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A DNA Segment Conferring Stable Maintenance on R6K γ-Origin Core Replicons

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The plasmid R6K γ origin consists of two adjacent modules, the enhancer and the core, and requires R6K initiator protein π for replication. While the core alone can replicate at a low level of wild-type π protein, we show here that host cells do not stably maintain core plasmids. The presence of the enhancer segment confers stable inheritance on core plasmids without a significant change in average plasmid copy number. Deletions and site-directed mutagenesis indicated that the stability of core plasmids is not mediated by binding sites or consensus sequences in the enhancer for DnaA, π protein, gyrase, Fis, or Dcm methylase. Proper segregation of core plasmids requires only the R6K *stb* or stability-related region, which includes the 20-bp segment of the 100-bp enhancer adjacent to the core. The use of the π116 mutant protein, which increases plasmid copy number fourfold, does not stabilize core plasmids lacking the enhancer. We also show that at an elevated level of wild-type π, the γ-origin plasmid is unstable, even in the presence of the enhancer. We discuss the differences and similarities between the R6K stability system and those found in other plasmids.

Naturally occurring plasmids generally perform three replication-related functions, namely, (i) initiation of DNA replication, (ii) control of DNA replication (exemplified by a relatively constant copy number), and (iii) stable maintenance of themselves throughout successive cell divisions. Some laboratory derivatives of these plasmids may only replicate under some circumstances or at an altered plasmid copy number, suggesting that control elements are deleted. Other derivatives may be able to control replication but be incapable of proper partitioning.

The three replication functions of plasmids are often mediated by distinct segments. While replication initiation and control functions are sometimes only subtly distinguished, partitioning is generally controlled separately. Partitioning is independent of control of plasmid copy number (2, 5, 34, 39, 44). Nonreplicating *par* segments of one plasmid can stabilize plasmids replicating from an unrelated origin (39, 45). For example, the *par* segment of pSC101 can stabilize the p15A derivative pACYC184 (39), and the R1 *par* region can stabilize an unstable ColE1 derivative (44).

Plasmid R6K carries three origins of replication, α, γ, and β (see Fig. 1). Each origin is activated by the R6K specific initiator protein π, encoded by the R6K *pir* gene. All three origins require the 277-bp core segment and a unique additional *cis*-acting segment (29, 30, 50, 51, 53). When the flanking α- and β-origin sequences are deleted, the remaining γ origin can replicate autonomously (18, 52, 55) if the *pir* gene is provided in *cis* or in *trans* (24, 30).

While the intact R6K uses primarily the α and β origins (6), the three origins share many important properties, and the small γ origin contains all of the elements sufficient for R6K replication control (13). The γ origin consists of the core segment and the adjacent enhancer (59); the enhancer is not required for replication of the α and β origins (42, 50, 51, 53). The core contains seven 22-bp binding sites for π protein (iterons) (14, 16, 19, 38) and binding sites for the host proteins integration host factor (IHF) (8, 11) and DnaA (59); the enhancer carries a single DnaA binding site, DnaA box 1 (59).

While π triggers replication, it can also inhibit replication above a certain intracellular concentration (15). The γ-origin plasmid (enhancer-plus-core) copy number is lower at a high π level (4,000 to 10,000 dimers per cell) than at a low level (10% of the high level) (15). It is believed that copy number reflects the relative strengths of positive and negative activities of π protein (15) and intracellular concentration of iterons (37, 38). At the low level of wild-type (wt) π, the core alone is able to replicate (59). Replication at the high level of wt π requires not only the core segment but also host protein IHF (7) and a DnaA box 1 in the enhancer (60). Thus, the DnaA box 1 in the enhancer and IHF together participate in replication control by counteracting the negative effect of π on R6K replication.

We wished to determine if R6K γ-origin derivatives previously used in replication control studies were stably maintained. We also hoped to determine what R6K segment(s) might be necessary for plasmid segregation. Even though the enhancer is not absolutely required for replication at the low level of wt π (59), we found in this study that the enhancer is required for stable plasmid maintenance. Stability is not mediated by the DnaA box 1. Sequence analysis of the enhancer revealed consensus sequences for π, gyrase, Fis, and Dcm methylase. Deletions and site mutations, however, showed that none of these sites mediated stability. Rather, perfect inheritance of core plasmids required only the *stb*, or stability-related segment, which includes only a 20-bp segment of the enhancer.

MATERIALS AND METHODS

Strains, plasmids, and mutagenesis. Strains W3110 *thy* (9) and C2110 (30) have been described previously. Strains GM2159 (*dam-13*::Tn*9*) (35) and GM2163 (*dam-13*::Tn*9 dcm-6*) (47) were also described. Strain RLG1679 is MG1655 Δ*lacX74* λi^{21}(*rrnB* P1-*lac-lacZ*) (48). RLG1677 is RLG1679 *fis*::*kan* (19a); the *fis* allele was obtained from R. Kahmann (28).

Plasmids producing the low level of wt π (Δ14), the low level of π copy-up mutant (Δ14-*pir*116), and the high level of wt π (Δ22) were described previously (7, 15).

Plasmids pACYCY184 (3), R6K (31), pMF39 (16), and pRK419 (30) were described previously.

The γ-origin plasmids used in this study are diagrammed in Fig. 1. Of these, pFW12 (60), pFW18 (60), pMF35 (59), and pMF36 (15) were described previously. Except for minor differences in the multicloning site and the presence or

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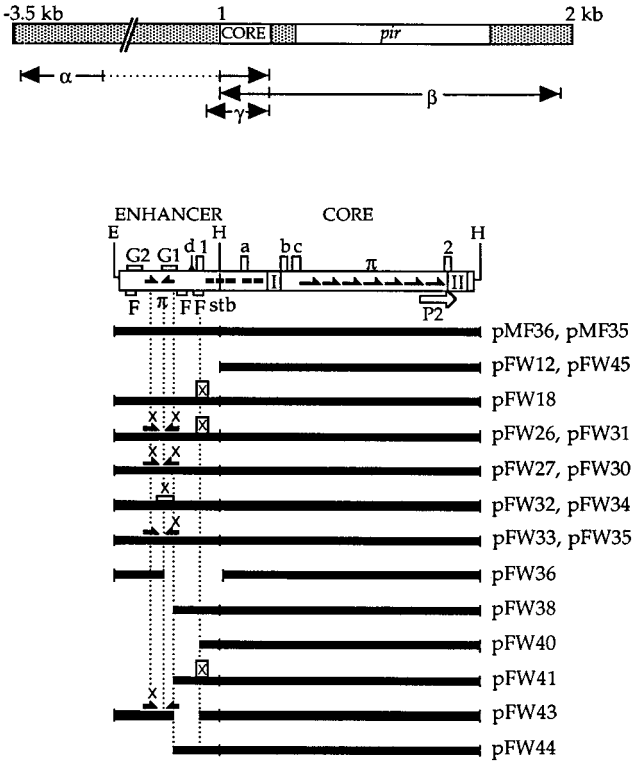


FIG. 1. Replication region of *E. coli* plasmid R6K. (Top) The 5.5-kb replication region. The two R6K factors absolutely required for replication are indicated, namely, the core segment and the *pir* gene, which encodes π protein. Double-headed arrows indicate *cis*-acting DNA segments required for replication from the α, γ, and β origins; the location of each Greek letter marks the approximate site of replication initiation for each origin. A dotted line separates the two *cis*-acting DNA segments required for α-origin replication (50). (Middle) Enlargement of the γ origin, which consists of the enhancer and core segments. The following binding sites and consensus sequences are indicated: Fis (F, brackets), gyrase (G2 and G1, open rectangles), π (head-to-head arrows represent site in the enhancer, and sequence of arrows represents iterons in the core), Dcm (d); partitioning site repeats (*stb*, closed rectangles); IHF sites (I and II, open squares); P2 promoter (open arrow); and DnaA (boxes 1, a, b, c, and 2). Restriction site abbreviations: E, artificial *Eco*RI site in vector; H, *Hin*dIII site between enhancer and core and artificial site adjacent to core. (Bottom) Portions of the γ origin contained in various R6K derivatives. X indicates mutations in π half-repeats, gyrase consensus G1, or DnaA box 1. Sequences for various binding sites and mutations in the enhancer and part of the core are diagrammed in Fig. 3. Schemes for construction and mutagenesis are described in Materials and Methods.

absence of the pUC origin, all of the γ-origin plasmids used in this study (except pDX101) have identical vector sequences.

Mutagenesis and construction of plasmids pFW26, pFW27, pFW30, pFW31, pFW32, pFW33, pFW34, and pFW35. In the USE method of mutagenesis (10), the target plasmid was pMF34 (16), which carries the wt γ origin inserted into pUC9. One oligonucleotide (previously described [60]) was used to change the *Afl*III site (nucleotide [nt] 806) near the pUC origin to an *Age*I site, while simultaneously one or two new oligonucleotides were used to alter protein binding sites in the enhancer. The oligonucleotide used to alter the DnaA box 1 in the enhancer (introducing a *Sca*I site) was also described previously (60). The oligonucleotide 5′CTGAAAATTGCTT**TCACTG**GCTCTAAGGGC**TTCGAA**G TGCGTTACATC3′ was used to alter both halves of the π binding site in the enhancer (the inverted repeats are in bold and the altered bases are underlined). These mutations introduced *Bsr*I and *Bst*BI sites to aid in screening for mutants. The pMF34 derivative with both the *Afl*III site and both halves of the π site mutated was named pFW27; the pUC origin, including the new *Age*I site, was then deleted (*Hae*II fragment at nt 680 to 1050, pUC9 coordinates) from pFW27, creating pFW30. The pMF34 derivative with the *Afl*III site, both halves of the π site, and the DnaA box 1 mutated was designated pFW31; the pUC origin *Hae*II fragment was then deleted to create pFW26.

In a parallel round of mutagenesis of pMF34, the *Afl*III site was altered, and oligonucleotide 5′GAGAG**GCTCTAAAGTTAACTCAGTG**CGTTACATCC3′ was used to mutate the gyrase consensus sequence (bold) and introduce an *Hpa*I site (all mutations are underlined). The pMF34 derivative with the *Afl*III site and the gyrase site mutated was named pFW32; the *Hae*II pUC origin fragment was deleted, creating pFW34.

In another separate round of mutagenesis of pMF34, the *Afl*III site was altered and the oligonucleotide 5′GCTCTAAGGGC**TTCCCCGGG**CGTTACATCC3′ was used to alter the π right half-site (RHS; in bold type) and introduce a *Sma*I site (all mutations introduced are underlined). The pMF34 derivative with the *Afl*III site and the π RHS mutated is pFW33; when the pUC origin was deleted, the new construct was labeled pFW35.

Other constructs. To build pFW36, pFW34 was digested with *Hpa*I, partially digested with *Dra*I (which cuts, among other places, adjacent to the *Hin*dIII site which marks the enhancer-core boundary), blunt-ended with mung bean nuclease, and religated with T4 DNA ligase. To build pFW38, pFW35 was digested with *Eco*RI, blunt-ended with mung bean nuclease, cut with *Sma*I, and religated. Plasmid pFW40 was constructed by digesting pFW18 with *Eco*RI, treating the DNA with mung bean nuclease, recutting with *Sca*I, and religating. To build pFW41, plasmid pFW31 was digested with *Eco*RI and then *Bst*BI, treated with mung bean nuclease, and religated. Plasmid pFW43 was built by cleaving pFW31 with *Sca*I and then *Bst*BI, treating with mung bean nuclease, and religating. Plasmid pFW44 was derived from pFW30 digested with *Eco*RI and then *Bst*BI, treated with mung bean nuclease, and religated. Plasmid pFW45 was created by deleting the *Hae*II pUC origin fragment from plasmid pMF31 (60).

pDX101 contains the following three *Hae*II fragments: the p15A origin (nt 580 to 1726), the chloramphenicol resistance marker (nt 3500 to 580) segments from pACYC184 (3), and the γ-origin segment from pMF36 (15).

DNase I footprinting. Plasmids used in footprinting (pMF36, pFW30, and pFW35) were digested with *Eco*RI, dephosphorylated with calf intestinal alkaline phosphatase, and treated with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. DNA was then digested with *Sal*I, and purified γ-origin fragments were subjected to DNase I cleavage in the presence of purified π protein as described previously (16). The π purification protocol was published previously (16), as was the method for G+A sequencing reactions (36).

Stability. W3110 cells carrying a tetracycline-resistant π-producing plasmid (Δ14-*pir*WT, Δ22-*pir*WT, or Δ14-*pir*116) and a monomeric penicillin-resistant γ-origin plasmid (pMF36, pFW12, pFW18, pFW30, pFW31, pFW34, pFW35, pFW36, pFW37, or pFW38) were grown overnight in L broth (LB) containing 125 μg of penicillin G per ml (LB-pen). Cultures were diluted 1:50 and grown in LB-pen for 1 h. At this time, $T = 0$, dilutions were plated onto LB plates containing tetracycline (15 μg/ml) and thymidine (5 μg/ml; LB-tet-thy), and the cultures were reinoculated into LB-tet-thy. Cultures were reinoculated (1:40,000 dilutions) every 7 to 8 h, and dilutions were plated onto LB-tet-thy. Once the colonies were grown, colonies for each time point and each plasmid were picked onto LB plates containing 5 μg of thymidine per ml (LB-thy) plus penicillin or tetracycline to determine what percentage of cells remained penicillin resistant. Minipreps were performed on random colonies to ensure that penicillin-resistant colonies contained autonomously replicating monomeric plasmids.

In the experiments with pACYC184, pDX101, and R6K, no π-producing helper plasmid was used; dilutions were plated onto LB-thy and picked onto both LB-thy and LB-thy plus chloramphenicol (35 μg/ml) for pACYC184 and pDX101 or LB-thy and LB-pen plus thymidine (5 μg/ml; for R6K).

Transformations. Transformations were performed as described earlier (59, 60) and in the legend to Table 1.

Supercoiling assay. Overnight cultures of W3110 carrying a π-producing plasmid (Δ14-*pir*WT or *pir*116) and a γ-origin plasmid (pFW12 or pMF36) were grown aerobically in LB-pen. Cultures were diluted (1:40) and grown aerobically in LB-pen to an optical density at 600 nm of 0.6. DNA was CsCl purified (49) and electrophoresed on 0.7% agarose gels in 0.5×TBE (45 mM Tris base, 45 mM boric acid, 0.7 mM EDTA [pH 8.2]) containing 16.5 μg of chloroquine per ml at 4°C and 62.5 V for 15 h without recirculating the buffer. Gels were detained in double-distilled H_2O for 2 h, stained with ethidium bromide for 1 h, destained for 10 h, restained for 10 min, and photographed under UV illumination. Photographically reversed images are shown.

RESULTS

The enhancer, but not its DnaA box, allows stability of R6K γ-origin plasmids at the low level of wt π. In the absence of the enhancer, the isolated core segment can drive R6K replication at the low level of wt π (59). Plasmid copy number, when examined in cells grown under selective pressure, was also unaffected by the presence or absence of the enhancer (60). However, it was not known whether core plasmids were stably maintained or if the enhancer was involved in stability. We first tested two R6K derivatives, pFW12 and pMF36 (Fig. 1), for their stability at a low level of wt π (produced by compatible helper plasmid Δ14-*pir*WT) in *E. coli* W3110. Plasmid pFW12 carries only the core, while pMF36 carries the core and the enhancer. Stability tests (Fig. 2A and data not shown) indicate

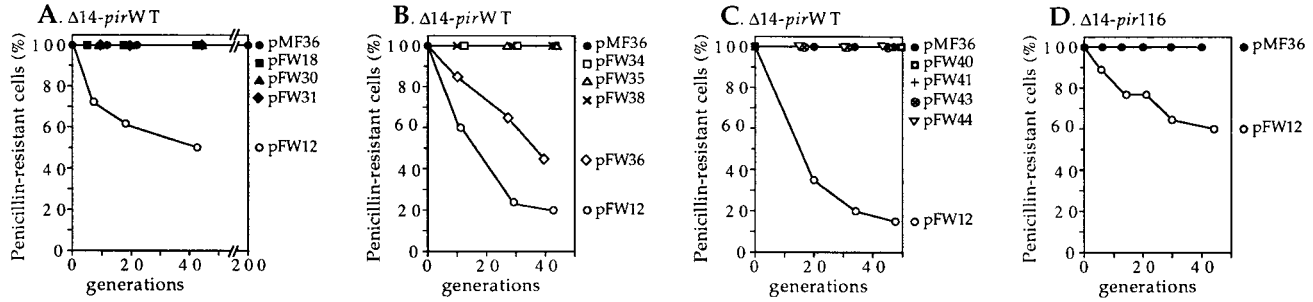


FIG. 2. Stability tests of various R6K derivatives. (A to C) Stability of γ-origin plasmids when helper plasmid Δ14-*pir*WT produced low levels of wt π; (D) stability of plasmids pFW12 and pMF36 at low levels of π copy-up mutant π116.

that cells stably maintained pMF36 for up to 200 generations (the last point tested), while cells quickly lost the pFW12 plasmid; after 40 generations, 50 to 80% of the host cells (depending on the experiment) had lost the core plasmid. Thus, the enhancer (Fig. 1) must somehow mediate the stability of R6K γ-origin plasmids.

We took advantage of the fact that the enhancer is not essential for replication at the low level of wt π to determine what portion(s) of it was involved in stability. We tested the stability properties of a series of plasmids, all containing the intact wt core but having different deletions and mutations in the enhancer.

DnaA was the only protein known to bind to a site (DnaA box 1) in the enhancer (59, 60). A possible role for the DnaA box 1 in γ-origin plasmid stability was suggested by both a finding that DnaA binds to the membrane (32) and the old hypothesis that membrane association might be required for plasmid partitioning (25).

The sequence of the DnaA box 1 matches the DnaA box consensus sequence in eight of nine nucleotides (17). In plasmid pFW18, the DnaA box 1 site was mutated to reduce the match to six of nine nucleotides of the consensus sequence (Fig. 3). This mutant site had been shown not to bind DnaA in the agarose gel retardation assay (60). Figure 2A shows that pFW18 is stable at a low level of wt π, indicating that the enhancer does not use DnaA box 1 for partitioning. To determine what sites in the enhancer do mediate this function, we first carried out detailed analysis of the enhancer sequence. This approach yielded surprising new information.

The enhancer carries a site which binds π protein. Sequence analysis of the enhancer revealed potential binding sites for π protein, gyrase, Fis, and Dcm methylase. These sites were analyzed individually for their possible role in stability by use of deletions or mutations in the sites in the enhancer or hosts with mutations in the genes encoding those proteins.

Like the π-binding site in the operator for the *pir* gene (12, 27), the potential enhancer π site (Fig. 3) contains a pair of inverted half repeats. Both these sites and the 22-bp iterons have in common the TGAGAG motif (12, 27, 54) (Fig. 3).

We performed the DNase I protection assay to determine if wt π bound to the enhancer site. As shown in Fig. 4A, the left half-site (LHS) was protected against DNase I, except for an enhancement of cleavage at the second G in the TGAGAG sequence. The RHS was completely protected, and an enhancement was detected at a G (nt −46) 3 bp downstream of this half site. This pattern of protection and cleavage is very similar to that detected when π bound to the *pir* operator (12, 27, 61); thus, π probably binds both pairs of inverted half sites similarly.

The π-binding site in the enhancer does not mediate stability. We next mutagenized most of the Gs of the TGAGAG motif in both half sites (Fig. 3). These Gs are important for π binding to 22-bp iterons (38). The DNase I protection assay was carried out with a DNA fragment containing these changes and isolated from plasmid pFW30. As shown in Fig. 4A, π protein does not protect the mutated site, although, as a control, it still protects the 22-bp iterons present on the same fragment. Moreover, π binding produces previously observed cleavage enhancements at nt 15 and 51 (16). The stability data (Fig. 2A) show that mutating both halves of the π-binding site in the enhancer (pFW30) does not impair segregation.

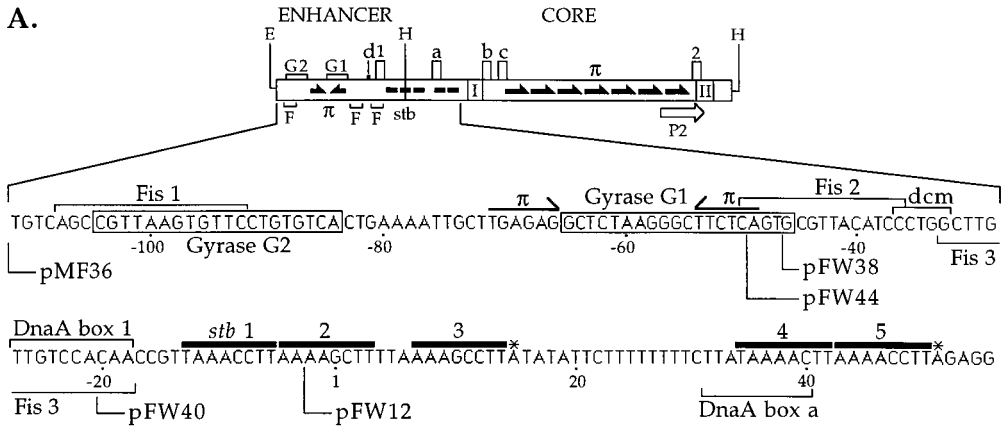
Because mutating both half sites may have introduced fortuitous changes of unknown effect, we also mutated only the π RHS (pFW35; mutations described in Fig. 3). DNase I footprinting showed that mutating just one half site was sufficient to prevent π binding to the inverted repeats (Fig. 4B). The stability assay showed that mutating the RHS, like mutating both half sites, did not destabilize the γ-origin plasmid (Fig. 2B). Finally, we combined the π site mutation in pFW30 with the DnaA box 1 mutation in pFW18, producing plasmid pFW31. This plasmid was also found to be stable (Fig. 2A). Thus, some factor other than the binding of DnaA and π proteins must be involved in stabilization of the γ origin.

Gyrase, supercoiling, and γ-origin plasmid stability. Gyrase and supercoiling have been implicated to be important in the segregation of the bacterial chromosome (56), pSC101 (1, 4, 40, 58), and other plasmids (40).

The enhancer carries a sequence, designated G1 (Fig. 3), which matches the gyrase consensus sequence in 10 of 13 nucleotides (33) and overlaps the π RHS. The mutations in pFW30 which altered the π-binding site fortuitously decreased the match of the G1 sequence to the consensus sequence to 9 of 13 nucleotides. Another sequence, designated G2, matches the gyrase consensus sequence in 9 of 13 nucleotides (Fig. 3).

To determine whether either gyrase consensus in the enhancer altered overall superhelicity, we purified plasmid DNA from W3110(Δ14-*pir*WT) strains producing a low level of wt π and carrying pFW12 or pMF36 and electrophoresed the DNA on an agarose-chloroquine gel to separate the topoisomers. As shown in Fig. 5, both γ-origin plasmids form approximately the same number of supercoiled isomers. Plasmid pFW12 runs faster than pMF36, but this is due only to the difference in size. Thus, the R6K enhancer does not seem to alter the overall plasmid superhelicity.

To determine if gyrase was involved in R6K stability, we mutated the G1 site, reducing it to a 7-of-13-nucleotide match to the consensus sequence, producing plasmid pFW34 (see Fig. 3 for the sequence). We also made several deletions in the R6K enhancer which removed most of this sequence. As diagrammed in Fig. 1, plasmid pFW36 contains a deletion right-



B.

DnaA consensus	TTAT$^{C}_{A}$CA$^{C}_{A}$A	Fis consensus	$^{G}_{T}$NN$^{CA}_{TG}$NN$^{A}_{T}$NN$^{CA}_{TG}$NN$^{A}_{C}$
DnaA box 1	TTGTCCACA	Fis 1	AGCCGTTAAGTGTTC
pFW18 mutant	TAGTACTCA	Fis 2	CAGTGCGTTACATCC
DnaA box a	TTATAAAAC	Fis 3	GCTTGTTGTCCACAA

π iteron consensus	A$^{AA}_{GC}$CATGAGAGCTTAGTACGT$^{T}_{G}$	*stb* repeats	
pir operator	AACATGAGAT-N9-CTCTCATGTT	1	TAAACCTT
π site	TGAGAG-N_{11}-TTCTCAGTG	2	AAAAGCTT
pFW30 mutant	TCACTG-N_{11}-TTCGAA	3	AAAGCCTT
pFW35 mutant	TGAGAG-N_{11}-TTCCCCGGG	4	TAAAACTT
		5	AAAACCTT
Gyrase consensus	$^{A}_{G}$NNN$^{A}_{G}$N$^{A}_{GG}$T$^{G}_{GT}$A$^{C}_{TT}$C$^{GC}_{TT}$N$^{C}_{T}$N$^{G}_{T}$N$^{C}_{T}$	consensus	$^{T}_{A}$AAANCTT
Gyrase G2	CGTTAAGTGTTCCTGTGTCA		
Gyrase G1	GCTCTAAGGGCTTCTCAGTG		
pFW34 G1 mutant	GCTCTAAAGTTAACTCAGTG		

FIG. 3. (A) Diagram of the γ origin, including enhancer and core (symbols are identical to those described in the legend to Fig. 1). The sequence of the enhancer and part of the core is shown (54). The following binding sites, consensus sequences, and repeats in the enhancer and part of the core are indicated: Fis (bracketed), gyrase (boxed), π (head-to-head arrows), Dcm (bracketed), DnaA (bracketed), and partitioning sequences (*stb*, overlined). The leftmost portion of the enhancer included in R6K derivatives pMF36, pFW44, pFW38, pFW40, and pFW12 are indicated by bent lines. (B) Consensus sequences, R6K enhancer sequences, and mutant sites for DnaA, π, gyrase, Fis, and *stb* sequences. Mismatches from consensus are underlined, whether present in the wt enhancer sequence or introduced by site-directed mutagenesis.

ward from the center of G1; plasmid pFW38 contains a deletion leftward from the π RHS, deleting both G1 and G2.

In the stability analysis using the host W3110(Δ14-*pir*WT), shown in Fig. 2B, mutating the gyrase-binding site G1 did not affect stability (plasmid pFW34) nor did deleting this site and the G2 gyrase consensus sequence (plasmid pFW38). In this and subsequent stability tests, plasmids pFW12 (the core) and pMF36 (core plus enhancer) were used as controls for unstable and stable replicons, respectively. Thus, these data suggest that the gyrase consensus sequences in the enhancer are not involved either in overall plasmid supercoiling or in stability at the low level of wt π.

In this stability test (Fig. 2B), a plasmid containing a large deletion of the right-hand portion of the enhancer (pFW36) was unstable. While this deletion removed half of the gyrase site, the stability data obtained with plasmids pFW34 and pFW38 suggested that a factor other than the gyrase was responsible for the instability of pFW36. The deletion in pFW36 removed the π RHS, the DnaA box, and also two previously unexamined sites, namely, a Dcm methylation site and a Fis consensus sequence. We tested the possible role of these sites in stability.

Stability is not mediated by either Fis or Dcm methylase. As mentioned earlier, membrane binding has long been suggested as being required for plasmid and chromosome partitioning (25). Hemimethylation by Dam methylase is required for *oriC* DNA binding to the membrane (46). Unlike Dam methylase, with its multiple roles (for a review, see reference 47), Dcm

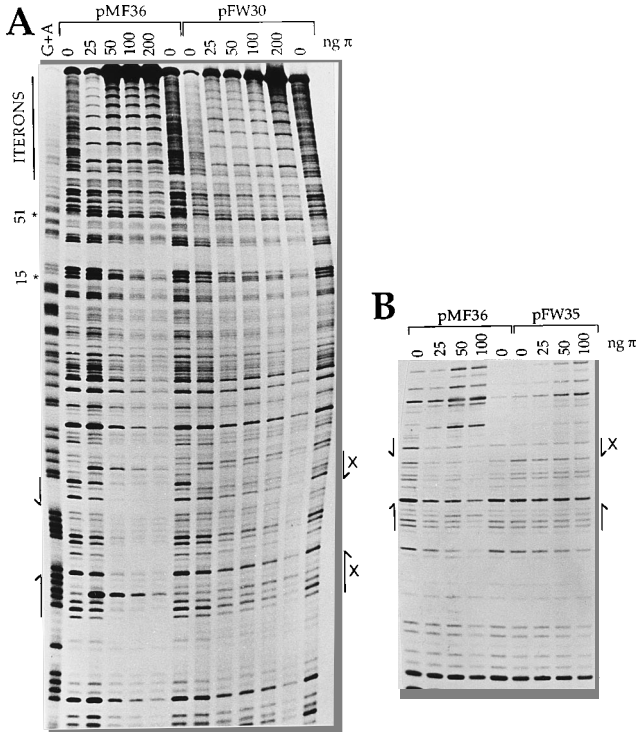


FIG. 4. Footprint of π binding to the enhancer. (A) γ origin with wt π site in enhancer (pMF36) or mutant site (designated by X, pFW30) treated with DNase I in the presence of indicated amounts of wt π. π site in enhancer is designated by inverted arrows (X marks mutant half-sites); DNase I cleavage enhancements at nt 15 and 51 (*) and iterons are also indicated. (B) Same as panel A, except that only one half site is mutated (X, pFW35).

methylase has no known function except blocking cleavage by the *Eco*RII, encoded by IncN plasmids (20). R6K is not a member of this group but, rather, is a member of the IncX group (21, 26, 31).

Nevertheless, we tested the stability of pFW12 (core alone) or pMF36 (enhancer plus core) in *dam* (GM2159) and *dam dcm* (GM2163) mutant strains carrying Δ14-*pir*WT, which supplied a low level of wt π. The absence of Dam did not affect plasmid stability (data not shown), which is not surprising because there are no Dam sites (GATC) in the γ-origin enhancer sequence (54). In the *dam dcm* mutant strain, the core plasmid (pFW12) remained unstable while the enhancer-plus-core plasmid (pMF36) remained stable (data not shown).

The enhancer contains three sequences (Fis 1, 2, and 3) (Fig. 3) which match the degenerate Fis consensus sequence in six of seven nucleotides (22). Plasmid pFW38, which lacked Fis 1, was stable, suggesting that this consensus was not necessary for stability. Instead of mutagenizing Fis 2 and Fis 3, we tested the stability of plasmids pFW12 (core) and pMF36 (enhancer plus core) in isogenic *fis*$^+$ (RLG1679) and *fis*::*kan* (RLG1677) strains, each carrying Δ14-*pir*WT. The core alone remained unstable, and the enhancer still mediated stability (data not shown), suggesting that Fis was not involved in R6K stability. Thus, we had found thus far that stability of the γ origin at the low level of wt π is mediated by the enhancer but not by the DnaA box 1, the π site, either gyrase consensus sequence (G1 or G2), the Dcm site, or any of the Fis consensus sequences in the enhancer.

Defining the R6K *stb* site. To delineate which segment(s) of the enhancer was involved in stability, we built and tested several plasmids containing large deletions in the enhancer (Fig. 1). To construct these, we took advantage of restriction sites introduced while mutating the various sites in the enhancer. These plasmids are isogenic, all containing identical vector sequences and the intact core, with differences only in the enhancer and the multicloning site.

Plasmid pFW44 contained a deletion of the leftmost portion of the enhancer up to the π RHS. Plasmid pFW41 contained the same deletion, and in addition, the DnaA box 1 was altered as it was in pFW18. A pair of plasmids was prepared to take advantage of the fact that pFW36 was unstable. In pFW43, the segment between the π RHS and the DnaA box 1 was deleted. Plasmid pFW40 has the entire enhancer to the left of the DnaA box 1 deleted.

When these plasmids were tested for stability (Fig. 2C), we found that plasmid pFW40 remained stable, even though it retained only a 20-bp portion of the enhancer. Indeed, all of the plasmids with this 20-bp segment retained were stable (pFW18, pFW30, and pFW31 [Fig. 2A]; pFW34, pFW35, and pFW38 [Fig. 2B]; pFW40, pFW41, pFW43, and pFW44 [Fig. 2C]). Both of the plasmids with this segment deleted were unstable (pFW12 and pFW36 [Fig. 2B]). There was no good correlation between the presence of any other portion of the enhancer and stability. Thus, the R6K *stb* site must include this 20-bp segment, the only portion of the enhancer needed for stability. We have yet to determine the right-hand boundary of the *stb*, which may differ from the *Hin*dIII site that marks the arbitrary enhancer-core boundary. Intriguingly, the *stb* region contains five repeats of the sequence 5′WAAANCTTT, where W is T or A and N is any base (Fig. 3).

Increasing the plasmid copy number with low levels of π copy-up mutants does not ensure stability of plasmids lacking the enhancer. Studies with pSC101 have shown that mutations in pSC101 RepA protein sometimes lower the rate of loss of *par*-deleted plasmids but are insufficient to restore complete stability, and there is no correlation between copy number and stability (5, 39, 57). To determine if the unstable core plasmid

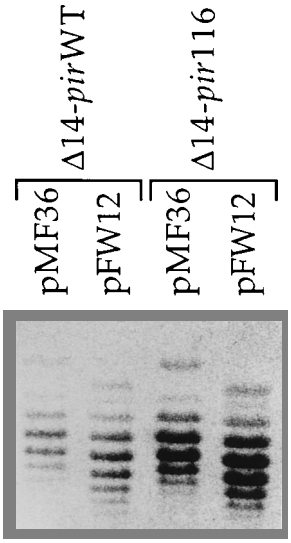


FIG. 5. Effect of the enhancer and π allele on γ-origin plasmid supercoiling. As described in Materials and Methods, W3110(Δ14-*pir*WT) or W3110(Δ14-*pir*116) host cells carrying γ-origin plasmids pFW12 or pMF36 were grown to the mid-log phase, and plasmid DNA was extracted. Plasmid topoisomers were separated by electrophoresis on agarose-chloroquine gels, and the gels were photographed. Only the portion of the gel showing monomeric γ-origin plasmid DNA is shown. The direction of migration is from top to bottom; more negatively supercoiled topoisomers migrate more rapidly.

pFW12 could be stabilized by increasing its plasmid copy number, we used copy-up π mutants. These mutants contain single amino acid substitutions and increase the copy number of core plasmids two- to fourfold relative to the copy number at the same level of wt π (60). We tested the stability of pFW12 and pMF36 in W3110 strains producing a low level of one π copy-up mutant (Δ14-*pir*116). The low level of π116 increases the copy number fourfold over that of the same level of wt π; also, the presence or absence of the enhancer does not affect copy number (60). As shown in Fig. 2D, the plasmid carrying the enhancer plus core (pMF36) is still stable. Interestingly, pFW12 at the low level of π116 is still unstable. Similar results were obtained with a low level of two other copy-up mutants, π113 and π200 (data not shown), which raise the plasmid copy number two- and fourfold, respectively, over that at the same level of wt π (60).

Separate binding sites in the enhancer are needed to (i) stabilize the R6K plasmids at a low level of wt π and (ii) counteract the negative effect of high levels of wt π on R6K replication. Distinct segments of basic replicons are often involved in the separate functions of replication control and stable maintenance. We wished to determine if different parts of the enhancer had separate roles. As shown above, the *stb* contains the only portion of the enhancer required for stability at the low level of wt π. As noted earlier, excess π has a negative effect on R6K replication (15). While the DnaA box 1 in the enhancer was needed to counteract the negative effect of a high level of wt π (60), this site was not needed for either replication (59, 60) or stability at the low level of wt π (this study). We also showed here that the gyrase consensus sequence, the π-binding site, Fis consensus sequences, and Dcm methylation site are not needed for stability. Were any of these sites involved in counteracting the negative effect of high levels of wt π on replication?

To test this, we transformed the γ-origin plasmids used in this study into W3110 host cells producing either the low or high level of wt π. The results, shown in Table 1, indicate that only the plasmids carrying the intact DnaA box 1 (pMF36, pFW30, pFW34, pFW35, pFW38, and pFW44) replicated at the high level of wt π. All of the plasmids with the DnaA box 1 mutated or deleted (pFW12, pFW18, pFW31, pFW36, pFW40, pFW41, and pFW43) failed to replicate at the high level of wt π. Furthermore, replication at the high π level was unaffected by any other mutation unless the DnaA box was also altered or deleted. Importantly, these data, combined with the stability studies, show that the DnaA box 1 in the enhancer is needed specifically for replication control (i.e., counteracting the negative effect on replication of the high level of wt π) but for neither replication initiation nor stability at the low level of wt π. Analogously, excess DnaA can compensate for the detrimental effects of excess RepA protein on pSC101 replication (23).

Plasmid pFW40 (which lacks the DnaA box 1 in the enhancer but retains the *stb*), is unable to replicate at the high level of π, although it both replicates and is stably maintained at the low level of wt π. In other words, the *stb* is needed for stability but is insufficient for replication control. Thus, the various functions of (i) replication initiation at the low level of π, (ii) counteracting inhibition by elevated levels of π, and (iii) stability at the low level of wt π are mediated by different segments of the R6K γ origin. The first function requires only the core (59); the second requires the core (59), IHF (7), and DnaA box 1 (60); and the third requires the *core* and *stb* but not the DnaA box 1 (this study).

Also, these studies help us to redefine the boundaries of the enhancer, which is itself an artificially delimited segment of over 100 bp. We have defined here the smallest γ origin described to date capable of both replication at the high level of wt π and stable maintenance at the low level of wt π. Plasmids pFW38 and pFW44 are capable of both functions; they have similar deletions, retaining only the rightmost 46 or 49 bp of the enhancer, respectively. Finally, these data reveal that, other than the *stb* and the DnaA box 1, none of the binding sites and consensus sequences in the enhancer have any known function.

TABLE 1. Effect of various lesions in the enhancer on transformation capability of γ-origin plasmids

Transforming γ-origin plasmid DNA	Lesion in enhancer[a]	No. of colonies[b] obtained on:	
		W3110 (Δ14-*pir*WT)	W3110 (Δ22-*pir*WT)
pMF36	None (wt)	1.1×10^4	4.2×10^2
pFW12	Δ Enhancer	2.8×10^2	0
pFW18	DnaA box 1	7.7×10^3	0
pFW30	π Site	9.6×10^3	6.0×10^2
pFW31	π Site, DnaA box 1	1.4×10^3	0
pFW34	Gyrase site	3.2×10^3	4.8×10^2
pFW35	π RHS	3.4×10^3	4.8×10^2
pFW36	Δ Gyrase site, π RHS, DnaA box 1	4.8×10^3	0
pFW38	Δ Gyrase site, π LHS	1.7×10^3	48
pFW40	Δ Gyrase site, π site, DnaA box 1	2.0×10^3	0
pFW41	Δ Gyrase site, π RHS; DnaA box 1 mutated	4.3×10^3	0
pFW43	Δ π site RHS, DnaA box 1	5.0×10^3	0
pFW44	Δ Gyrase site, π site	6.8×10^3	1.6×10^2
pFW45	Δ Enhancer	2.4×10^2	0

[a] Δ, deletion.
[b] W3110 was transformed with helper plasmids, producing the low level of wt π protein (Δ14-*pir*WT) or the high level (Δ22-*pir*WT) of wt π. These strains were made competent, and 1.5×10^8 cells of each were transformed with 0.25 μg of closed circular plasmid DNA. Numbers indicate colonies obtained per microgram of DNA per 4×10^7 cells plated on LB-pen plates containing 5 μg of thymidine per ml.

DISCUSSION

The core segment of the R6K γ origin is necessary for replication under all conditions tested to date; this segment alone is sufficient for replication when a low level of R6K initiator protein π is provided (59). We show here that, at this level of wt π, core plasmids are not stably maintained unless the adjacent enhancer is present. Using a reductionistic method, we determined whether factors which stabilize other plasmids were involved in R6K segregation; most of these were not. Only the rightmost 20-bp portion of the enhancer is required for stability of the core plasmids at a low level of π; this 20-bp segment is included in what we define here as the R6K *stb*. We discuss below the similarities and differences between the R6K stability mechanism and that in other plasmids.

Stability of R6K γ-origin plasmids. The region of the *stb* of R6K contains five repeats with the consensus nucleotide sequence 5′-WAAANCTT3′, where W is A or T and N is any base. The repeats lie in two sets. The first set contains three tandem repeats (with 3 bp between the second and third repeat) centered around the *Hin*dIII site that marks the (arbitrary) boundary between the core and enhancer. The second set, which lies 21 bp away in the AT-rich portion of the core, contains two tandem repeats. These repeats are numbered 1 to 5 in Fig. 3.

Interestingly, in the presence of π, enhancements of DNase I cleavage were seen at positions 15 and 51 (16), which lie 1 bp downstream of the first and second sets of *stb* repeats, respectively. However, the significance of this is not clear. In cells not producing π, the pACYC184 replicon, which is normally unstable (39), could be stabilized by the addition of the γ origin (enhancer plus core, including the *stb* site; construct pDX101 [58a]). This preliminary finding suggests that π is not needed for stability functions; rather, an unidentified host protein may bind to the *stb* repeats. An alternative explanation is that *stb* mediates partitioning by altering the structure of the DNA.

Similarities and differences between the R6K stability system and other mechanisms of stability. Like the *stb* of R6K, the partition (*par*) locus of pSC101 stabilizes the parent plasmid, although neither segment is essential for replication (39, 59). The enhancer does not alter R6K γ-origin plasmid copy number (60). The pSC101 *par* site, while having an effect on plasmid copy number, does not mediate its effect through copy number (2, 5, 34). Also, neither segment contains significant open reading frames (41, 54, 57), and both contain sequence repeats. The pSC101 *par* site carries three 13-bp partition-related sequences. Deleting different repeats yields various degrees of partition deficiency (57). It remains to be seen, however, what effect (if any) deleting repeats in the *stb* will have on R6K stability.

The pSC101 *par* region contains a gyrase-binding site, and a small deletion that interferes with partitioning also reduces gyrase binding (58). Furthermore, mutations in gyrase which reduce negative supercoiling destabilize Cmp^- pSC101 derivatives, which contain deletions of one repeat in the pSC101 *par* locus but are normally stable (40). We have shown here that the R6K enhancer carries two gyrase consensus sequences (G1 and G2 [Fig. 3]). However, unlike pSC101, mutations which alter or delete these R6K gyrase consensus sequences do not cause instability (Fig. 2).

The pSC101 *par* site does not mediate stability through altering overall plasmid superhelicity (40, 58). Instead, Beaucage et al. (1) and Conley and Cohen (4) have suggested that the pSC101 *par* sequence may mediate stability partially through altering the local superhelicity of pSC101 plasmids. While the R6K enhancer does not alter overall plasmid superhelicity, as monitored by chloroquine-agarose gels (Fig. 5), what effects the enhancer has on local superhelicity are not known. Mutations which increase the stability of *par*-deleted pSC101 plasmids include the introduction of an appropriately placed strong promoter (1). In contrast, reorienting the γ origin relative to the strong *lacZ* promoter in the vector does not change plasmid stability characteristics. Plasmids containing the enhancer and core are stable whether *lacZ* transcription enters the origin from the right (pMF36) or the left (pMF35). Similarly, core plasmids are unstable whether transcription enters the core from the right (pFW45) or the left (pFW12) (Fig. 2) (58a).

Cohen and coworkers have shown that a mutation in pSC101 RepA protein which allows replication in the absence of IHF also stabilizes pSC101 plasmids lacking *par* (2). Conley and Cohen (5) have recently shown that many RepA mutations that increase stability in the absence of *par* also increase plasmid copy number (although there is not a direct correlation between the increase in plasmid copy number and stability). In the R6K system, three copy-up π mutants can both drive γ-origin replication in the absence of IHF (7) and increase the plasmid copy number of core origin plasmids severalfold (60). However, none of these mutant π proteins are able to stabilize these core plasmids (Fig. 2D and data not shown). It is important to note, however, that stability data obtained with copy-up π mutants may not be easily comparable to those obtained with wt π, because the wt and mutant proteins may have very different biochemical characteristics. Unlike wt π, copy-up π mutants can tolerate the absence of IHF protein (7) or many different mutations in the γ origin (7a, 57a, 59, 60) and overcome incompatibility (37).

Thus, despite some superficial similarities (e.g., the presence of repeats and the probable role of a host factor), the partitioning systems of R6K and pSC101 differ from each other in many aspects.

Stability and replication. We have noted the similarity between the average copy number (60) of pFW12, which lacks the enhancer and is unstable, and that of pMF36, which carries the enhancer and is stable. Despite this similarity, we cannot exclude the possibility that the role of *stb* is primarily in replication and its effects on plasmid stability are secondary. A large spread in plasmid copy number, despite a high average copy number, can have a deleterious effect on stability (43). Also, plasmids which are slow to initiate replication may be unstable. In this case, a cell which receives few plasmids may have too little time to replicate sufficient plasmid copies prior to the next cell division. To further determine the relationship between R6K replication and stability, both in vitro and in vivo studies are needed to determine what role (if any) the *stb* has on plasmid replication rates and spread in copy number.

Stability of the parent R6K plasmid. The examination of R6K stability described above concerned only the role of two small segments, the core and the enhancer, at the low level of R6K initiator protein π. At a high level of wt π (the level produced by the intact R6K or 10 times the low level [15]), the γ-origin plasmid is unstable, even when the enhancer is present (58a). The simplest explanation for this instability is that the copy number of pMF36 is decreased threefold when the π is supplied at the high level (15). A similar study with plasmid pSC101 revealed that partitioning and replication of pSC101 are inhibited by high levels of the initiator protein RepA (23).

Not surprisingly, the entire R6K plasmid is stable (58a). This is most likely due to the presence of two additional origins, α and β, on the intact R6K, but it also remains possible that R6K contains other mechanisms for stability which the isolated γ origin lacks.

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