DnaAcos is insensitive to inhibition by Hda and the β clamp.

The mutant DnaAs vary in their *in vitro* response to Hda and the β clamp

We then measured the effect of Hda and the β clamp on the activity of the mutant DnaAs in an assay that has two stages of incubation (Figure 4). As DnaAcos was described to be insensitive to IdaA, a partially purified factor later identified as the β clamp (55), we also asked whether DnaAcos is insensitive to regulation by the β clamp and Hda. In the first stage, Hda, the β clamp, and DNA polymerase III*, which contains the clamp loader or DnaX complex and the core subassembly (56,57), were incubated with the mutant DnaAs or wtDnaA as a control. For Hda and the β clamp to stimulate the hydrolysis of ATP bound to DnaA, which is dependent on Hda under conditions similar to those previously described [(26); Figure 4] the clamp loader must load the β clamp onto duplex DNA. Hence, reactions also contained a supercoiled plasmid, which carried *oriC* for the second stage. At the end of the first incubation, we supplemented each reaction with DnaB, DnaC, HU, SSB, primase and DNA gyrase (or a crude enzyme fraction for DnaAcos), and other components needed to measure DNA replication of the *oriC*-containing plasmid in the second stage. Compared with wtDnaA, we found that Hda only weakly inhibited DnaAcos (Figure 4A). We also found that G79D, E244K, V303M and E445K were less sensitive, whereas H202Y was refractory to inhibition by Hda (Figure 4B–D). For example, the curves for G79D and V303M are shifted to the right compared with that for wtDnaA, indicating that these mutants are partially inhibited. As the *hda* plasmid was able to maintain viability when their levels were elevated (Table 3), the effect of elevated gene dosage of *hda* suggests a weaker interaction. For these four mutants, the higher *in vitro* levels of Hda needed to attain comparable levels of inhibition as for wtDnaA supports this expectation. These results suggest that, except for V292M, DnaAcos and the remaining mutants overinitiate *in vivo* because they either respond weakly or are refractory to negative control by Hda and the β clamp. Of interest, the curve for V292M is comparable with that for wtDnaA if one sets aside the data point for which no Hda was added, suggesting that V292M is as sensitive as wtDnaA to inhibition by Hda *in vitro* (Figure 4C). However, this mutant DnaA is resistant *in vivo* (Table 3). We suggest that this mutant DnaA remains able to respond to Hda complexed with the β clamp, but resists an unknown regulatory factor *in vivo*.

Inhibition by Hda is dependent on the β clamp and the clamp loader of DNA polymerase III*

The experiments of Figure 4 presume the involvement of the β clamp bound to DNA. If so, inhibition should depend on the β clamp and the clamp loader. Under conditions in which DNA replication relied on the crude enzyme fraction in the second stage, we found that inhibition of DnaA was strongly dependent on Hda (Figure 4A) but was partially dependent on the β clamp and the clamp loader or DnaX complex contained in DNA polymerase III* (Figure 4E). Similar results were obtained when purified replication proteins were used in place of the enzyme fraction (data not shown). We suggest that this partial dependence is due to the requirement for the β clamp and clamp loader in the second stage of incubation. Despite their absence in the first stage, their inclusion during the second stage for DNA replication also permits their interaction with Hda to inhibit DnaA.



E

Component Omitted	DNA Synthesis (pmol)
Hda, β clamp, Pol III*	235
none	40
Hda	220
β clamp	125
Pol III*	145

Figure 4. DnaAcos and H202Y are refractory to Hda, whereas G79D, E244K, V303M and E445K are partially sensitive and V292M is sensitive. In (**A**), DnaAcos or wtDnaA was incubated under conditions described in 'Materials and Methods' section with the β clamp, DNA polymerase III* as the source for the clamp loader or DnaX complex, M13*oriC*2LB5 (200 ng; 46 fmol), and the indicated amounts of Hda. After incubation for 20 min at 30°C, a crude enzyme fraction and other components were added to measure DNA synthesis during a second stage of incubation for 20 min with wtDnaA, or 30 min with DnaAcos. Assays in (**B**), (**C**) and (**D**) were performed as above but with purified components in both the first and second incubations, each for 20 min at 30°C, with wild-type or mutant DnaA (100 ng; 2 pmol) and the indicated amounts of Hda. Preliminary studies did not indicate a dependence on ADP, which we presume is due to the presence of about 1% ADP contained in the ATP used for the experiment. Assays in (**E**) were performed as described in (A) with Hda (100 ng; 3.8 pmol), and wtDnaA (64 ng; 1.2 pmol). Where noted, DNA polymerase III*, the β clamp and Hda were omitted.

The Hda-β clamp complex only weakly enhances the ATPase activity of H202Y and E445K compared with its function with wtDnaA and the other novel mutants

The relative resistance of the mutants to inhibition by Hda in Figure 4 may correlate with the reduced ability of the Hda-β clamp complex to stimulate the intrinsic ATPase activity of DnaA. The experiments typified in Figure 5 explore this possibility and show that ATP hydrolysis by H202Y and E445K compared with wtDnaA was only marginally stimulated. In contrast, the remaining mutants

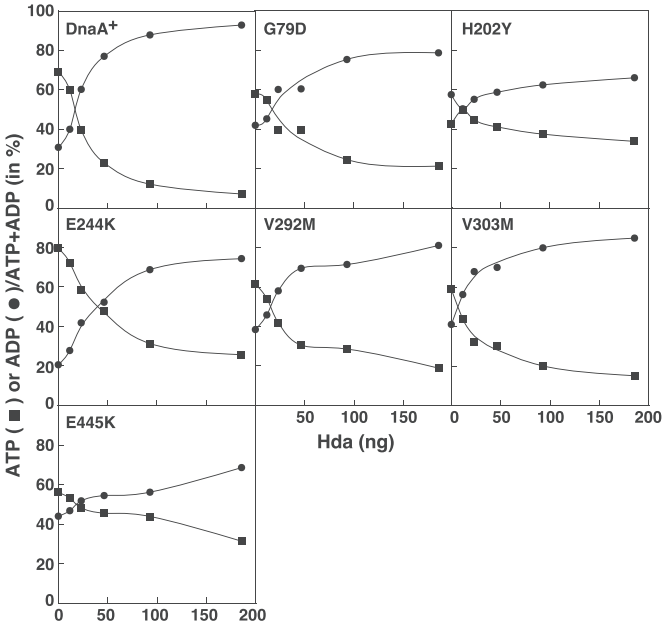


Figure 5. Hda and the β clamp remain able to stimulate the hydrolysis of ATP bound to G79D, E244K, V292M and V303M, but are only weakly effective with H202Y and E445K. Assays were performed as described in 'Materials and Methods' section. The amounts of ATP and ADP bound to DnaA have been plotted relative to the total nucleotide and are expressed in percentage.

appeared to be relatively responsive, whereas DnaAcos was not active under these assay conditions presumably because of its weak affinity for adenine-containing nucleotides (data not shown) (21). These observations may relate to an early study, which showed that DnaA-ADP added to a subsaturating level of DnaA-ATP stimulates *in vitro* DNA replication of an *oriC*-containing plasmid (22). Hence, the relative proportion of DnaA molecules bound to ATP or ADP and assembled at *oriC* governs initiation. As the Hda-β clamp complex varied in its ability to stimulate the ATPase activity of the mutants (Figure 5), the relative ratio of bound ADP to ATP in response to the Hda-β clamp complex in Figure 4 presumably reflects the activity of this mixture in initiation.

The mutant DnaAs retain their ability to physically interact with Hda

The results described earlier in the text suggest that the respective amino acid substitutions reside in a domain that interacts with Hda, or indirectly alters the conformation of the interacting domain to impede this interaction. To test these possibilities, we developed an assay that measures the binding of Hda when added either alone or in a complex with the β clamp to DnaA immobilized on a microtiter plate (Figure 6A). Under the assay conditions, we confirmed previous observations that DnaA and HU interact (11) but were unable to detect an interaction between DnaA and either the β clamp or bovine serum albumin. Thus, DnaA interacts specifically with Hda. With the exception of E445K, which at its highest level is reduced in its interaction with Hda, most of the remaining mutants bind as well as wtDnaA to Hda (Figure 6B).

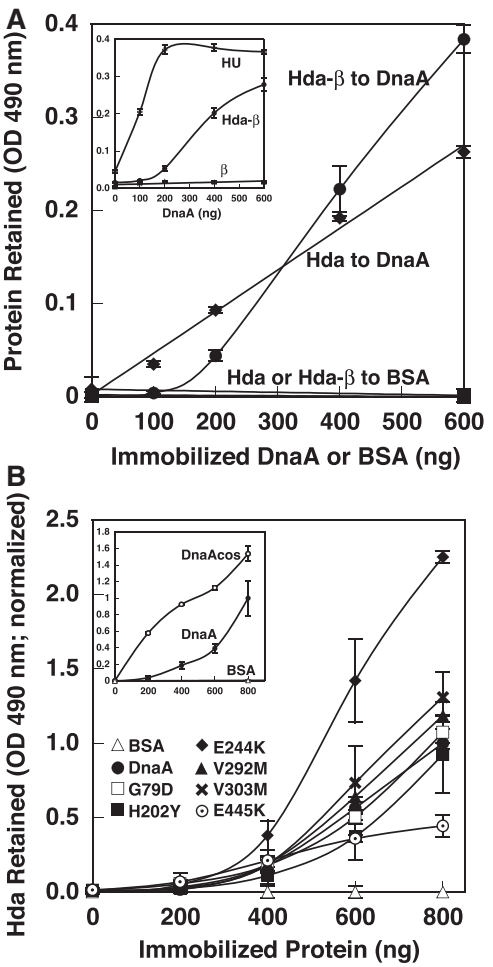


Figure 6. Hda interacts with immobilized DnaAcos and E244K more effectively than wtDnaA and the other mutant DnaAs. Enzyme-linked immunosorbent assays were performed as described (10), but with the following modifications. Briefly, wtDnaA, a mutant DnaA or bovine serum albumin in 100 µl of buffer containing 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 2.7 mM KCl and 0.137 M NaCl (PBS) was added in triplicate at the indicated amounts to the wells of a microtiter plate. After binding, the wells were washed as described. In (**A**), the Hda-β clamp complex at an amount corresponding to 0.1 µg of Hda, or Hda (0.1 µg) was added. In the inset of (A), the Hda-β clamp complex at an amount corresponding to 0.15 µg of the β clamp, the β clamp (0.15 µg), or HU (0.15 µg) was added. In (**B**), the Hda-β clamp complex at an amount corresponding to 0.15 µg of Hda was added to the washed wells. Appropriate dilutions of antiserum that specifically recognizes HU (A inset), the β clamp (A, A inset, and B), or the polyhistidine sequence fused to Hda (Amersham; A) were then added followed by incubation at 4°C overnight. After washing the plates as described earlier in the text, immune complexes were detected colorimetrically with goat anti-rabbit antibody or goat anti-mouse antibody conjugated to horseradish peroxidase (Pierce Biotechnology).

These observations suggest that the respective amino acid substitutions do not interfere with the interaction between DnaA and Hda. For H202Y and E445K, these substitutions clearly affect the ability of Hda to stimulate their ATPase activity. Remarkably, E244K or DnaAcos binds more effectively to Hda. Considering also that these mutants hyperinitiate, their greater avidity for Hda may limit its availability, which is estimated to be 20- to 40-fold less abundant than DnaA in log phase cells

(27,58). Such a mechanism allows for free DnaA to initiate unscheduled rounds of DNA replication. For E244K, its weak stimulation by the Hda-β clamp complex in ATP hydrolysis may also lead to more of the mutant bound to ATP relative to ADP.

DISCUSSION

The Hda-β clamp pathway

The mutant DnaAs characterized in this study appear to vary in their sensitivity to regulation by the Hda-β clamp complex (see Table 4 for a summary). H202Y and DnaAcos are similar in that the *hda*, *dnaN* and *datA* plasmids fail to suppress the toxic phenotype caused by their overexpression, and these mutants resist inhibition by the Hda-β clamp complex *in vitro*. Like the A184V substitution, which is responsible for the hyperactive initiation activity of DnaAcos and greatly lowers the affinity of the mutant protein for ATP (16,21), the H202Y substitution resides in the ATP binding domain. This substitution apparently alters the conformation of this domain based on the ~10-fold reduced affinity of H202Y for ATP compared with wtDnaA (Supplementary Table S2). In addition, the relative insensitivity of H202Y to the Hda-β clamp complex in stimulating the hydrolysis of ATP evidently results in this mutant remaining persistently active in initiation.

An interesting difference between H202Y and DnaAcos is that the latter apparently binds with greater affinity to Hda. So does E244K. We note that the cellular abundance of Hda and DnaA is estimated at 50 dimers and 800–2100 molecules per cell, respectively (27,58). By forming a more stable complex with Hda, DnaAcos and E244K may lower the availability of Hda. Hence, the mutant DnaA molecules that evade Hda are able to initiate extra rounds of DNA replication.

Like H202Y, E445K responds weakly to the Hda-β clamp complex in assays that measure the hydrolysis of ATP bound to DnaA, and DNA replication of an *oriC*-containing plasmid. Although it is not definitive that E445K is impaired in its interaction with Hda, these observations suggest that this amino acid substitution interferes with the interaction between DnaA and Hda. However, the increased abundance of Hda can apparently fortify this interaction as indicated by the ability of an *hda* plasmid to suppress the toxicity caused by an increased level of E445K.

Although V292M, like H202Y and DnaAcos, resists the neutralizing effect of the *hda* plasmid *in vivo*, this mutant is comparably sensitive as wtDnaA with inhibition by the Hda-β clamp complex *in vitro*, which stimulates its ATPase activity. We suggest that V292M remains responsive to Hda complexed with the β clamp but resists a novel regulatory factor *in vivo* that regulates the frequency of initiation. The identification of this factor is the objective of future work, which may lead to important new insight into how DNA replication is regulated.

G79D and V303M no longer caused toxicity when their abundance is elevated in the presence of the *hda* plasmid. Although this gene dosage effect suggests that the mutants remain able to respond to Hda complexed with the β clamp, but more weakly compared with wtDnaA, we were unable to show that their binding to Hda is

Table 4. Summary of mutant DnaAs that cause overinitiation[a]

	Hyperinitiation (*in vivo*)	Multicopy suppression of the toxicity caused by hyperinitiation[b]				ATP binding	Inhibition by the Hda-β clamp complex in DNA replication *in vitro*	Stimulation of ATP hydrolysis by the Hda-β clamp complex	Direct interaction with Hda	Reference
		seqA	*hda*	*dnaN*	*datA*					
wtDnaA	–	NA	NA	NA	NA	+	+	+	+	This work
DnaAcos	+	+	–	–	–	Greatly reduced	–	ND	+ +	This work
G79D	+	+	+	+	+	+	±	+	+	This work
H202Y	+	+	–	–	–	10-fold reduced	–	±	+	This work
E244K	+	+	+	+	–	+	±	+	+ +	This work
V292M	+	+	–	–	–	+	+	+	+	This work
V303M	+	+	+	+	+	+	±	+	+	This work
E445K	+	+	+	+	–	+	±	±	±[c]	This work
N44A	ND	ND	ND	ND	ND	+	ND	–	–	(36)
K54A	ND	ND	ND	ND	ND	+	ND	±	ND	(36)
A184V	+	ND	ND	ND	ND	Greatly reduced	ND	ND	ND	(20)
R334A	+	ND	ND	ND	ND	+	–	–	–	(32,59)
L422A/G	ND	ND	ND	ND	ND	+	ND	–	±	(37)
P423A	ND	ND	ND	ND	ND	+	ND	±	±	(37)
DnaA219 (A184V, H252Y, R342C)	+	ND	ND	ND	ND	ND	ND	ND	ND	(60)

[a]Abbreviations are –: not hyperactive, not suppressed, not inhibited or stimulated, or not interactive; +: hyperactive, suppressed, active, inhibited or stimulated, or reactive; ± partially active, inhibited or stimulated, or reactive; ND, not determined; and NA, not applicable.
[b]Suppression measured at 30°C (Table 3).
[c]It is not certain if E445K is defective in interaction with Hda.

impaired, and observed only slight defects in their Hda-stimulated ATPase activity. Perhaps, more sensitive methods will reveal the mechanism(s) whereby they initiate excessively.

For G79D, E244K, V303M and E445K, suppression by the plasmid encoding *dnaN* varied (Table 3). Except for G79D, this suppression was ineffective at the higher temperatures. If the increased gene dosage of *dnaN* leads to a greater abundance of the Hda-β clamp complex either free or bound to Okazaki fragments on the lagging strand template, the respective amino acid substitution may hamper the interaction between the mutant DnaA and the complex more effectively at the elevated temperatures. We have not examined the *in vitro* interaction of DnaA with Hda at different temperatures.

The SeqA and *datA* pathways

Our results show that the *seqA* plasmid suppresses the toxic effect of the *dnaA* alleles, albeit with varying efficiency (Table 3). Suppression to inhibit new initiations may involve the binding of SeqA to hemi-methylated *oriC* to sequester this site from DnaA (24). Alternatively as *in vitro* replication of an *oriC*-containing plasmid requires that it is supercoiled, inhibition of topoisomerase IV by SeqA may reduce the negative superhelical density of the chromosomal domain containing *oriC*, causing less frequent initiation (61).

The *datA* locus is estimated to titrate several hundred DnaA molecules (62). A recent study describes that this site also stimulates the hydrolysis of ATP bound to DnaA (63). This and other evidence supports the idea that this site is where DnaA binds when in excess to inhibit unscheduled initiations (30,64). When the mutant DnaAs are elevated in the presence of the *datA* plasmid, the measure of viability reflects not only the effect of elevated gene dosage of *datA* but also of other regulatory pathways. Our results show that the *datA* plasmid neutralizes the toxicity of G79D and V303M (Table 3). In contrast, the inability of this plasmid to suppress H202Y, V292M, E244K and E445K suggests that these mutants are able to resist negative regulation by other regulatory pathways. In agreement, the *in vitro* results suggest that H202Y is refractory to inhibition by Hda, whereas G79D, E244K, V303M and E445K are partially sensitive (Figure 4).

Hyperinitiation in eukaryotes

This work may have significance that extends beyond the area of DNA replication in *E. coli*. In eukaryotes, the general mechanism of initiation of DNA replication is remarkably similar to this process in *E. coli* [reviewed in (65)]. In mammals, re-initiation can be induced by overexpression of licensing factors (Cdt1 and Cdc6), or by depletion of inhibitors of licensing (geminin and Emi1) [reviewed in (66,67)]. As in *E. coli* (17), these unscheduled initiations lead to head-to-tail fork collisions and the production of DSBs (68). The DSBs not fixed by the DNA damage response lead to aneuploidy, chromosome fusions and other types of DNA rearrangements that can lead to tumorigenesis. Thus, the study of mutant DnaAs that evade regulatory factors that normally control the initiation process has health implications and can lead to new insight into mechanisms that control the frequency of initiation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online, including [69–74].

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