A *dnaC* Mutation in *Escherichia coli* That Affects Copy Number of ColE1-Like Plasmids and the PriA-PriB (but Not Rep-PriC) Pathway of Chromosomal Replication Restart

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ABSTRACT

Escherichia coli nusG and *rho* mutants, which are defective in transcription termination, are killed following transformation with several ColE1-like plasmids that lack the plasmid-encoded copy-number regulator gene *rom* because of uncontrolled plasmid replication within the cells. In this study, a mutation [*dnaC1331*(A84T)] in the *dnaC* gene encoding the replicative helicase-loading protein was characterized as a suppressor of this plasmid-mediated lethality phenotype. The mutation also reduced the copy number of the plasmids in otherwise wild-type strains. In comparison with the isogenic *dnaC*$^+$ strain, the *dnaC* mutant was largely unaffected for (i) growth on rich or minimal medium, (ii) tolerance to UV irradiation, or (iii) survival in the absence of the PriA, RecA, or RecB proteins. However, it was moderately SOS-induced and was absolutely dependent on both the Rep helicase and the PriC protein for its viability. A *dnaC1331*(A84T) *dam* mutant, but not its *mutH* derivative, exhibited sensitivity to growth on rich medium, suggestive of a reduced capacity in the *dnaC1331*(A84T) strains to survive chromosomal double-strand breaks. We propose that DnaC-A84T is proficient in the assembly of replication forks for both initiation of chromosome replication (at *oriC*) and replication restart via the Rep-PriC pathway, but that it is specifically defective for replication restart via the PriA-PriB pathway (and consequently also for replication of the Rom$^-$ ColE1-like plasmids).

THE replication fork represents a complex of enzymes that act in concert to achieve unidirectionally processive semiconservative replication of the template DNA, notwithstanding the opposing polarity of its two strands. In *Escherichia coli*, the replication fork that is involved in replication of the chromosome as well as of various plasmids and coliphages is composed of the 5′–3′ replicative helicase DnaB, the primase DnaG, and two catalytic polymerase subassemblies of the DNA polymerase III holoenzyme (one each for the leading and lagging template DNA strands; reviewed in Kornberg and Baker 1992; Marians 1996; Messer and Weigel 1996).

Initiation of replication fork assembly on the chromosome involves the delivery by DnaC protein of the DnaB helicase to a specific region *oriC* at which the initiator protein DnaA is bound. This assembly is also known as the ABC primosome (Marians 1996). DnaB then recruits DnaG (Tougu and Marians 1996) and DNA polymerase III (Kim *et al.* 1996) to constitute the replication fork. Two replication forks so constituted at *oriC* then travel in opposite directions to achieve replication of the entire chromosome.

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In the past few years, it has been recognized that replication forks initiated at *oriC* often do not progress to completion around the chromosome but instead collapse or disintegrate spontaneously at, for example, sites of template DNA damage. In such cases, the fork may be reassembled at the site of its collapse in a process called replication restart, which in turn is intimately connected to the process of RecA-dependent recombinational repair of the damaged DNA (reviewed in Kogoma 1997; Kuzminov 1999; Cox *et al.* 2000; Michel 2000; Sandler and Marians 2000; Cox 2001; McGlynn and Lloyd 2002).

Although the mechanism of replication restart is not understood in all its detail, it is DnaA independent and shares many features with phage ϕX174 replication. Biochemical studies with purified proteins *in vitro* have suggested that during replication restart, a restart primosome (also called the ϕX-type primosome) is constituted at a three-way-junction DNA structure, the D-loop, which is an intermediate in recombinational repair (McGlynn *et al.* 1997; Liu and Marians 1999; Liu *et al.* 1999; Xu and Marians 2003). The restart primosome is formed by the sequential action of three proteins, PriA, PriB, and DnaT, at the D-loop. PriA is a bifunctional protein that both initiates ϕX-type primosome assembly and acts as a 3′–5′ DNA helicase, but studies *in vitro* (Liu *et al.* 1999) and *in vivo* (Sandler *et al.* 1996, 2001; Jaktaji and Lloyd 2003) have suggested that the

latter function is dispensable for replication restart at D-loops; PriB is a structural component of the primosome whose precise role in replication restart is not established, while DnaT apparently promotes the transfer of DnaB from the DnaB-DnaC complex in solution to the PriA-PriB-DNA complex (Marians 1996). The subsequent steps of replication fork formation are similar to those at DnaA-bound *oriC*.

Genetic evidence not only has provided corroboration for the involvement of PriA and PriB in replication restart at D-loops (in the so-called PriA-PriB pathway), but also has suggested that two additional pathways exist for this process (Sandler *et al.* 1999, 2001; Sandler 2000). Both proposed pathways require the PriC protein (which, like PriB, was originally identified as a factor required for ϕX174 replication *in vitro* but has otherwise not been well characterized), acting with either PriA (PriA-PriC pathway) or another 3′–5′ DNA helicase Rep (Rep-PriC pathway); however, biochemical data in support of either pathway are not available.

Finally, replication fork assembly is also important for plasmid replication. In the case of the ColE1-like plasmids (see Cesareni *et al.* 1991; Del Solar *et al.* 1998 for reviews), an RNA primer generated by the processing of the plasmid-specified transcript RNA-II is extended by DNA polymerase I to form a D-loop that is then believed to serve for replication fork assembly in much the same way as that described above for replication restart on the chromosome. RNA-II processing to generate the primer is modulated by an antisense inhibitor RNA-I, and the interaction between RNA-II and its inhibitor is stabilized by the Rom protein, encoded by the plasmid-borne *rom* gene. Null mutants in *priA* do not support ColE1 plasmid replication, and point mutations in *priA* as well as a triple substitution mutant allele of *priB* that confer a reduced plasmid-copy-number phenotype have been described (Lee and Kornberg 1991; Nurse *et al.* 1991; Sandler *et al.* 1996, 2001; Berges *et al.* 1997; Jaktaji and Lloyd 2003).

Although it is apparent from the description above that DnaC-mediated delivery of DnaB is the first common step in all mechanisms of replication fork assembly, it is not clear whether the same domain of DnaC is involved in interacting with the different structures (such as DnaA-bound *oriC* or the restart primosome assembled at a D-loop). *dnaC* mutants that are temperature sensitive (Ts) for growth have been characterized (Wechsler 1975; Maisnier-Patin *et al.* 2001), consistent with the protein's essential role in replication fork assembly at *oriC* (and perhaps also in replication restart). Other *dnaC* mutations have been recovered as suppressors of sickness or inviability associated with *priA* disruption (Kogoma *et al.* 1996; Sandler *et al.* 1996, 1999, 2001; Gregg *et al.* 2002; Rangarajan *et al.* 2002; Jaktaji and Lloyd 2003). In this article, we report the identification and characterization of a novel *dnaC* mutant [*dnaC1331*(A84T)] that exhibits both a reduced-copy-number phenotype with Rom$^-$ ColE1-like plasmids and synthetic lethality with certain genes, such as *rep* and *priC*, implicated in replication restart. Our results suggest that DnaC-A84T is affected for the PriA-PriB pathway but not for the Rep-PriC pathway of replication restart or for replication fork assembly at *oriC*.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids: Genotypes of *E. coli* K-12 strains are listed in Table 1. The routinely employed rich and minimal growth media were, respectively, Luria broth (LB) and glucose-minimal A (Miller 1992). Antibiotics were used at the following final concentrations (micrograms per milliliter): ampicillin (Amp), 100; rifampicin (Rif), 100; chloramphenicol (Cm), 25; kanamycin (Kan), 50; spectinomycin (Spec), 50; and tetracycline (Tet), 15. Unless otherwise indicated, the growth temperature was 30°.

Plasmids previously described include (salient features in parentheses): (i) p15A-derived: pACYC184 (Cm^R Tet^R; Chang and Cohen 1978); (ii) pMB9-derived: pBR322 (Amp^R Tet^R; Bolivar *et al.* 1977), pUC19 (Amp^R; Yanisch-Perron *et al.* 1985), pBR329 (Amp^R Cm^R Tet^R; Covarrubias and Bolivar 1982), and pHYD762 (Amp^R; Harinarayanan and Gowrishankar 2003); and (iii) pSC101-derived: pLG339 (Kan^R; Williams *et al.* 1996), and pCL1921 (Spec^R; Lerner and Inouye 1990).

Plasmid pHYD554 is a pCL1921 derivative carrying a 5-kb chromosomal fragment that includes the *rep*$^+$ gene and was obtained by subcloning of a *Kpn*I-*Pst*I fragment from the phage clone λ556 of the ordered genomic library of Kohara *et al.* (1987) into the corresponding sites of pCL1921.

DNA methods: Standard protocols were followed for experiments involving recombinant DNA (Sambrook *et al.* 1989). Intracellular content of test plasmids in different strains was estimated following agarose gel electrophoresis of plasmid preparations that were made from constant numbers of cells withdrawn from the cultures; prior to the plasmid isolation procedure, a fixed volume of culture of DH5α carrying plasmid pHYD762 (2.2 kb) was added as loading control to normalize for efficiency of plasmid recovery (Harinarayanan and Gowrishankar 2003). The *dnaTC* locus (see Figure 1) was PCR amplified from chromosomal DNA preparations of isogenic strains GJ1541 (*spl-16*) and GJ1556 (*spl*$^+$) with the aid of primers DNACFP (5′-GGCAATGGCACAGCACAG-3′) and DNATRP (5′-GTGCGTTATCCATCGGTC-3′); both primers were used also for sequencing the PCR products (each 1.54 kb long), along with a third internal primer, DNACIP (5′-CATACTGGCGCGCTTTGC-3′).

Transposon tagging of the *spl-16* locus: Random transpositions of the Kan^R-encoding mini-Tn*10* derivative Tn*10*dKan into the chromosome of the *nusG spl-16* (*dnaC1331*) strain GJ1538 were generated following infection with phage λ1316, as described (Kleckner *et al.* 1991; Miller 1992). A P1 lysate prepared on a pool of 10^6 Kan^R clones was used to transduce the *nusG spl*$^+$ strain GJ1504 to Kan^R. The Kan^R transductants were pooled and transformed with plasmid pACYC184 to identify derivatives of GJ1504 that, by virtue of their coinheriting the *spl-16* allele (from GJ1538) with a linked Tn*10*dKan insertion, survived transformation with pACYC184. One clone (GJ1539) so identified was subsequently shown to have a Kan^R insertion (designated *zji-901*::Tn*10*dKan) ~90% cotransducible with the *spl-16* locus.

UV survival tests: Sensitivity of a strain to UV was determined essentially as described by Miller (1992). Cells from a 50-ml volume of LB culture (grown at 37° to an A_{600} of ~0.5) were chilled on ice for 30 min and resuspended in an equal volume

TABLE 1

List of *E. coli* K-12 strains

Strain[a]	Genotype[b]
CAG18619	*zji-3188*::Tn*10*kan
DH5α	Δ(*argF-lac*) *U169 supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1* (ϕ80*lacZ*ΔM15)
MC4100	Δ(*argF-lac*) *U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*
SS135	*argE3 his-4 thi-1 rpsL31 lac*BK1 Δ*priB302*
GJ11	MC4100 *proU224::lac*
GJ1313	GJ11 *nusG*-G146D *zja-900*::Tn*10*
GJ1504	MC4100 *nusG*-G146D
GJ1538	GJ1313 *dnaC1331*(A84T)[c]
GJ1539	GJ1538 *zji-901*::Tn*10*dKan
GJ1541	GJ1504 *dnaC1331*(A84T) *zji-901*::Tn*10*dKan
GJ3127	MC4100 *dnaC1331*(A84T) *mdoB*::Tn*10*[d]
GJ3128	MC4100 *mdoB*::Tn*10*
GJ3185	MC4100 *galEp3 pcnB1 zad-981*::Tn*10*dKan
GJ5201[e]	GJ3127 Δ*rep*::Cm
GJ5202	GJ3128 Δ*rep*::Cm
GJ5203	MC4100 Δ*rep*::Cm
GJ5204	MC4100 *priC303*::Kan
GJ5205[f]	*thr leu arg his sulA mdoB*::Tn*10* (λ*sulA*::*lac*)
GJ5206	GJ5205 *dnaC1331*(A84T)
GJ5207	GJ5205 *recA*::Kan
GJ5208	GJ5206 *recA*::Kan
GJ5209	GJ5205 *lexA3 malB*::Tn*9*
GJ5210	GJ5206 *lexA3 malB*::Tn*9*
GJ5211	GJ3127 *dam*::Tn*9*
GJ5212	GJ3127 *mutH471*::Kan
GJ5213	GJ5212 *dam*::Tn*9*
GJ5220	GJ3127 *priA1*::Kan
GJ5221	GJ3128 *priA1*::Kan
GJ5222	GJ3127 *recA*::Kan
GJ5223	GJ3128 *recA*::Kan
GJ5224[g]	GJ11 *dnaC1331*(A84T) *recB268*::Tn*10*
GJ5225	GJ11 *recB268*::Tn*10*

[a] The following strains have been described earlier: CAG18619 (Berlyn *et al.* 1996); DH5α (Sambrook *et al.* 1989); MC4100 and GJ11 (Gowrishankar 1985); SS135 (Sandler *et al.* 2001); GJ1313 (Jayashree and Gowrishankar 1995, wherein the *nusG*-G146D allele was designated as *rpoB364*); and GJ1504 and GJ3185 (Harinarayanan and Gowrishankar 2003). All other strains were obtained or constructed in this study.

[b] Genotype designations are as in Berlyn *et al.* (1996). All strains are F^-. The following mutations were transduced from the parenthetically indicated strains described in SaiSree *et al.* (2000): *lexA3 malB*::Tn*9* (GJ1999) and Δ*rep*::Cm (GJ2127); Reddy and Gowrishankar (2000): *dam*::Tn*9* (GJ2282), *mutH471*::Kan (GJ2284), and *recA*::Kan (GJ2293); and Sandler *et al.* (2001): *priC303*::Kan (SS145). Mutation *priA1*:Kan was transduced from strain PN105 obtained from the *E. coli* Genetic Stock Center and *recB268*::Tn*10* from strain JJC315 obtained from B. Michel.

[c] The *dnaC1331*(A84T) mutation has also been referred to as *spl-16* in this study.

[d] The *mdoB*::Tn*10* insertion was previously identified as *zji-202*::Tn*10* (Berlyn *et al.* 1996; Nichols *et al.* 1998) and was transduced from strain CAG18430. Although the Tn*10*kan insertion in CAG18619 was reported to be at the same site as the Tn*10* insertion in CAG18430 (Berlyn *et al.* 1996), our transductional mapping data suggest otherwise (see text and data not shown).

[e] GJ5201 was recovered and maintained as a transformant derivative with the plasmid pHYD554 carrying rep^+.

[f] GJ5205 is a derivative of strain GJ1992 (SaiSree *et al.* 2000). Not all mutations in the strain have been listed here.

[g] GJ5224 was constructed in several steps of P1 transduction from strain GJ1538.

of 0.1 M $MgSO_4$. Five-milliliter aliquots of the suspension were placed in 85-mm petri dishes and exposed to UV (at 0.4 J/M^2/sec) for different lengths of time, after which they were protected from visible light. Appropriate dilutions of each aliquot were then plated on LB for estimation of the viable count.

Other methods: All strain constructions were by P1 transduction (Gowrishankar 1985); when the recipient strain carried an Amp^R plasmid, transductants were recovered at limiting dilutions to minimize the extent of Amp degradation by the recipient lawn on the selection plates. The mutator phenotype of a strain was scored by spotting ~10^7 cells of LB-grown

cultures on LB-Rif plates; typically, the *mut*$^+$ and *mut* strains yielded, respectively, <3 and 50–100 Rif^R colonies per spot. Procedures for nitrosoguanidine mutagenesis and β-galactosidase assays were as described (Miller 1992).

RESULTS

Identification of *dnaC1331*(A84T) as a suppressor of *nusG*-pACYC184 lethality: We have recently described a novel lethality phenotype associated with runaway replication (that is, uncontrolled increase in copy number) of ColE1-like plasmids such as pACYC184 in *E. coli nusG* or *rho* mutants that are defective in factor-dependent transcription termination (Harinarayanan and Gowrishankar 2003). Following nitrosoguanidine mutagenesis of a *nusG* strain GJ1313, an extragenic suppressor mutant GJ1538 was identified that survived transformation with pACYC184. The mutation was designated *spl-16* (for *s*uppressor of *p*lasmid *l*ethality). To facilitate mapping and transfer of the suppressor mutation into other strain backgrounds, it was tagged with a transposon (Tn*10*dKan) marker as described in materials and methods. Conjugational and transductional mapping experiments (data not shown) demonstrated that the Tn*10*dKan insertion (*zji-901*::Tn*10*dKan) and the *spl-16* mutation are at 98 min on the *E. coli* linkage map and that the latter is 42 and 97% cotransducible, respectively, with the previously described transposon insertions *zji-3188*::Tn*10*kan and *mdoB*::Tn*10* (previously called *zji-202*::Tn*10*; Berlyn *et al.* 1996; Nichols *et al.* 1998).

On the basis of our finding (see below) that the mutation affects copy number of ColE1-like plasmids in otherwise wild-type strains, we reasoned that it may be situated in the *dnaTC* locus [which is 3 kb from *mdoB* (Blattner *et al.* 1997)] whose products are known to be involved in replication fork assembly. Accordingly, the *dnaTC* operon from a pair of isogenic *spl*$^+$ and *spl-16* strains was PCR amplified and sequenced. A single mutation was identified in the *spl-16* strain that altered codon 84 of *dnaC* from GCG to ACG (which is deduced to cause the change of a conserved Ala residue to Thr in the protein). The mutation has been designated below as *dnaC1331*(A84T).

The fact that the mutation also results in loss of a *Bsm*I site allowed us to test for cosegregation of the *Bsm*I polymorphism with suppression of pACYC184-mediated lethality in the *nusG spl-16* strain. A P1 lysate prepared on strain CAG18619 (which has the Tn*10*kan marker 42% linked to *spl*$^+$) was used to transduce GJ1538 (*nusG spl-16*) to Kan^R, and a complete concordance between presence (or absence) of the *Bsm*I site and lethality (or survival) following pACYC184 transformation was observed among 20 transductants that were tested (Figure 1). These results established that the *dnaC1331*(A84T) mutation (or another closely linked mutation) is responsible for suppression of pACYC184-mediated lethality in the *nusG* strain.

We have shown previously that a *rho*-A243E mutant is also killed following transformation with pACYC184 (Harinarayanan and Gowrishankar 2003). With the aid of the linked Tn*10*dKan marker, the *dnaC1331* allele was introduced into the *rho* strain, and here, too, it was able to suppress the plasmid-mediated lethality (data not shown).

***dnaC1331*(A84T) reduces copy number of Rom$^-$ ColE1-like plasmids:** Harinarayanan and Gowrishankar (2003) have shown that lethality in pACYC184 transformants of the *nusG* and *rho* strains was accompanied by a substantial increase in plasmid copy number that is most prominent in the early stationary phase of growth; furthermore, mutations in several loci (including *hns*, *rpoB*, *pcnB*, and *polA*), as well as in multicopy *recG*$^+$ or *rom*$^+$, were identified as suppressors of lethality that were all also associated with reversal of the copy number alteration in the mutants.

The *dnaC1331*(A84T) suppressor mutation, characterized in the present study, also behaved like the suppressors previously described in that it was able to reverse the increase in pACYC184 content otherwise observed in cultures of the *nusG* strain in the early-stationary growth phase (Figure 2). Densitometric analysis of the band intensity of pACYC184 (relative to that of the loading control) in each of the lanes in Figure 2 indicated that the pACYC184 content (per unit of biomass) in the *nusG* mutant in the exponential and stationary phases of growth was, respectively, two- and ninefold higher than that in the corresponding culture aliquots of either the wild-type strain or the *nusG dnaC1331* double mutant. The latter two strains themselves exhibited an approximate doubling in pACYC184 content as the cultures progressed from the log phase to the stationary phase.

Two mechanisms have been identified by which the different suppressors act to suppress *nusG*- or *rho*-pACYC184 lethality. The first is by reversing the transcription termination defect in the mutants, and the second is by a primary effect on plasmid copy number even in the *nusG*$^+$ *rho*$^+$ strain (Harinarayanan and Gowrishankar 2003). The *dnaC1331*(A84T) mutation did not alleviate the defect in transcription termination in the *nusG* and *rho* strains, as judged by the extent of transcriptional polarity relief at different loci such as *galP3* (an IS*2* insertion in the interval between the promoter and the first structural gene of the *gal* operon) or *trpE* (Oc; a nonsense mutation in the first gene of the *trp* operon; data not shown). On the other hand, the mutation was associated with a pronounced decrease in plasmid pACYC184 content even in the *nusG*$^+$ *rho*$^+$ background (Figure 3).

As mentioned in the Introduction, the plasmid-encoded Rom protein serves to downregulate the copy number of ColE1-like plasmids by stabilizing the interaction between RNA-II and its antisense inhibitor RNA-I. When plasmids such as pUC19, pBR329, or pBR322 were ex-

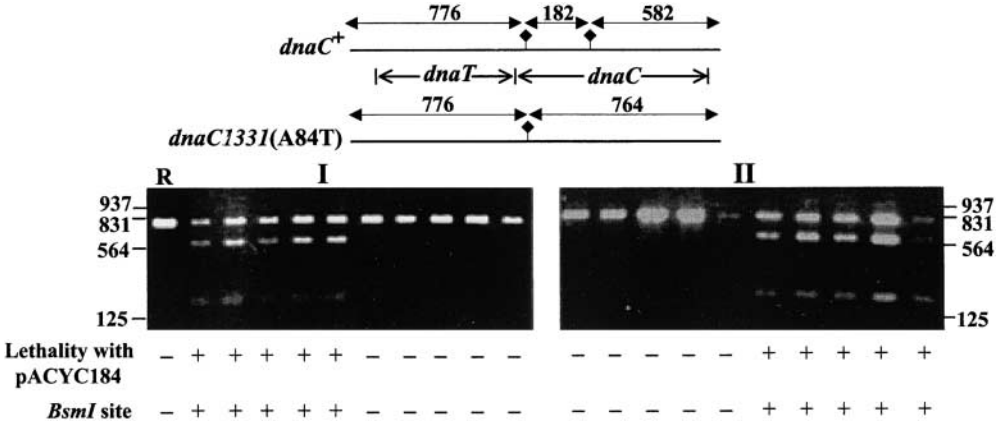

FIGURE 1.—Association of the *spl-16* [*dnaC1331*(A84T)] mutation with loss of the *Bsm*I site in *dnaC*. The *dnaTC* locus was PCR amplified from individual Kan^R transductants of a cross [P1(CAG 18619) × GJ1538] in which the *spl*$^+$ and *spl-16* alleles were segregating and the PCR products were analyzed by agarose gel electrophoresis following digestion with *Bsm*I. Positions of DNA size markers (in base pairs) are marked beside the gels. (Top) Linear depictions of the PCR products from the *dnaC*$^+$ and *dnaC1331*(A84T) strains with the expected sizes (in base pairs) of the *Bsm*I fragments obtained therefrom marked (*Bsm*I recognition sites shown as solid diamonds). Marked beneath the gel lanes are the phenotypes of the transductants with respect to lethality (+, that is, *spl*$^+$) or survival (−, that is, *spl-16*) following pACYC184 transformation and the deduced presence (+) or absence (−) of the *Bsm*I site overlapping the codon 84 in *dnaC*. Transductants in gel I were first classified as *spl*$^+$ or *spl-16* and subsequently tested for *Bsm*I polymorphism, while those in gel II were first classified on the basis of *Bsm*I polymorphism and then tested for pACYC184-mediated lethality. R, strain GJ1538 (*spl-16*) used as recipient in the transduction.

amined in isogenic *dnaC*$^+$ and *dnaC1331*(A84T) mutants, it was observed that the content of the first two (which, like pACY184, are Rom$^-$) is also markedly reduced in *dnaC* relative to *dnaC*$^+$, whereas that of the third (which is Rom$^+$) is only marginally affected in the mutant (Figure 3). Densitometric analysis of the test plasmid band intensities in Figure 3 (after normalization against the corresponding loading controls) indicated that the ratio of plasmid content in the *dnac1331* mutant to that in *dnaC*$^+$ was, respectively, 0.19, 0.06, and 0.27 for the plasmids pACYC184, pBR329, and pUC19 and that it was 0.77 for plasmid pBR322. These results also established that there is no correlation between plasmids conferring lethality in the *nusG* or *rho* mutants and those whose copy number is reduced by the *dnaC1331*(A84T) mutation [since pBR329 is an example of a plasmid that does not kill the *nusG/rho* strains (HARINARAYANAN and GOWRISHANKAR 2003) and yet whose copy number is reduced in the *dnaC* strain].

Suppression by *dnaC1331*(A84T) or *priB* of the Ts growth phenotype of pUC19 transformants: LIN-CHAO *et al.* (1992) have shown that (i) the very-high-copy-number phenotype of plasmid pUC19 is the result of a mutation in its gene encoding RNA-II, which destabilizes the latter's interaction with the inhibitor RNA-I; (ii) the interaction of pUC19-encoded RNA-II with RNA-I is rendered even less stable at 42°, so that cultures that have been shifted from 30° to 42° exhibit a further increase in plasmid copy number and a reduction in growth rate; and (iii) Rom protein expression rescues the slow-growth phenotype at 42°.

We confirmed in this study that pUC19 transformants of the wild-type strain GJ3128 exhibit a pronounced increase in plasmid copy number upon temperature upshift to 42° (Figure 4A).The transformants also exhibited a Ts growth phenotype on LB agar medium (Figure 4B). The isogenic *dnaC1331*(A84T) mutant GJ3127 exhibited neither the plasmid copy number increase at 42° (Figure 4A) nor the Ts growth phenotype (Figure 4B).

As expected, the pUC19-associated Ts phenotype was also suppressed in a *pcnB* strain [in which plasmid copy number is reduced because of an increased stability of the inhibitor RNA-I (XU *et al.* 1993; Figure 4B)]. When

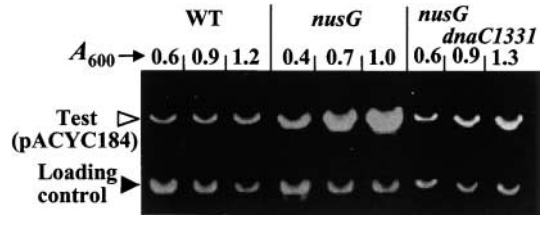

FIGURE 2.—Intracellular content of test plasmid pACYC184 in derivatives of wild-type (MC4100), *nusG* (GJ1504), and *nusG dnaC1331* (GJ1541) strains as a function of growth phase. Plasmid preparations were made from a constant number of cells that had each been withdrawn at the indicated values of A_{600} of the cultures. See MATERIALS AND METHODS for description of loading control. Plasmid preparations were digested with *Eco*RI before gel electrophoresis.

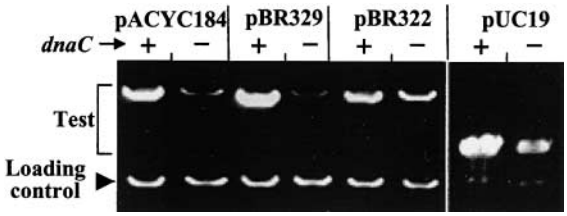

FIGURE 3.—Content of test plasmids pACYC184, pBR329, pBR322, and pUC19 in *dnaC*$^+$ (+) or *dnaC1331*(A84T) (−) derivatives GJ3128 and GJ3127, respectively. See MATERIALS AND METHODS for description of loading control. Plasmid preparations were digested with *Eco*RI before gel electrophoresis.

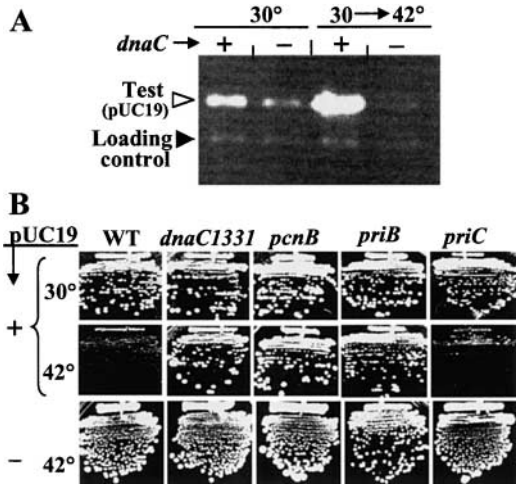

FIGURE 4.—Suppression of Ts phenotype associated with increased pUC19 copy number at 42°. (A) Content of test plasmid pUC19 in cultures of *dnaC*$^+$ (+) or *dnaC1331*(A84T) (−) derivatives GJ3128 and GJ3127, respectively, either upon continuous incubation at 30° or following upshift (at an A_{600} of ~0.5) to 42° for 4 hr (30 → 42°). See MATERIALS AND METHODS for description of loading control. Plasmid preparations were digested with *Eco*RI before gel electrophoresis. (B) Growth phenotypes on LB medium at 30° and 42° of wild-type (GJ3128), *dnaC1331* (GJ3127), *pcnB* (GJ3185), *priB* (SS135), and *priC* (GJ5204) strains with (+) or without (−) pUC19, as indicated. Inocula were taken from LB medium plates that had been incubated at 30°.

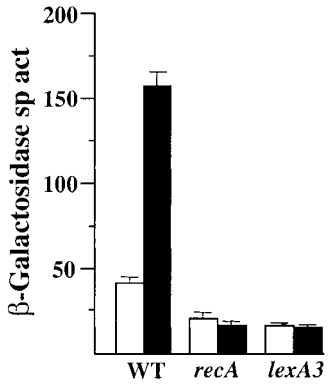

FIGURE 5.—Chronic SOS induction in *dnaC1331*(A84T) mutant. Expression of *sulA-lac* was measured in isogenic *dnaC*$^+$ (open bars) and *dnaC1331* (solid bars) derivatives without any additional mutation (wild type) or with *recA* or *lexA3* as indicated. β-Galactosidase specific activity values (each the average of at least three individual measurements, with standard error of the mean as marked) are given in Miller units (MILLER 1992). Strain pairs employed were (in the order, *dnaC*$^+$ and *dnaC1331*) wild type, GJ5205 and GJ5206; *recA*, GJ5207 and GJ5208; and *lexA3*, GJ5209 and GJ5210.

null mutations in *priB* or *priC* were tested, the former but not the latter suppressed the Ts phenotype (Figure 4B). [In the absence of pUC19, the wild type and all the mutant strains were unaffected for growth at 42° (Figure 4B).] A *priB* missense mutant with reduced copy number of Rom$^-$ ColE1-like plasmids has previously been reported (BERGES *et al.* 1997).

Chronic SOS induction in the *dnaC1331*(A84T) mutant and synthetic lethality with *rep* or *priC* mutations: The growth rates of the wild-type strain GJ3128 and its *dnaC1331*(A84T) derivative GJ3127 were indistinguishable in both LB (2.47 and 2.44 hr^{-1}, respectively, at 37°; see also Figure 4B and Figure 6) and glucose-minimal A (0.87 and 0.88 hr^{-1}, respectively, at 37°), suggesting that the mutant protein is unaffected in its ability to load DnaB helicase at replication forks initiated at *oriC*. At the same time, the mutation conferred a moderate level of chronic SOS induction with a threefold increase of *sulA-lac* expression in the *dnaC1331*(A84T) strain; the increase was abolished, as expected, upon introduction of *recA* or *lexA3* mutations that render the SOS regulon noninducible (WALKER 1996; Figure 5). These observations suggested that the *dnaC1331* mutant might be affected for replication fork assembly at restart primosomes.

As a test of this possibility, we looked for synthetic lethal interactions between *dnaC* and other genes previously implicated in recombinational repair and replication restart pathways. In the first approach, double mutants of *dnaC1331*(A84T) with each of the other mutations were sought to be constructed by P1 transduction. As shown in Figure 6, viable double mutants were recovered with *recA*::Kan, *recB*::Tn*10*, or *priA*::Kan; likewise, double mutants were also obtained at the expected frequencies for three other loci: *recG258*::Kan, Δ*ruvABC*::Cm, and Δ*priB302* (data not shown). [Consistent with an earlier report (GREGG *et al.* 2002), the *priA* single mutant was itself very sick and gave rise to many faster-growing suppressor clones (see Figure 6).] Growth of each of the double mutants was in general no more significantly impaired than that of the respective single-locus mutants, although the *recB dnaC* strain alone did exhibit a 40% slower growth rate than *recB* (see Figure 6). On the other hand, no transductants were obtained when we sought to introduce the *rep*::Kan, Δ*rep*::Cm, or *priC*::Kan alleles into the *dnaC1331*(A84T) strain (data not shown).

Two additional experiments were undertaken to establish that *rep* is synthetically lethal with *dnaC*-A84T. The strategy for the first was analogous to that employed by SANDLER (2000) for demonstrating *rep-priA* lethality and involved an attempt to introduce *dnaC1331*(A84T) in a nonselective fashion into the Δ*rep* strain. A P1 lysate prepared on a strain (GJ3127) carrying the *dnaC1331* mutation along with the closely linked *mdoB*::Tn*10* marker was used to transduce a pair of isogenic *rep*$^+$ and Δ*rep*::Cm strains (MC4100 and GJ5203, respectively) to Tet^R. The recipient strains also carried plasmid pUC19 that rendered them Ts for growth (see above). As expected, the vast majority (39/40) of the Tet^R transductants in the cross into the *rep*$^+$ strain had become temperature resistant, indicating coinheritance of *dnaC1331*(A84T), whereas none of 40 transductants in the

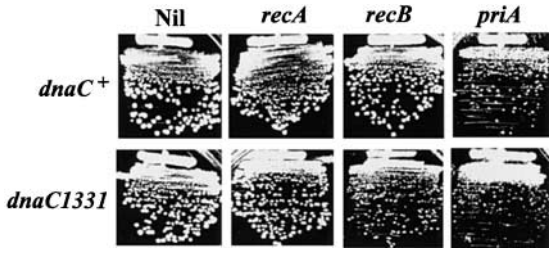

FIGURE 6.—Viability of double mutants of *dnaC1331*(A84T) with *recA*::Kan, *recB*::Tn*10*, or *priA*::Kan. Growth was scored on LB agar plates of the following pairs of strains (in the order, *dnaC*$^+$ and *dnaC1331*): with no additional mutation (Nil), GJ3128 and GJ3127; *recA*, GJ5223 and GJ5222; *recB*, GJ5225 and GJ5224; and *priA*, GJ5221 and GJ5220.

Δ*rep*::Cm strain had become temperature resistant. In a variation of this experiment, the recipient strains did not carry plasmid pUC19 and inheritance of the *dnaC1331* mutation in the transductants was scored directly by testing each of them (with the PCR-based method described above) for concomitant loss of the *Bsm*I site in the gene. Of 11 transductants tested from the cross into the *rep*$^+$ strain, 10 had lost the *Bsm*I site in *dnaC*, whereas none of 12 from the cross into the Δ*rep*::Cm strain had lost this site (Figure 7).

In the second experiment, the frequency of loss of a Spec^R plasmid carrying *rep*$^+$ (pHYD554) in either a Δ*rep*::Cm *dnaC*$^+$ (GJ5202) or a Δ*rep*::Cm *dnaC1331* (GJ5201) strain was scored following introduction of a second plasmid, pLG339 (Kan^R), that is incompatible with pHYD554. In the former strain, after 20 generations of growth in the absence of Spec, 26 of 30 Kan^R transformants had become Spec^S even as they retained the Δ*rep*::Cm marker. On the other hand, in the *dnaC* strain, only 5 of 30 were Spec^S and in all these cases, the strains had also become *rep*$^+$ on the chromosome (that is, Cm^S) presumably by recombination between the plasmid and the chromosome. These results indicated that a Δ*rep dnaC* mutant cannot survive in the absence of the plasmid with *rep*$^+$.

That *priC* is synthetically lethal with *dnaC1331* (A84T) was also established by the experiment of P1 transduction with the lysate of the *mdoB*::Tn*10 dnaC* strain (GJ3127) into isogenic *priC*$^+$ and *priC*::Kan recipients (MC4100 and GJ5204, respectively) carrying plasmid pUC19. As with *rep*, none of 50 Tet^R transductants in the *priC* mutant had lost the Ts phenotype (as compared to 47/50 in the *priC*$^+$ strain), indicating the absence of inheritance of the *dnaC* mutation in any of them. By the PCR-based *Bsm*I polymorphism test as well, none of 12 Tet^R transductants examined of GJ5204 (without pUC19) had inherited the *dnaC1331* allele (Figure 7).

***mutH*-suppressible broth sensitivity of the *dnaC1331* (A84T) mutant:** The synthetic lethality of *dnaC1331* (A84T) with *rep* or *priC* mutations suggested that the former may be compromised for one or more pathways of replication restart on the chromosome. We reasoned that the mutant may also consequently be unable to tolerate conditions that increase the need for replication restart, and to test this possibility we introduced a *dam* mutation into the *dnaC1331* strain. Mutants deficient in the Dam methylase suffer an increased frequency of double-strand breaks (DSBs) by a process that is dependent on a functional mismatch repair system encoded by the *mutHLS* genes (RADMAN and WAGNER 1986; WANG and SMITH 1986; KUZMINOV 1999; MARINUS 2000). In general, the need for replication restart is also increased during growth of cultures in rich medium (when the time interval between successive replication forks is considerably reduced) when compared to that in minimal medium, and *priA* mutants that are defective in replication restart or *priB gyrB* strains exhibit rich-medium sensitivity (KOGOMA 1997; GREGG *et al.* 2002; GROMPONE *et al.* 2003).

Transductants recovered from the cross in which a *dam*::Tn*9* mutation was introduced into the *dnaC1331* (A84T) strain were very sick on LB but not on glucose-minimal agar plates (see Figure 8) and gave rise to spontaneous fast-growing suppressors. Five of eight such suppressors that were tested had acquired a mutator phenotype, suggestive of the loss of mismatch repair functions in them. *dam*::Tn*9* transductants that were recovered in a *dnaC1331*(A84T) *mutH* strain GJ5212 also did not exhibit the rich-medium-sensitive phenotype (Figure 8).

These data provide strong support for the notion that the *dnaC1331* mutant is rendered growth sensitive by the increased frequency of replication-directed DSBs that are generated in the absence of Dam methylase. On the other hand, the *dnaC1331*(A84T) single mutant was no more sensitive to UV irradiation than was its isogenic *dnaC*$^+$ parent (Figure 9), suggesting that the mutant protein is able to function as effectively as the wild type to satisfy the increased need for replication restart on UV-damaged DNA templates.

DISCUSSION

The *dnaC1331*(A84T) mutation was identified in this study by its ability to suppress the lethality associated with runaway replication of Rom$^-$ ColE1-like plasmids in transcription termination-defective *nusG* and *rho* mutants and was shown to do so by a primary effect on plasmid copy number even in otherwise wild-type strains. The amino acid alteration in the mutant protein has occurred in a short stretch of identified sequence similarity with the λP protein (which is functionally analogous to DnaC in phage λ replication; NAKAYAMA *et al.* 1987). The *dnaC1331*(A84T) allele was also synthetically lethal with the *rep* or *priC* null mutations. These results are interpreted below in terms of a model in which the

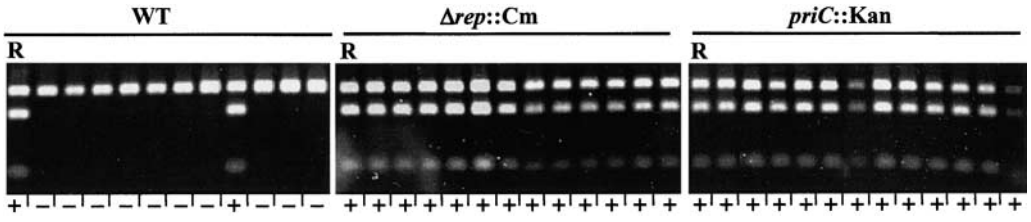

FIGURE 7.—Synthetic lethality of *dnaC1331*(A84T) with Δ*rep*::Cm or *priC*::Kan. Lanes in the three panels represent individual Tet^R transductants obtained from crosses into the recipient strains wild type (MC4100), Δ*rep*::Cm (GJ5203), or *priC303*::Kan (GJ5204), as indicated, with a P1 lysate grown on GJ3127 (*dnaC1331* linked 95% to *mdoB*::Tn*10*). Chromosomal DNA from each transductant was subjected to PCR of and *Bsm*I polymorphism testing at the *dnaC* locus, as described in the text and in the legend to Figure 1. R, chromosomal DNA from the corresponding recipient strain. Marked beneath each lane is the *dnaC* genotype (+, *dnaC*$^+$; −, *dnaC1331*) of the strain, as inferred from the *Bsm*I polymorphism analysis.

dnaC mutant is specifically defective in the PriA-PriB pathway of replication restart.

DnaC and assembly of replication forks by different pathways: As mentioned above, DnaC delivers the DnaB helicase for replication fork assembly during the processes of (i) initiation of chromosome replication at *oriC*, (ii) replication restart following the collapse of previously assembled forks, and (iii) plasmid replication. The *dnaC1331*(A84T) mutant exhibits chronic SOS induction, which suggests some perturbation in chromosomal DNA replication in the strain. The fact that the mutant has a normal growth rate in both rich and minimal media argues that it is not affected for replication fork assembly at the ABC primosome (that is, DnaA-bound *oriC*). That, on the other hand, it may be affected for replication restart (that is, at the ϕX-type primosome at D-loops) is suggested by the findings that (i) *dnaC1331*(A84T) confers rich-medium sensitivity in combination with *dam* (that is, when the requirement for replication restart functions is expected to be increased because of frequent DSBs) and (ii) this inviability is suppressed by mutation in the gene *mutH* for mismatch repair.

Sandler and co-workers have obtained genetic data to suggest that three alternative pathways exist for replication restart at D-loops in wild-type cells, which have been designated (on the basis of respective gene product requirements) as the PriA-PriB, PriA-PriC, and Rep-PriC pathways (SANDLER *et al.* 1999, 2001; SANDLER 2000). Of these, it is only for the PriA-PriB pathway that supporting biochemical evidence is available at present (XU and MARIANS 2003).

Many phenotypes associated with the *dnaC1331* (A84T) mutation (for example, that it is synthetically lethal with *priC* but not with either *priA* or *priB*) may be explained by this multiple-pathway model of replication restart by making the additional assumptions that (i) the ability to restart replication at D-loops is important for viability and (ii) in the *dnaC1331* strain, the PriA-PriB pathway of replication restart at D-loops is rendered largely, if not completely, nonfunctional; consequently, the mutant is dependent on the functionality of the PriA-PriC and Rep-PriC pathways for its survival. The fact that the *dnaC1331* mutant is sensitive to Dam methylase deficiency (Figure 8), but not to UV irradiation (Figure 9), would then suggest that there is a much greater need for the PriA-PriB pathway of replication restart on DNA templates with DSBs than on those with single-strand lesions. MCGLYNN and LLOYD (2000) have also provided evidence that replication-generated DSBs are infrequent in UV-irradiated *E. coli*.

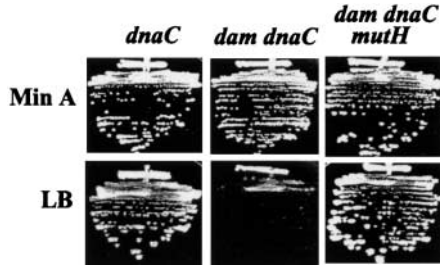

FIGURE 8.—Rich-medium sensitivity in *dnaC1331*(A84T) *dam* mutant and its suppression by *mutH*. Strains GJ3127 (*dnaC*), GJ5211 (*dam dnaC*), and GJ5213 (*dam dnaC mutH*) were scored for growth on glucose-minimal A (Min A) and LB agar plates.

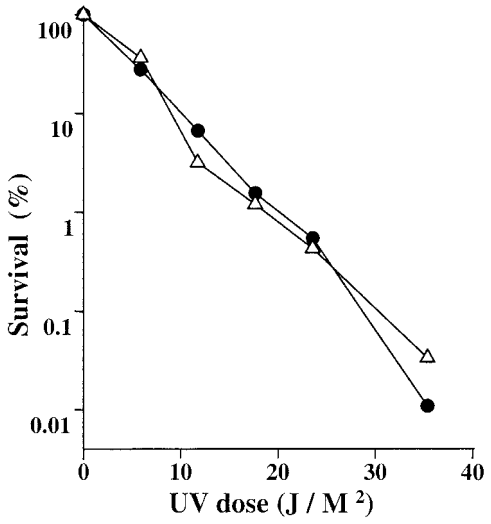

FIGURE 9.—UV tolerance of *dnaC*$^+$ (GJ3128, △) and *dnaC1331* (GJ3127, ●) strains. The percentage fraction of surviving cells, following exposure to UV for the indicated time periods, was determined. Two independent experiments yielded similar results, and the data from one of them are plotted. The initial culture densities for the two strains were, respectively, 1.2×10^8 and 9.2×10^7/ml.

Consistent with the model that the PriA-PriB pathway is rendered nonfunctional in the *dnaC1331*(A84T) mutant is the finding that the *dnaC1331* strain shares several phenotypic features in common with the null *priB* mutant (Sandler *et al.* 1999; Sandler 2000; Grompone *et al.* 2003; this study): minimal or no growth phenotype as single mutants, no change in UV tolerance, reduced copy number of ColE1-like plasmids, viability in combination with *priA*, and severe sickness or lethality in combination with *rep* or *priC*. On the other hand, the *dnaC1331* mutant, but not the *priB* mutant, is SOS induced, which suggests that the former might harbor some reversible trapped intermediates of the PriA-PriB pathway that activate the coprotease activity of RecA but do not compromise growth.

One finding of ours that is not readily accounted for by the three-pathway replication restart model of Sandler and co-workers is that of *rep-dnaC1331* synthetic lethality (because the PriA-PriC pathway would still be expected to be functional in the double mutants). One possibility is that the PriA-PriC pathway requires the Rep helicase (as has also been speculated earlier; Sandler and Marians 2000); in such a case, however, *rep* would be expected to exhibit synthetic lethality with *priB*, but the double mutants have been reported to be viable (Sandler 2000). The alternative possibility is that PriA-PriC may not represent a major pathway of replication restart, as is further discussed below.

Two lines of genetic evidence were earlier interpreted as support for a PriA-PriC pathway of replication restart: (i) *priB* or *priC* single mutants are reasonably healthy whereas a *priBC* double mutant is even more sick than *priA* (Sandler *et al.* 1999) and (ii) the *priA300* mutation, which specifies an altered PriA protein that is competent for ϕX-type primosome assembly but is defective for the 3′–5′ DNA helicase activity, behaves like the *priA* knockout when combined with a *priB* but not a *priC* null mutation; it was proposed that the mutant protein is proficient for replication restart via the PriA-PriB but not via the PriA-PriC pathway (Sandler *et al.* 2001). However, both these findings may also be explained by an alternative model in which the helicase activity of PriA participates in an additional, PriB- and PriC-independent pathway of replication restart (see Figure 10). Indeed, a pathway of direct resetting of regressed replication forks (that can occur following blockage of normal fork progression; Seigneur *et al.* 1998; McGlynn and Lloyd 2000) that is mediated by the combined actions of the RecG (or RecBCD) and PriA proteins (including the latter's 3′–5′ helicase activity) has been proposed by Lloyd and co-workers (McGlynn and Lloyd 2000; Gregg *et al.* 2002; Jaktaji and Lloyd 2003). Furthermore, Flores *et al.* (2002) have shown that in a strain with increased frequency of replication fork regression because of a DNA polymerase III ψ-subunit mutation, replication restart is compromised by the *priA300* mutation but not by a *priC* null mutation [suggesting that the pathway rendered defective in the *priA300* strain is one that does not require PriC, which is contrary to the proposal made by Sandler *et al.* (2001)].

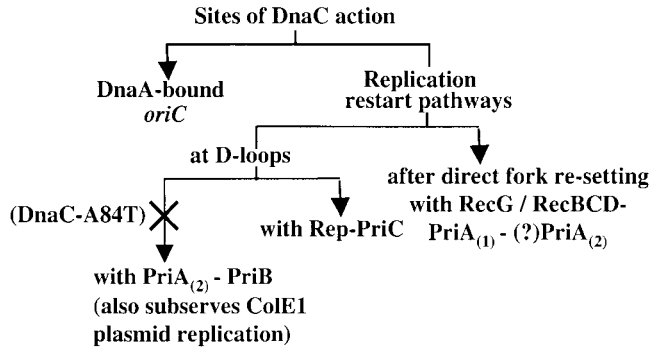

Figure 10.—A depiction of DnaC's role in different postulated pathways of replication fork assembly and of the defect associated with DnaC-A84T. $PriA_{(1)}$ and $PriA_{(2)}$ refer to the 3′–5′ DNA helicase and ϕX-type primosome assembly activities, respectively, of PriA.

On the basis of these arguments, therefore, we suggest that replication restart events occur primarily by the PriA-PriB and Rep-PriC pathways at D-loops, as well as by the direct resetting of regressed replication forks (Figure 10) and that, in the *dnaC1331*(A84T) mutant, the PriA-PriB pathway is rendered nonfunctional.

***dnaC1331*(A84T) and replication of ColE1-like plasmids:** The *dnaC1331*(A84T) mutation was associated with a reduction in content of the Rom^- ColE1-like plasmids pACYC184, pBR329, or pUC19 (and consequently with suppression of the Ts phenotype of pUC19 transformants), but not with that of the Rom^+ plasmid pBR322. One way to explain these results is to assume that DnaB loading by the mutant DnaC protein at the D-loop formed in the plasmid *ori* region occurs with reduced efficiency and that it consequently becomes the rate-limiting step for replication of Rom^- plasmids, but that for Rom^+ plasmids, it is the number of replication primers that remains rate limiting for replication [since the Rom protein reduces the proportion of RNA-II molecules that are processed to generate them (Cesareni *et al.* 1991; Del Solar *et al.* 1998)]. Consistent with this explanation, we have found that the *dnaC1331* mutation also does not affect the copy number of Rom^- plasmids in a *pcnB* strain [in which the half-life of the inhibitor RNA-I is increased (Xu *et al.* 1993), so that once again fewer replication primers are generated from RNA-II (data not shown)]. Our hypothesis is very similar to that proposed by Berges *et al.* (1997), who showed that a missense *priB* mutant harbors reduced copy numbers of Rom^- but not of Rom^+ ColE1-like plasmids.

The effects on plasmid replication of *dnaC1331*(A84T), *priB* null, or *priC* null mutations, as inferred from the data obtained in this study (Figure 4), are also in accord with a model in which replication fork assembly on the plasmid template is served primarily by the components of the PriA-PriB pathway of replication restart.

Need for and relative efficiency of the different replication restart pathways: When one considers the multiple replication restart pathways (Figure 10), two major questions are: What fraction of replicating chromosomes in wild-type cells (in the absence of exogenous DNA-damaging agents) suffers fork collapse necessitating replication restart? And what is the relative efficiency of each of the pathways in the process? The estimated values, in answer to the first question, have varied from 18 to 50 to 100% by different approaches, as discussed below.

i. The lower-end value was obtained in experiments involving growth of *dnaC*(Ts) mutants at the restrictive temperature (Maisnier-Patin *et al.* 2001). However, our own observations with *dnaC1331*(A84T) suggest that *dnaC* mutants may be differentially affected for DnaB loading at different fork-precursor structures, and it is therefore possible that, in the work of Maisnier-Patin *et al.* (2001), all pathways of replication restart were not inactivated at the restrictive temperature. The *dnaC*(Ts) mutants employed in their study were "slow stop" for DNA synthesis (Wechsler 1975), suggestive of a block mainly for fork assembly at *oriC*.
ii. The midrange value was the estimate obtained from the proportion of viable cells in cultures of *recA* strains defective in recombinational repair (reviewed in Kuzminov 1999; Cox *et al.* 2000), but once again there is evidence for the existence of RecA-independent pathways of replication restart (McGlynn and Lloyd 2001, 2002; Miranda and Kuzminov 2003).
iii. Finally, the argument that the capacity for replication restart is essential in every cell was based on both the extreme sickness or inviability of *priA*, *priBC*, or *priAC* mutants and the fact that these phenotypes could be suppressed by *dnaC* mutations (Sandler *et al.* 1999, 2001; Sandler 2000). As mentioned above, our finding in this study that the combination of *dnaC1331*(A84T) with *priC* is inviable also supports the notion that replication restart is required in every cell.

A related question in this context has been on the role of the Rep helicase in *E. coli* chromosome replication. Simultaneous deficiency of Rep and PriA is associated with inviability, but two alternative explanations have been offered for it. According to one model, Rep deficiency leads to increased frequency of replication fork collapse so that PriA becomes essential for survival (Seigneur *et al.* 1998). According to the second model, Rep and PriA participate in redundant pathways for replication restart (Sandler 2000). According to the second model, therefore, cell viability is again absolutely dependent on the capacity to restart replication, and our finding that *rep dnaC1331*(A84T) double mutants are inviable is in accord with it. Maisnier-Patin *et al.* (2001) showed also that 100% of *rep dnaC*(Ts) cells failed to complete replication initiated at *oriC* following a temperature upshift, but whether this was because of increased fork collapse or decreased restart associated with the *rep* mutation is not clear.

With regard to the relative efficiency of different pathways for restart, the extreme sickness of *priA* mutants has again been taken to mean that the PriA-independent pathways are at best minor contributors to replication restart. On the other hand, our finding in this study that the *dnaC1331*(A84T) single mutant is quite normal for growth but is inviable in combination with *priC* [which, unlike *priA* and *priB*, has so far been implicated only in "minor" pathway(s) of replication restart (Sandler 2000; Sandler *et al.* 2001; Flores *et al.* 2002; Grompone *et al.* 2003)] strongly suggests that the Rep-PriC pathway may indeed potentially be as efficient as the "major" PriA-PriB pathway.

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