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Genetics

Mutations in direct repeat sequences and in a conserved sequence adjacent to the repeats result in a defective replication origin in plasmid R6K

(DNA binding of initiator protein π/bisulfite mutagenesis/γ-origin mutant bank)

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ABSTRACT **Plasmid pMM3 is a pBR322 derivative carrying the γ origin of replication of the naturally occurring plasmid R6K. We have produced a γ-origin mutant bank of this plasmid using the single-strand-specific mutagen sodium bisulfite. Members of this bank contain single or multiple mutations in the seven direct repeats and the flanking sequences in the γ origin. Three mutants with defective γ origins have been isolated from this mutant bank. Two of these direct repeat mutants, γ117 and γ120, are unable to replicate and also have lost the ability to bind the R6K initiation protein π *in vitro* at one of the seven 22-base-pair direct repeats within their respective origins. Precise deletion of the damaged repeat of either of these mutants restores origin function, suggesting that the primary defect of these mutants involves a disruption of the normal spacing of π binding and flanking sequences within the γ origin. The third mutant, γ111, binds π normally but replicates at a greatly reduced copy number due to a mutation near the seventh repeat. This mutation falls within a short sequence that appears to be conserved among a number of other plasmids that contain direct repeats within their origins of replication.**

R6K is a 38-kilobase-pair (kb) self-transmissible antiobiotic resistance plasmid that maintains itself at a copy number of 12–18 per chromosome equivalent in *Escherichia coli* (1). The basic R6K replicon is contained within a 4-kb region that contains three origins of replication α, β, and γ, and the *pir* structural gene that encodes the π protein, an R6K-specific replication initiation protein. The π protein is required for the activity of all three origins both *in vivo* and *in vitro* and is also involved in the negative regulation of R6K replication (2–4). The α and β origins both appear to require part or all of the γ-origin sequences in *cis* in order to function.

A prominent feature of the minimal γ origin is the presence of seven 22-base-pair (bp) direct repeats that have been demonstrated to bind π protein (5–7). Deletion of three or more of these direct repeats results in an inactivation of the γ origin (8). It has been proposed that the binding of π protein to the direct repeats of the γ origin is essential for γ-origin functionality (9).

A variety of other plasmid and phage replicons including F (10), P1 (11), RK2 (12, 13), Rts1 (14), pSC101 (15, 16), and λ (17) have an arrangement similar to that of R6K. Each has an origin of replication that contains direct repeats and each codes for a replicon-specific initiation protein. The initiation proteins of pSC101 and λ, like R6K, have been shown to bind to the direct repeats of their respective origins (18, 19). It is of great interest then to discover the role that direct repeats, and the binding of proteins to these repeats, have in the initiation of replication.

We report here that mutations within the repeats of R6K that prevent binding of π protein also eliminate the ability of the γ origin to replicate. We also report the identification of a short nucleotide sequence that is present next to the repeats of several plasmids including R6K. A single base change in this sequence results in a substantial decrease in the copy number of an R6K γ-origin plasmid.

MATERIALS AND METHODS

Strains. Strains used in this study were C2110 (*polA his rha*), C600 (*thr leu thi lacY tonA supE44*), BD1391 (*his arg leu thr rpsE trpA446 ungl*) (20), DK100 TS214 (*his argG metB leu strA thy xyl lacY polA214*-Ts) (21), *φl1* (*C2110* λ*pir*) (8).

Plasmids. pRK690 was constructed by the insertion into pBR322 of an *Eco*RI linked *Hae* III restriction fragment from pRK526 containing the γ origin (22). Plasmid pMM4 was constructed by ligating an *Eco*RI fragment from pRK665 (22) containing the *pir* gene into the RK2 derivative pRK291 (G. Ditta, personal communication). The γ111 revertants were constructed by replacement of either of the two *Pst* I–*Sna*BI fragments of γ111 with the corresponding fragment of wild-type pMM3. The constructions were confirmed by restriction analysis and by running G-specific DNA sequencing reactions. Plasmid DNA was obtained by using the procedure of Currier and Nester (23). Restriction enzymes were purchased from New England Biolabs or Bethesda Research Laboratories and used according to the manufacturers' specifications.

Mutagenesis. Gapped circular DNA molecules were constructed by denaturing 100 μg of each of the large *Hin*dIII–*Bgl* II fragment of pMM3 and *Hin*cII linearized pMM3 in 440 μl of 1 M NaOH/20 mM EDTA at 20°C for 10 min. Four-hundred microliters of deionized formamide was added and the mixture was allowed to rehybridize at 37°C for 15 min. The DNA was then passed over a 12-ml Sephadex G-100 column equilibrated with 6 mM NaCl/6 mM Tris·HCl, pH 8.0/0.1 mM EDTA, and the DNA-containing fractions were pooled together. Approximately 20–30% of input DNA was converted to gapped circular molecules that ran slightly ahead of nicked circular pMM3 molecules on a 1% agarose gel. Sodium bisulfite mutagenesis was carried out according to Shortle and Nathans (24) except that the reaction was rapidly stopped prior to dialysis with the addition of an equal volume of cold 1 M NaOH, which raised the pH to ≈8.0. Three micrograms of DNA from each of seven time points of bisulfite treatment was digested with *Hin*dIII and *Bgl* II and transformed into strain BD1391. The digestion minimizes transformants from linear DNA and the ung recipient prevented excision of bisulfite-induced uracil residues from the gapped circles.

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Abbreviations: bp, base pair(s); kb, kilobase pair(s).

Revertants of replication-defective mutants were obtained after nitrous acid mutagenesis according to Miller (25). DK100 *polA214*-Ts containing both pMM4 and a mutant pMM3 plasmid (γ117 or γ120) were mutagenized and then incubated at 43°C in the presence of penicillin. Plasmid DNA was isolated from cultures that grew overnight at 37°C and used to transform strain φ11 to Pen^r. Transformants that appeared were checked for being cointegrates of pMM3 and pMM4 by screening for resistance to tetracycline. Plasmid DNA from tetracycline-sensitive clones was used for further analysis.

Sequencing and DNase Protection Experiments. DNA sequencing and DNase protection experiments were performed as described (5, 26, 27). Purification of the π protein will be described elsewhere. Restriction fragments containing the γ origin of plasmid pMM3 or its mutants were labeled at the 5′ end with polynucleotide kinase (Bethesda Research Laboratories) and [γ-^{32}P]ATP. Labeled fragments were separated on polyacrylamide gels and eluted from appropriate slices of polyacrylamide by diffusion.

RESULTS

Construction of a γ-Origin Mutant Bank. Tn*5* mutagenesis has established that the minimal γ origin of plasmid R6K resides within a 277-bp *Hin*dIII–*Bgl* II restriction fragment (8). To more precisely determine the structural requirements of the γ origin we have isolated point mutations within this 277-bp region by constructing a sodium bisulfite-induced mutant bank. An outline of the scheme for making this γ-origin mutant bank is shown in Fig. 1. Plasmid pMM3 is a joint replicon, carrying the pBR322 origin of replication in addition to the R6K γ origin, each of which can be studied separately by using appropriate genetic backgrounds. The pBR322 origin is nonfunctional in a strain lacking DNA polymerase I activity (28), whereas the γ origin is nonfunctional in any strain that does not provide a source of the R6K-encoded π protein (2). This feature allows the isolation of replication-defective γ-origin mutants. pMM3 also contains unique *Hin*dIII and *Bgl* II restriction sites that flank the γ origin and facilitate the construction of circular DNA molecules that are single stranded in the γ-origin region. We have constructed such gapped circles and subjected them to the single-strand-specific mutagen sodium bisulfite in order to obtain a variety of single and multiple C → T transitions within the γ origin. Similar procedures for constructing gapped circles have been described (29–31). The extent of mutagenesis was controlled by varying the length of time the gapped circles were exposed to bisulfite, with lengths of mutagenesis being 0, 5, 10, 20, 40, 90, and 180 min. The mutagen-treated DNA was transformed into BD1391, a *polA*$^+$ *ung*$^-$ strain that does not excise bisulfite-induced uracil residues and allows pMM3 to replicate using the unmutagenized pBR322 origin. Approximately 150,000 transformants were isolated from each of the early time points, decreasing to ≈100,000 transformants for the 180-min time point. The colonies representing a given time point of mutagenesis were pooled together and plasmid DNA was isolated from the mixture to generate the γ-origin mutant bank.

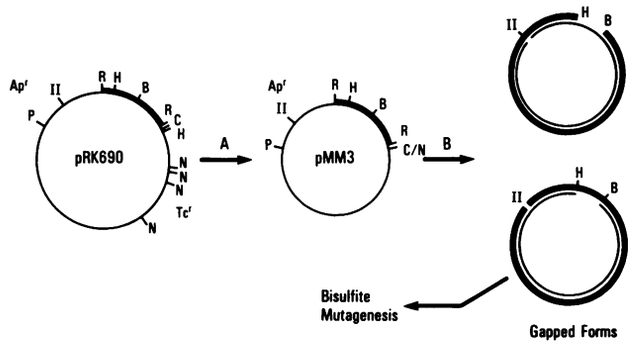

FIG. 1. Construction and mutagenesis of pMM3. (*A*) pRK690 was digested with *Cla* I and *Nar* I and religated to generate pMM3. H, B, II, C, N, R, and P refer to *Hin*dIII, *Bgl* II, *Hin*cII, *Cla* I, *Nar* I, *Eco*RI, and *Pst* I restriction sites, respectively. (*B*) *Hin*cII-linearized pMM3 was combined with an equimolar amount of the large *Hin*dIII–*Bgl* II fragment of pMM3, denatured in NaOH, and renatured together to yield a mixture of renatured linear fragments and circular molecules that are single stranded in the γ-origin region. This mixture was treated with sodium bisulfite for 0–180 min and transformed into an ung$^-$ recipient. Plasmid DNA was isolated from the resulting colonies and used to transform a *polAt214*-Ts strain containing pMM4 as a source of π at 30°C. Individual colonies were picked twice at 43°C to detect pMM3 mutants with defective γ origins.

Isolation and Characterization of Defective γ-Origin Mutants. Plasmid DNA from each time point of the γ-origin mutant bank was transformed into the *E. coli* strain DK100 *polA214*-Ts harboring plasmid pMM4 and plated at 30°C. Under these conditions either origin of pMM3 can function since the π protein is provided by pMM4 and since 30°C is a permissive temperature for replication of the pBR322 origin. Individual colonies were then screened for the ability to grow in the presence of penicillin at 43°C, where the pBR322 origin is nonfunctional. This tests whether or not a given pMM3 clone contains a functional R6K γ origin. One-hundred to 2000 clones from each time point of bisulfite treatment were screened for a defective γ origin. The earliest time point of mutagenesis where strongly defective mutants were isolated was 20 min. Three of the defective mutants from this time point, γ111, γ117, and γ120, were chosen for further analysis. DNA from each mutant was transformed into the control strain C600, where only the pBR322 origin of pMM3 is functional, and into the strain C2110 (*polA*$^-$) containing pMM4, where only the γ origin of pMM3 can replicate. Results of these transformations showed that γ117 and γ120 are unable to form colonies in C2110 (pMM4) and that γ111 could form colonies, though these were distinctly smaller and less numerous than transformants with wild-type pMM3 (data not shown). When plated on penicillin G at 250 μg/ml in C600 (polA$^+$), colonies formed by γ111, γ117, and γ120 were indistinguishable from those formed by wild-type pMM3. Clonal analysis demonstrated that γ111 maintained itself in C2110 pMM4 at a substantially reduced copy number compared to wild-type pMM3 (data not shown).

To determine which mutation or mutations were responsible for the observed phenotypes of the mutants, the origin region of each mutant was sequenced. Shown in Fig. 2 are the positions of the mutations in each of the three mutants. As expected, all of the mutations are C → T transitions confined to one or the other strand of the mutagenized region. γ117 has three base changes, all of which are in the first 22-bp direct repeat, at the 7th, 9th, and 22nd positions of that repeat. γ120 has five base changes, distributed among three of the direct repeats. These are at the 10th and 11th positions of the fourth repeat, at the 7th and 9th positions of the sixth repeat, and at the 9th position of the seventh repeat. γ111 has two base changes, both outside of the repeats, one near the *Hin*dIII site and one near the *Bgl* II site.

π DNase Protection of γ-Origin Mutants. The seven direct repeats within the γ origin are known to be essential for R6K replication and to be binding sites for the π protein (6, 7). It was therefore of interest to determine whether or not any of the defective mutants had an altered affinity for π protein at one or more of their repeat sequences. To address this question DNase I protection experiments were performed with π bound to ^{32}P-end-labeled fragments from each γ-origin mutant. As shown in Fig. 3, γ111, which has unaltered repeats,

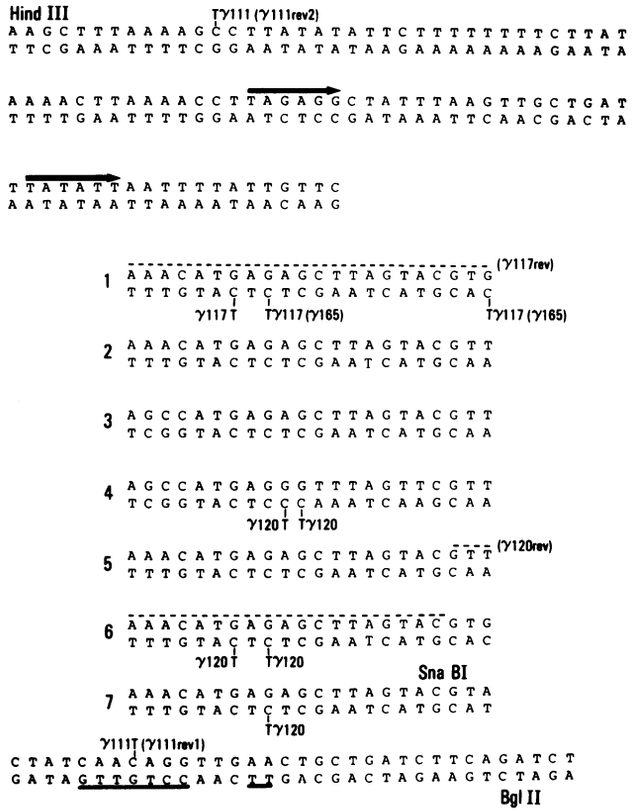

FIG. 2. Nucleotide sequence of the R6K γ origin and the positions of base changes in γ117, γ120, γ165, γ111, and revertants of γ111. The bases deleted in the γ117 revertant and the γ120 revertant are shown under the dashed lines. The underlined bases are the conserved sequence found in other repeat-bearing plasmids (see *Results*). The arrows represent the −35 and −10 regions of an RNA polymerase binding site (32).

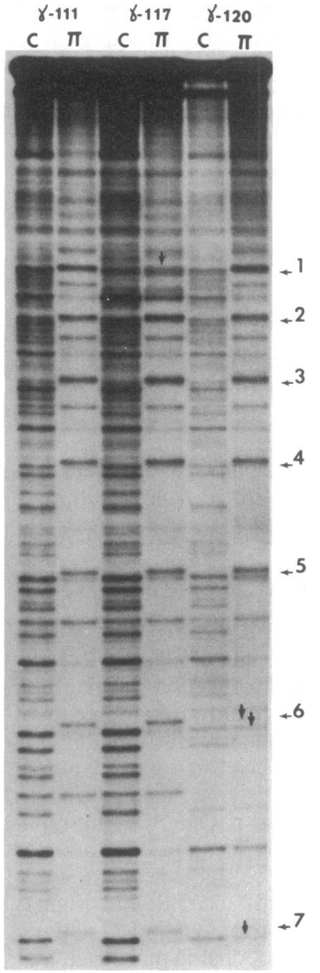

FIG. 3. DNase protection patterns of π bound to the repeat region of γ111, γ117, and γ120. The *Eco*RI–*Bgl* II fragment containing the γ origin of each mutant, labeled with ^{32}P on the 5′ end at the *Bgl* II site, was preincubated with purified π protein and subjected to a limited DNase I digestion. Samples marked with C are control DNase digestions with no added π. Samples marked with π were preincubated with 20 ng of π protein for 15 min at 37°C prior to DNase treatment. Numbered arrows point to the prominent π-induced DNase cleavage enhancement produced in each repeat. Vertical arrows show where mutants have altered protection patterns.

displays the protection pattern characteristic of a wild-type origin—that is, π binding strongly and equally to each of the seven repeats (6, 7). γ117 with three mutations in the first repeat is unable to bind π protein at this repeat while showing normal binding to the remaining six repeats. γ120 is unable to bind π at its sixth repeat (containing mutations at the 7th and 9th positions) and shows weaker protection and hence weaker binding at its seventh repeat (containing a single mutation at the 9th position) but shows an unaltered protection pattern for the other five, including the fourth repeat that contains two mutations at the 10th and 11th positions, respectively. A common feature of the repeats of γ117 and γ120 that are unable to bind π is the presence of C → T transitions at the 7th and 9th positions of their respective repeats. These two positions within each repeat are among those that are known to interact with a π–β-galactosidase fusion protein (6).

Isolation and Characterization of Revertants of γ117 and γ120. To understand which base change or changes are responsible for preventing π binding and for inactivating γ-origin function, revertants of γ117 and γ120 were isolated after nitrous acid mutagenesis by selecting for the ability of the mutant to replicate in strain *polA214*-Ts (pMM4) at 43°C as described in *Materials and Methods*. One revertant of γ117 and six independent revertants of γ120 were isolated in this manner. In every case the revertant contained a deletion of a 20- to 25-bp segment. Because a C → T transition at the ninth position of a repeat, as in the first repeat of γ117 and the sixth and seventh repeats of γ120, creates a *Hin*dIII restriction site it was possible to use *Hin*dIII digestion to analyze these revertants. The γ117 revertant no longer contained the newly generated *Hin*dIII site, and all of the γ120 revertants lost one of the two *Hin*dIII sites acquired by the mutations. This strongly suggested that the γ117 revertant contained a deletion of the damaged first repeat and that the γ120 revertants contained deletions of either their sixth or their seventh repeat. The γ117 revertant and one of the γ120 revertants were sequenced in order to confirm this (Fig. 2). The γ117 revertant was, in fact, found to be a precise 22-bp deletion of the damaged first repeat, the end points of which cannot be exactly determined due to the degeneracy of the repeats. The γ120 revertant was found to be a perfect deletion of the damaged sixth repeat, strongly suggesting that the mutations in the sixth repeat that abolish π binding are the mutations responsible for inactivating the origin of γ120. To obtain a point revertant of γ117 another strategy was adopted. Because the base change in the 9th position in the first repeat of γ117 creates a *Hin*dIII restriction site it became theoretically possible to remove a 102-bp *Hin*dIII fragment that contained two of the three base changes of γ117 and replace that fragment with an equivalent fragment that has only the base change that creates the *Hin*dIII site. To obtain such a replacement fragment, random clones of pMM3 from 10 min of bisulfite treatment were screened for containing an extra small *Hin*dIII fragment. A mutant with a *Hin*dIII fragment of 102 bp in size, whose extra site mapped to the first 22-bp repeat, was isolated and designated γ164. This mutant, which contains a functional γ origin, was digested with *Hin*dIII and its 102-bp fragment was used to replace the 102-bp *Hin*dIII fragment of γ117. The resulting plasmid, pMM3 γ165, was sequenced and found to contain two base changes: C → T transitions at the 9th and 22nd positions of the first repeat. γ165, therefore, is equivalent to a point revertant of γ117 with the 7th position of the first repeat now containing the wild-type base pair. The origin activity and π binding

ability of this revertant were then examined. It was found that γ165 does contain a functional γ origin but that its copy number, like that of γ164, appeared to only be about one-half that of a wild-type pMM3 (data not shown). DNase protection experiments demonstrated that the addition of π protein to the γ165 origin fragment did not lead to full protection of the first repeat against DNase cleavage; however, it did lead to partial protection. A DNase protection gel comparing γ117, γ165, and wild-type pMM3 is shown in Fig. 4. We conclude that the first repeat of γ165 is capable, albeit weakly, of interacting with π protein *in vitro*. It can be concluded that the mutation at the 7th position of the first repeat in γ117 plays a role in both the loss of π binding to that repeat and in the loss of origin function.

The γ111 Mutation. γ111, the mutant containing two base changes outside of the repeats (base pair positions 11 and 254, respectively) binds π protein at each of its repeats, suggesting that it represents another class of origin-defective mutants. To determine which of two base changes in γ111 is responsible for the greatly reduced copy number it was necessary to separate the two mutations. This was accomplished by making use of the restriction enzyme *Sna*BI that cleaves pMM3 once at a location between the two γ111 base changes (see *Materials and Methods*). γ111rev1, which contains only the mutation at bp 254, was found to have a copy number in C2110 (pMM4) slightly higher than the γ111 mutant but still well below that of wild-type pMM3. γ111rev2, which contains only the mutation at bp 11, was found to have a copy number indistinguishable from wild-type pMM3. Although the mutation at bp 11 apparently contributes to the phenotype of γ111, it is clear that the mutation at bp 254 is largely responsible for the poor replicating ability of the mutant, indicating that the short region between the seventh repeat and the *Bgl* II site is important for replication of the γ origin.

When the sequence of this region of R6K was compared to other plasmid origin sequences it was found that several of the *E. coli* plasmids that contain repeats within their origin share a short stretch of apparent homology adjacent to the repeats. Fig. 5 shows the location and sequence of this homology for six different plasmids. The apparent consensus

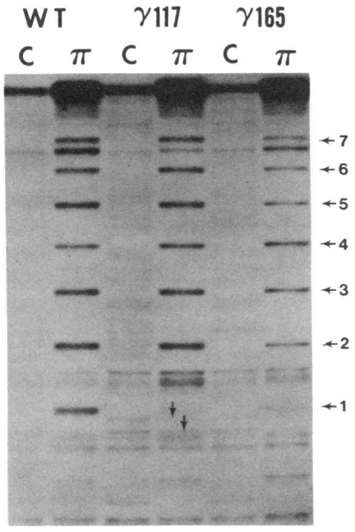

FIG. 4. DNase protection patterns of γ117 and its point revertant γ165. The *Eco*RI–*Bgl* II γ-origin fragments of wild-type pMM3, γ117, and γ165 were 5′ labeled at the *Hin*dIII site. Samples marked with C were not preincubated with π protein and samples marked with π were preincubated with 50 ng of π for 15 min at 37°C prior to DNase cleavage. Arrows indicate bands within the first repeat that are protected from DNase I cleavage by π protein in γ165 but not in γ117.

R6K	t a c t a t C a A C A G G t t g A A c t g c t
R485	g t a c a t C C A C A G G c t a A A a a a a t
pSa	a c c c g g a g A C A G G c a t A A t a a t a
RK2	g g c t g t C C A C A G G c a g A A a a t c c
Rts1	g c c a a a C C A C A G G c a c A c t t a t t
F	a a t t t g t C A C A G G g t t A A g g g c a

FIG. 5. Short conserved sequence present adjacent to the nucleotide repeats within the origin of several plasmids. The base altered in γ111 rev1 is underlined. The sequences were taken from refs. 26 (R6K), 33 (pSa), 11 (RK2), 13 (Rts1), and 9 (F). The sequence for plasmid R485 was obtained by D. Stalker (unpublished observations).

sequence of this homology is C-C-A-C-A-G-G-N-N-N-A-A with the mutation of γ111 occurring at the 4th position of the R6K sequence. In the case of each plasmid examined the position of this A-C-A-G-G sequence is adjacent to the last repeat of the replication origin of its respective plasmid, except for the F plasmid, where the A-C-A-G-G sequence occurs between two of the direct repeats.

DISCUSSION

We have generated a bank of mutations within the γ origin of plasmid R6K using sodium bisulfite mutagenesis. This involved construction of circular DNA molecules specifically gapped at the γ origin followed by exposure of these molecules to the single-strand-specific mutagen sodium bisulfite. The objective for constructing this mutant bank was to obtain a large number of both single and multiple point mutations within the γ origin to insure that mutants with altered replication and incompatibility properties would be obtained. The mutant bank that was generated contains ≈1 million clones representing seven different times of bisulfite treatment. Ideally this bank would contain every possible single C → T transition as well as a large variety of multiple transitions, all confined to the γ origin. It is possible, however, that certain base changes are absent in the mutant bank due to the formation of bisulfite-resistant secondary structures within the single-stranded region of the gapped circles. Although we cannot rule out this possibility, extensive stem loop structures are not likely to be formed within the mutagenized region and therefore a great majority of the possible mutations are probably represented in this mutant bank.

We have screened the pMM3 mutant bank for clones that have a diminished ability to replicate using the R6K γ origin. Three such defective γ-origin mutants were isolated and characterized. Two of these mutants, γ117 and γ120, are unable to replicate in an *E. coli polA*$^-$ *pir*$^+$ strain and, therefore, contain nonfunctional γ origins. DNase I protection analysis demonstrated that the first 22-bp repeat of γ117 and the sixth 22-bp repeat of γ120 are unable to bind purified π protein. Each of these mutants was found to have C → T transitions at the 7th and 9th positions of their damaged repeats. Reversion of the mutation at the 7th position of the first repeat of γ117 restores origin activity as well as partial π binding to that repeat. This demonstrates that the mutation at the 7th position of the first repeat in γ117 is involved in the loss of both π binding and origin functionality. However, data from other mutants indicate that a C → T transition at the 7th position of other repeats by itself is insufficient to eliminate either origin activity or π binding to that repeat (unpublished observations). Therefore, alteration of the G-C base pairs at both the 7th and 9th positions of the first repeat of γ117 and the sixth repeat of γ120 appear to be necessary for the loss of either origin activity or π binding.

Isolation of revertants of γ117 and γ120 demonstrated that precise deletion of either the damaged first repeat of γ117 or

the damaged sixth repeat of γ120 restored γ-origin function. This strongly suggests that of the five base changes in γ120, the two in the sixth repeat that disrupt π binding are the most crucial. These revertants, as well as earlier deletion mutants, demonstrate that the γ origin does not require all seven repeats in order to function (8, 34) and that neither the first nor the sixth repeat is absolutely essential. This suggests that the primary defect of γ117 and γ120 can be viewed as a disruption of the normal spacing requirements of the γ origin. γ117, for example, could be viewed as having a nonfunctional repeat spacer between six functional repeats and essential neighboring sequences of the origin. Interestingly, there is a relatively weak promoter immediately adjacent to the first repeat that transcribes in the direction of the repeats (32). The role that this promoter plays in replication is unknown; however, a rifampicin-sensitive transcriptional step is known to be required for R6K *in vitro* replication (3). These facts combined with recent genetic evidence for an interaction between π and RNA polymerase (unpublished data) suggest the possibility that the primary defect of γ117 may be the inability of π to properly interact with RNA polymerase when each is bound to the origin region. Deletion of the damaged first repeat or point revertants such as γ165 that restore partial π binding would be expected to again allow π and RNA polymerase to interact.

The γ120 mutant also appears to have a disrupted spacing requirement, though probably of a nature that is different than the γ117 mutant since there is no disruption of the region near the promoter. At least two possibilities are worth considering. One possibility is that the binding of π protein to the γ origin must be contiguous over all of the repeats if the origin is to be functional. An argument against this is that γ120 contains five contiguous repeats that are functional at binding π that in turn are properly spaced with upstream origin sequences. Origins with five repeats have been shown to still be active. A second possibility is that π protein must interact with a host protein that binds specifically at a region adjacent to the seventh repeat. In favor of this hypothesis is the presence of a short conserved sequence adjacent to this repeat that was identified by analysis of the γ111 mutant. An argument against is the fact that π can bind, albeit weakly, to the seventh repeat of γ120. These two possibilities are not mutually exclusive, and it may be that each plays a role in the defectiveness of γ120. Yet another factor that conceivably could be important for the γ117 and γ120 mutants is the role of adjacent repeats. If the binding of π to the seven direct repeats is cooperative it might be expected that the ability of π to bind to a mutant repeat would be influenced by π bound at adjacent repeats. It is worth noting that the damaged repeat of γ117 is bordered by another repeat on only one side and that the damaged sixth repeat of γ120 is bordered by a mutant seventh repeat that binds π poorly.

Though the data from γ117, γ120, and their revertants indicate a tight correlation between the ability of π to bind to a repeat and origin functionality, other explanations for the loss of origin function in these mutants cannot be ruled out. The C → T transitions at the 7th and 9th positions of the repeats that are associated with loss of π binding also fall within a conserved hexanucleotide sequence that is present in the repeats of a number of different plasmids (7). It is therefore possible that a host-encoded protein also binds tc the direct repeats of R6K and that γ117 and γ120 are defective because thay have improper spacing between this bound hypothetical host factor and nearby origin sequences.

The third mutant that we have analyzed, γ111, is capable of replication but maintains itself at a greatly reduced copy number. The two base changes of the original mutant have been separated and the properties of the single mutants indicate that the C → T transition near to, but outside of, the seventh repeat is primarily responsible for the observed phenotype of γ111. This base change falls within a short nucleotide sequence that appears to be present adjacent to the nucleotide repeats within the origin of several other plasmids (Fig. 4). This suggests that this sequence might be a binding site for a host-encoded protein important for the replication of R6K and perhaps other plasmids as well.

The mutants described here contain γ origins that are partially or completely defective at replication. It will be necessary to investigate the effect that these mutations have on the replicative ability of the two primary *in vivo* origins of R6K, α and β, to further determine the sequence requirements of these origins.

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