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Structural and functional analysis of a replication enhancer: Separation of the enhancer activity from origin function by mutational dissection of the replication origin γ of plasmid R6K

(origin activation at a distance/initiation of replication/DNA looping)

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ABSTRACT **The plasmid R6K possesses three distinct origins of replication: α, β, and γ. The replication origin γ of plasmid R6K performs a dual function: (*i*) as an origin itself and (*ii*) as an enhancer element required in cis for the activation at a distance of the other two replication origins α and β. We have dissected the γ origin/enhancer by site-directed mutagenesis and have reached the following conclusions. The origin function can be specifically inactivated without impairing the enhancer function by insertion and/or deletion mutations near the opposite ends of the origin γ sequence. One such mutation deleted sequences that included the left DnaA site I. The second mutation involved insertion of linker sequences that resulted in a spatial alteration between the right DnaA site II and the VIIth π binding iteron (tandemly repeated binding sites). Other mutations that either partly or completely deleted the A+T-rich sequence adjacent to, but not including, the π binding iterons also abrogated enhancer and origin function and suggested that π binding sites were necessary but not sufficient for enhancer activity. Finally, the functional analysis of a set of mutants of the γ origin/enhancer suggested that a continuous stretch of 300 base pairs is necessary for origin γ function and that the sequences that included the binding sites for π, DnaA, and integration host factor proteins are required in the correct stereochemical alignment to impart origin activity.**

The plasmid R6K of *Escherichia coli* has been developed as a model system for the study of selective origin activation in a chromosome that contains multiple replication origins. R6K possesses three known origins of replication termed α, β, and γ (1, 2). The plasmid-encoded initiator protein, π, acts in trans with other host-encoded proteins to trigger replication from any one of the three origins *in vivo* or *in vitro* in a mutually exclusive mode (1, 3–6). Recent work has shown that the DnaA protein is required for the activation of the α and γ origins but not for the β origin. In addition, host-encoded integration host factor (IHF) protein functions as an accessory factor in controlling copy number of the γ replicon, and under certain conditions, is essential for replication from the γ origin (7–9). Thus, differential protein requirements in trans by the three origins have contributed to at least a partial understanding of the mechanism of selective origin activation in the R6K system.

Sequences spanning the minimal origin γ perform a dual function as an origin and as an essential cis element required for the activation of the distant origins α and β (10–12). Since the α and β origins are located at distances of up to 3 kilobases and in opposite directions from the γ origin sequence, the γ origin has been functionally defined as a replication enhancer element (13). A model for origin activation at a distance that involved initiator-protein-mediated DNA looping has been proposed (13). The model predicts the existence of at least three types of cis mutations at the enhancer and the origins that are (*i*) defective in γ origin function but able to activate α and β origins at a distance, (*ii*) defective in α and/or β origin function but retain the ability to replicate from γ, and (*iii*) defective in all three origin functions. The model also predicts the existence of looping-defective mutations in the trans-acting π protein that are able to initiate replication from origin γ but not from origins α or β.

We now report the isolation and analysis of the first category of predicted cis mutations. Our experiments were designed to address primarily the following questions. Can the origin activity of γ be separated from its replication enhancing function? Is the π protein, when bound to the iterons at the γ origin/enhancer, both necessary and sufficient to activate the distant origins? Finally, do the known binding sites for DnaA, IHF, and π protein have to be in a specific stereochemical alignment with respect to each other to elicit origin γ function?

Our results show that indeed mutations at two regions of origin γ inactivated origin function while simultaneously maintaining its replication enhancer function. The π protein and its binding sites were necessary, but not sufficient, for enhancer function. Finally, the correct stereochemical and spatial alignments between elements of the γ origin/enhancer sequence were essential for origin activity.

MATERIALS AND METHODS

E. coli CM1793 and EH3827 (14), PolA12:MM383 *polA12*(Ts) (15), DH5α (16), and GM2159 (17) have been described. Plasmids containing *Bam*HI linker additions at unique sites within the origin β region and directed mutagenesis of the origin β region looping target site are described elsewhere (10). All recombinant constructions were propagated in strain DH5α. The presence of specific lesions in the $\gamma^-\beta^+$ mutants was followed by sequencing, restriction enzyme digestion, or, for the repaired *Bam*HI sites, *Ban* III cleavage of unmethylated DNA prepared in GM2159. The following oligonucleotides were used for site-directed mutagenesis (18) with the relevant plasmid harboring the mutation indicated in parenthesis: StyI (pWK234), 5′-TCATGTTTCCTAGGAACAATAAAATT-3′; *Bam*HI-III (pWK236), 5′-CTCATGGCTGGATCCAACGTACT-3′; *Bam*HI-VII (pWK238), 5′-CTGTTGATAGGGATCCTACGTACTAA-3′; ΔDnaAII (pWK240), 5′-TCAGCAGTTCAACTACGTACTAAGCTCT-3′; DPRA1 (pWK232), 5′-ACTTAAATAAAGGTTTTAAG-3′. Protocols for molecular cloning and sequencing

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Abbreviation: IHF, integration host factor.

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have been described in detail (19, 20). Unless otherwise indicated, antibiotic concentrations used were ampicillin at 50 μg/ml and kanamycin at 50 μg/ml.

RESULTS

Extensive Stretches of the γ Region Are Required for Origin Activation. To analyze mutants generated within the γ origin/enhancer element for their ability to activate origins γ or β, we designed a plasmid system that would conveniently permit the detection and analysis of differential origin usage. Plasmids were constructed in a pBR322 vector background that either lacked (pγA series) or contained (pγβA) sequences corresponding to the origin β region present as a *Hin*dIII–*Bam*HI insert. Mutations within the γ origin/enhancer element were first subcloned into an R6K *Hin*dIII fragment cassette that contained the contiguous *Hin*dIII fragments 9 and 15, a functional pR6K-1 promoter, and the gene encoding the π protein. The resulting hybrid plasmid replicons contained the pBR322 origin and either the R6K origin γ alone or origins γ and β. Replication from the R6K origins could then be selectively assayed in the presence of the pBR322 vector origin by propagation in a *polA*(Ts) strain since R6K origins do not require DNA polymerase I (21). Alternatively, the plasmids could be cleaved at unique *Sal* I and *Pst* I sites to dissociate the R6K origin fragment and test it for autonomous replication after ligation to an ampicillin-resistant non-self-replicating selectable marker.

The results of our analysis of the pγA series of plasmids are shown in Fig. 1. We observed that most of the lesions inactivated the γ origin. Wild-type origin γ sequences present in pWK138 or pWK139 were used as positive controls. Plasmid pWK138 contained an *Eco*RI site inserted in place of the *Bgl* II site at coordinate 277 and was used for convenient cloning and substitution of mutant origin sequences. In all cases, the *in vivo* requirements for replication of the plasmids pWK138 and pWK139 were indistinguishable.

Previous *in vivo* and *in vitro* analyses had established that the host-encoded DnaA protein was essential for origin γ activation (10, 22). Consistent with this observation and the known localization of the binding sites for the DnaA protein within the γ origin (T. W. MacAllister, W.L.K., and D.B., unpublished data), we observed that plasmids that lacked the 174-base-pair (bp) *Eco*RI–*Hin*dIII fragment containing the DnaA binding site I adjacent to the *Hin*dIII site at coordinate 1 (pWK55a) or the site-specific deletion of the DnaA binding site II adjacent to the VIIth repeat and spanning coordinates 249–258 (pWK240) inactivated the γ origin.

Deletions of the A+T-rich region from coordinates 1 to 53 or 1 to 69 also inactivated the γ origin (Fig. 1, pWK230d or pWK231d, respectively). A binding site (ihf) for the host-encoded IHF has been mapped within the A+T-rich region spanning coordinates 61–75 (8, 9). We have shown (9) that IHF is not strictly required for replication from the γ origin (9) and, therefore, the failure of plasmids pWK230d and pWK231d to replicate in our assay likely results from sequences that are distinct from the embedded ihf site.

We observed that a site-specific deletion of 6 bp (coordinates 53–58) within the A+T-rich region adjacent to the ihf site in pWK232 also abolished replication. This 6-bp sequence corresponds to a region of altered hydroxyl-radical cleavage chemistry and hypersensitivity induced by the binding of either IHF or π protein to the origin region (9). This site may represent a region necessary for partially unwinding the origin, or alternatively, could have abrogated origin function by changing the stereospecific alignment of flanking sequences by introducing less than an integral multiple of the helical repeat unit.

Linker insertion mutations of a 6-bp *Sty* I site at coordinate 94 (pWK234), 6-bp *Bam*HI sites between the IIIrd and IVth repeat (pWK236) or immediately adjacent to the VIIth repeat

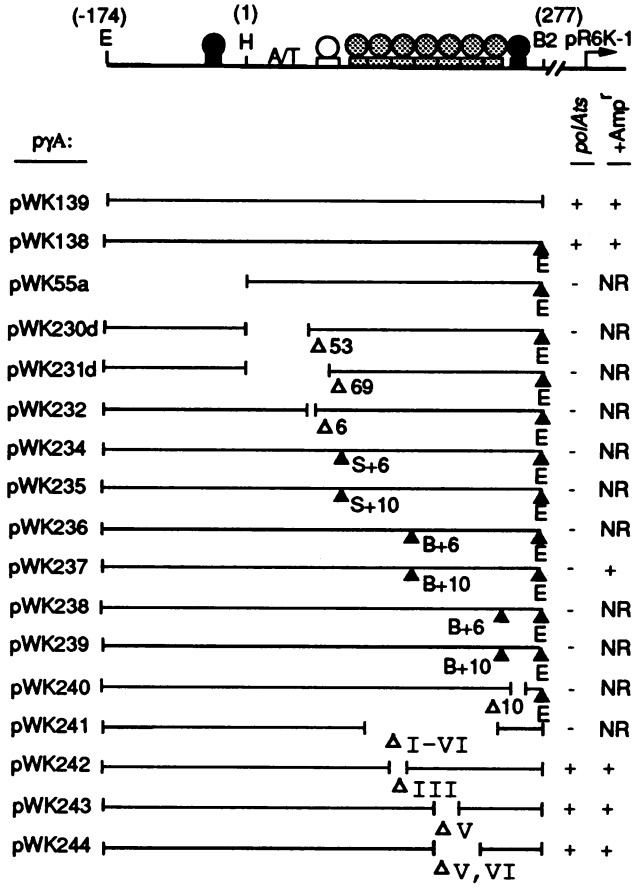

FIG. 1. Results of the *in vivo* transformation assays for the pγA series plasmids and schematic diagram of the γ origin/enhancer mutations tested. The top of the figure is an expanded view of the γ origin/enhancer region showing the location of known protein binding sites. Solid circles, DnaA initiator protein sites I (to the left) and II (to the right); shaded circles, π initiator protein sites I–VII; open circle, IHF; A/T, A+T-rich region from coordinates 1 to 93; arrow, pR6K-1 promoter; open triangles, deletions; solid triangles, insertions; numbers, length of the linker insertion or deletion in bp. Data are presented as follows: +, Plasmids were recovered through multiple replicate experiments; −, no transformants recovered at the restrictive temperature in the *polA*(Ts) assay; NR, no viable replicons recovered. B, *Bam*HI; B2, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; S, *Sty* I. Coordinates are numbered as described (9).

at coordinate 248 (pWK238) inactivated the γ origin. Subsequent cleavage at these unique linker sites followed by repair and religation resulted in a set of plasmids containing 10-bp inserts that partially restored the helical phase disrupted by the original linker insertion. We observed that plasmids pWK235 and pWK239 that represented the repaired *Sty* I site of pWK234 or *Bam*HI site of pWK238, respectively, did not restore the ability of the origin to replicate. Interestingly, repair of the *Bam*HI site of plasmid pWK236 between the IIIrd and IVth repeats resulted in a plasmid, pWK237, that could not be recovered by the *polA*(Ts) transformation assay but could be recovered as an independent replicon linked to an ampicillin-resistance marker.

Since our results from the panel of mutations studied revealed that extensive regions of the γ origin flanking the seven 22-bp iterons were essential for origin function, we wished to examine the role of the repeat sequences themselves in origin function. We designed a mutation that eliminated all but one iteron (VIIth) to test the hypothesis that the π initiator protein when bound to a single iteron could direct other proteins of the replication machinery to the γ origin to activate it or, alternatively, that the repeat sequences and the

concomitant topological changes associated with π protein binding (23, 24) contributed to origin activation. The plasmid that contained this six-iteron deletion failed to replicate in either of our test systems (Fig. 1, pWK241). Our results, therefore, suggested that although the capacity of the π protein to bind to the origin was retained and other origin features such as the binding sites for the DnaA and IHF proteins were unaltered in sequence or context, multiple binding sites for the π protein were required to recover viable γ origin replicons.

We did note, however, that plasmids that contained deletions of one or two repeats [pWK242 (ΔIII), pWK243 (ΔV), and pWK244 (ΔV, VI)], could be tolerated and recovered as viable γ origin replicons consistent with previous observations (25).

Analysis of the γ Origin/Enhancer Reveals Cis Sequences Essential for Origin β Activation at a Distance. To determine whether any of the mutations that we had characterized as lethal for origin γ could nevertheless support replication initiation from origin β, we designed a second series of pBR322-based plasmids, termed p$\gamma\beta$A, that contained the 1018-bp *Hin*dIII–*Cla* I fragment of the origin β region positioned adjacent to the *Hin*dIII fragments 9–15 in the wild-type configuration. Specific lesions within the γ origin/enhancer were introduced by subcloning and the resulting p$\gamma\beta$A plasmids were tested by both transformation assays.

Our results, shown in Fig. 2, revealed that most mutations within the γ origin/enhancer did not support replication in the presence of origin β sequence; however, two separate regions that contained specific mutations at the extremities of the minimal γ origin/enhancer supported replication when origin β sequences were present. Since our previous analysis of the

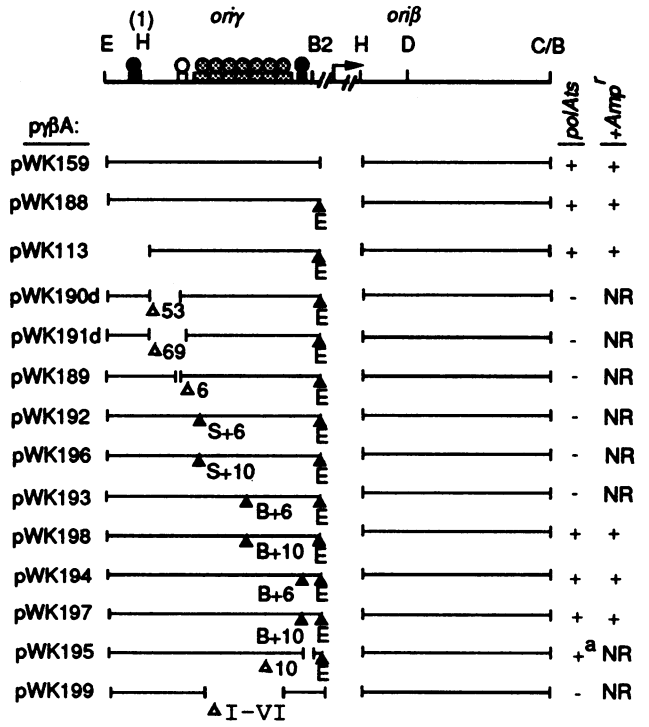

FIG. 2. Results of the *in vivo* transformation assays for the p$\gamma\beta$A series plasmids. A schematic diagram of the γ origin/enhancer mutants tested and the origin β region included in the plasmid are shown. At the top of the figure is an expanded view of the γ origin/enhancer region with details as described in the legend to Fig. 1. B, *Bam*HI; B2, *Bgl* II; C/B, *Cla* I (*Ban* III) converted to *Bam*HI site; D/B, *Dde* I site converted to *Bam*HI site; E, *Eco*RI; H, *Hin*dIII; S, *Sty* I.

[a]Only a few transformants were recovered that were the correct plasmid.

consequence of these mutations in the pγA vector showed that origin γ was inactivated by each one of these lesions, we termed those γ origin/enhancer mutations that were permissive for β origin replication as $\gamma^-\beta^+$ mutants.

The first region identified that conferred a $\gamma^-\beta^+$ phenotype was defined as a deletion of the 174-bp *Eco*RI–*Hin*dIII fragment that contained the DnaA binding site I. Since our previous analysis of the *in vivo* dependence of R6K origins upon DnaA had shown that origin γ required DnaA whereas origin β did not require DnaA (10), we reasoned that deletion of the 174-bp *Eco*RI–*Hin*dIII fragment from the γ origin/enhancer might permit replication of plasmids containing origin β provided that other essential regions were not present in the 174-bp fragment that were distinct from DnaA site I. Indeed, our results showed that plasmid pWK113 was able to replicate in both *in vivo* assays, provided that origin β sequence was present on the test plasmid (compare pWK113 in Fig. 2 and pWK55a in Fig. 1).

A second region of the γ origin/enhancer that conferred a $\gamma^-\beta^+$ mutant phenotype mapped proximal to the VIIth repeat. The *Bam*HI linker insertion mutation at coordinate 248 (pWK194) and the linker insertion repaired (pWK197) at the end of the VIIth repeat supported replication when origin β sequence was present (Fig. 2), in contrast to the failure of the same mutations to support replication when only origin γ sequence was present (compare with Fig. 1, plasmids pWK238 and pWK239). Our observation that the 10-bp deletion of the DnaA site II spanning coordinates 249–258 and adjacent to this linker insertion could not be recovered as an independent replicon (Fig. 2, pWK195) suggested that the DnaA site II was an essential sequence element necessary *in vivo* for the function of both origin γ and origin β. Since DnaA protein is not needed *in vivo* for origin β function, the DnaA site II must function in ways other than as a binding site for DnaA protein. Also, the observed results with linker insertions between the VIIth iteron and DnaA site II yield the $\gamma^-\beta^+$ phenotype, regardless of whether the two flanking sites are on the same or opposite helical face, and indicate that the contiguity and alignment of the VIIth iteron and the adjacent DnaA site II is not essential for origin β function.

We note that the plasmid containing the *Bam*HI linker insertion between the IIIrd and IVth repeats (pWK198) also replicated when origin β sequence was present, although it was not classed as a $\gamma^-\beta^+$ mutant since the same mutation was able to replicate origin γ alone (compare Fig. 1, pWK237, and Fig. 2, pWK198). All other mutants tested did not support the replication of origin β even under the conditions when p$\gamma\beta$A plasmids containing the wild-type γ origin/enhancer sequences as controls (pWK159 and pWK188) could be recovered easily. Our results, therefore, suggested that the A+T-rich region, the stereospecific alignment of the A+T-rich region with the 22-bp repeats, sequences spanning the DnaA site II, and the necessity of iterated π binding sites were all critical determinants for the activation of origin β at a distance.

Origin Usage Analysis with the $\gamma^-\beta^+$ Mutations. Do the mutants classified here as $\gamma^-\beta^+$ truly initiate replication from origin β as shown by our established criteria (10)? Specifically, we wished to determine (*i*) whether the $\gamma^-\beta^+$ lesions could be introduced into plasmids that contained a minimal origin β sequence previously determined by deletion mapping and direct electron microscopic analysis to harbor a *bona fide* origin β; (*ii*) whether our prediction that the $\gamma^-\beta^+$ lesions should not be recovered if the 9-bp deletion of an essential looping target site within origin β was present on either the extended or minimal origin β fragment is correct; and (*iii*) whether the $\gamma^-\beta^+$ mutants could support replication from origin β *in vivo* in the absence of DnaA, as predicted by our previous observation of differential DnaA requirements of the γ and β origins.

The results of our analysis of $\gamma^-\beta^+$ mutants with a minimal origin β sequence and sequences that contained a specific deletion of an essential looping target site are shown in Fig. 3. Surprisingly, we were able to recover independent replicons representing only one of the three $\gamma^-\beta^+$ mutations studied, pWK297D that contained the repaired *Bam*HI linker. Interestingly, these results suggested that there could be altered and expanded compensatory sequence requirements for the β sequence minimal limits if the γ origin/enhancer element was mutated and not present in the wild-type form. When the origin β *Hin*dIII–*Bam*HI fragment used in the p$\gamma\beta$A vector contained the 9-bp looping target site mutant essential for origin β activation, we did not recover any viable replicons with the $\gamma^-\beta^+$ mutants under conditions where we were consistently able to recover replicons using wild-type γ origin/enhancer sequences (Fig. 3, pWK188 D and pWK288 D).

Table 1 shows the results of transformation of minireplicons that contained the $\gamma^-\beta^+$ mutants into the isogenic strains CM1793 (*dnaA*$^+$) and EH3827 (*dnaAD*). In every case our results showed that $\gamma^-\beta^+$ mutations could replicate in the absence of DnaA, provided that origin β sequence was present on the plasmid. Thus, our results confirmed that indeed the β origin was the active origin in our $\gamma^-\beta^+$ mutant screen.

DISCUSSION

The observations reported in this paper have the following implications with regard to activation of the β origin by the γ origin/enhancer. The extended sequences of γ that are required for β activation suggest that besides π protein, which is responsible for γ–β looping (13), probably several other proteins are first assembled at γ and then transferred to β. The mutational analysis further suggest that the nucleoprotein complex must be in a unique stereochemical alignment to have activator function. Our observations are consistent with previous work of Kolter *et al.* (25) that showed that most Tn*5* insertions into the γ sequence inactivated the γ origin. However, Kolter *et al.* (23) observed that several Tn*5* insertions into the region of DnaA site I did not abolish origin γ function. In contrast, our results show that the DnaA site I is necessary for origin γ function (but not origin β activity). These two apparently conflicting observations could be reconciled by suggesting that the Tn*5* sequence provided an alternative DnaA site, a protein, or a sequence that could bypass the requirement for DnaA site I.

The isolation of mutations that have the $\gamma^-\beta^+$ phenotype has helped us to dissociate the γ origin function from its enhancer function. That the deletion of the DnaA site I generates a $\gamma^-\beta^+$ phenotype is not inconsistent with the DnaA-independent nature of origin β *in vivo* (10). The $\gamma^-\beta^+$ mutant that disrupts the continuity of the VIIth iteron and the DnaA site II has a more complex interpretation that cannot be explained simply by DnaA dependence of origin γ and DnaA independence of origin β *in vivo* (10), because deletion of DnaA site II inactivates both origins. The phenotypes of mutation within the 30 bp immediately adjacent to the VIIth iteron suggest that this region plays a critical role in both the origin and enhancer functions of γ. A point mutation within this region at coordinate 256 is known to affect the copy number of the γ replicon (26). It is conceivable that DnaA site II, besides being a binding site for DnaA, plays other roles in imparting functional integrity to the γ origin.

Although the genetic analysis presented needs to be extended by biochemical analysis involving a fully reconstituted *in vitro* replication system using purified proteins, a preliminary comparison of the origin γ sequence with other known origins (e.g., λ ori; ref. 27) has enabled us to propose a model of activation of the β origin by the γ enhancer. Since origin β replication *in vivo* is independent of IHF (8, 9) and DnaA (10), the transfer of these two proteins to origin β or their participation in the assembly of a replication complex at origin γ must be nonessential for origin β activity. The requirement for the A+T-rich region immediately preceding the π iterons at γ for β activation and the fact that similar A+T-rich regions at other origins are the site of assembly for

Table 1. Transformation of plasmids containing γ origin/enhancer mutations into *dnaA* deletion strains

Plasmid	Relevant genotype	Transformants CM1793 *dnaA*$^+$	Transformants EH3827 *dnaA*Δ
pWK188A	Wild-type control (p$\gamma\beta$A)	+	+
pWK188ΔA	Δ9bp-β (C) (p$\gamma\beta$A)	+	−
pWK288DA	Wild-type minimal β control (p$\gamma\beta$A)	+	+
pWK288DΔA	Δ9bp-β (D) (p$\gamma\beta$A)	+	−
pWK113A	ΔDnaA site I [$\gamma^-\beta^+$] (p$\gamma\beta$A)	+	+
pWK194A	B VII + 6 [$\gamma^-\beta^+$] (p$\gamma\beta$A)	+	+
pWK197A	B VII + 10 [$\gamma^-\beta^+$] (p$\gamma\beta$A)	+	+
pWK198A	B III + 10 (p$\gamma\beta$A)	+	+*
pWK297D	B VII + 10 [$\gamma^-\beta^+$] (p$\gamma\beta$A)	+	+
pWK237A	B III + 10 (p$\gamma\beta$A)	+	−

Transformants represent summed counts from multiple replicate experiments. +, Consistent recovery of the plasmid; −, no plasmid recovered.
*Transformants are recovered at a 100 times lower frequency.

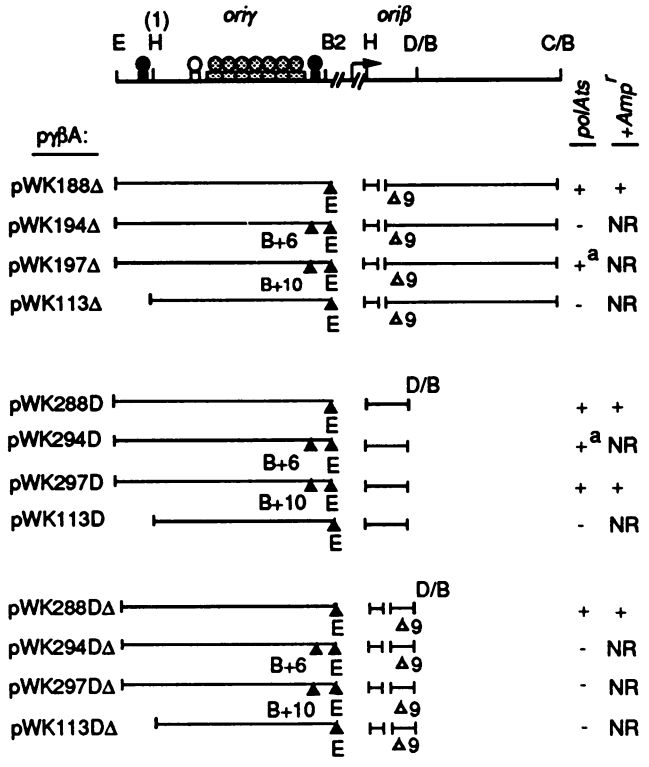

FIG. 3. Results of the *in vivo* transformation assays to test origin β viability for the candidate $\gamma^-\beta^+$ origin/enhancer mutations. The top of the figure shows an expanded view of the γ origin/enhancer region and the origin β region truncations or looping target site mutations used in the assay. Most details are described in Figs. 1 and 2. The notation Δ9 refers to a 9-bp deletion of a π protein looping target site within the β origin region that has been shown to be essential for origin β viability (10). The 9-bp lesion was introduced into the 1018-bp *Hin*dIII–*Cla* I (*Bam*HI) origin β fragment or into the 85-bp *Hin*dIII–*Dde* I (*Bam*HI) truncated origin β fragment that has been shown to contain a functional origin β.
[a]Only a few independent transformants were recovered, but they were the correct plasmid upon analysis.

DnaBC complex (e.g., λ ori; ref. 27) suggests that π protein, possibly with the help of other host proteins, unwinds the A+T-rich region at γ and allows the DnaBC complex to assemble at that site. The π-mediated γ–β looping then transfers this complex by contact with the β origin. What proteins, if any, assemble at origin β is presently unknown. It is also not known why γ specifically and productively loops to the 9-bp half iteron at the β origin (10, 13) to form an initiation complex although there are other potential looping sites in the vicinity that do not participate in the initiation reaction (10). We note especially that the looping model of origin activation predicts a class of mutations in the π protein that would cause a looping defect resulting in its failure to activate β and α while retaining its activity to initiate replication from origin γ. Recently, this laboratory has isolated such mutations (28) thus confirming the looping model of distant origin activation *in vivo*.

It is our hope that future work using purified proteins to reconstruct looping-mediated initiation of the β origin *in vitro* will help illuminate the questions discussed above.

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1. Crosa, J. H. (1980) *J. Biol. Chem.* **255,** 11075–11077.
2. Inuzuka, N., Inuzuka, M. & Helinski, D. R. (1980) *J. Biol. Chem.* **255,** 11071–11074.
3. Crosa, J. H., Luttropp, L. K., Heffron, F. & Falkow, S. (1975) *Mol. Gen. Genet.* **140,** 39–50.
4. Germino, J. & Bastia, D. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 5475–5479.
5. Kolter, R., Inuzuka, M. & Helinski, D. R. (1982) *Cell* **15,** 33–43.
6. Stalker, D., Kolter, R. & Helinski, D. R. (1982) *J. Mol. Biol.* **161,** 33–43.
7. Dellis, S. & Filutowicz, M. (1991) *J. Bacteriol.* **173,** 1279–1286.
8. Filutowicz, M. & Appelt, K. (1988) *Nucleic Acids Res.* **16,** 3829–3843.
9. Kelley, W. L. & Bastia, D. (1991) *J. Biol. Chem.* **266,** 15924–15937.
10. Kelley, W. L. & Bastia, D. (1992) *New Biol.*, in press.
11. Crosa, J. H., Luttropp, L. K. & Falkow, S. (1978) *J. Mol. Biol.* **124,** 443–468.
12. Kolter, R., Inuzuka, M., Figurski, D., Thomas, C., Stalker, D. & Helinski, D. R. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 91–103.
13. Mukherjee, S., Erickson, H. & Bastia, D. (1988) *Cell* **52,** 375–383.
14. Hansen, E. B. & Yarmolinski, M. B. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 4423–4427.
15. Monk, M. & Kinross, J. (1972) *J. Bacteriol.* **109,** 971–978.
16. Hanahan, D. (1983) *J. Mol. Biol.* **166,** 557–580.
17. Marinus, M. G., Carraway, M., Frey, A. Z., Brown, L. & Arraj, J. A. (1983) *Mol. Gen. Genet.* **192,** 288–289.
18. Carter, P., Boudelle, H. & Winter, G. (1985) *Nucleic Acids Res.* **13,** 4431–4443.
19. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
20. Sanger, F., Nicklen, S. & Coulson, A. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
21. Kingsbury, D. T. & Helinski, D. R. (1970) *Biochem. Biophys. Res. Commun.* **41,** 1538–1544.
22. MacAllister, T. W., Kelley, W. L., Miron, A., Stenzel, T. T. & Bastia, D. (1991) *J. Biol. Chem.* **266,** 16056–16062.
23. Germino, J. & Bastia, D. (1983) *Cell* **34,** 125–134.
24. Mukherjee, S., Patel, I. & Bastia, D. (1985) *Cell* **43,** 189–197.
25. Kolter, R. & Helinski, D. R. (1982) *J. Mol. Biol.* **161,** 45–56.
26. McEachern, M. J., Filutowitz, M. & Helinski, D. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1480–1484.
27. Dodson, M., McMacken, R. & Echols, H. (1989) *J. Biol. Chem.* **264,** 10719–10725.
28. Miron, A., Mukherjee, S. & Bastia, D. (1992) *EMBO J.* **11,** 1205–1216.