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Negative control of plasmid R6K replication: Possible role of intermolecular coupling of replication origins

(initiation protein/*Escherichia coli*/direct repeats/nucleoprotein/copy number)

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ABSTRACT **The γ origin binding sites of the replication initiator π protein, composed of seven 22-base-pair (bp) direct repeats and previously shown to be essential for replication of plasmid R6K, can also act as an inhibitor of R6K replication in *Escherichia coli* cells if provided in trans. Inhibition is dependent upon the ability of these repeats to bind the R6K-encoded π protein but is not overcome by increasing the intracellular π level. The insertion of a second repeat cluster in close proximity to the γ origin also can markedly inhibit replication. The severity of this effect is dependent upon the position, orientation, and number of repeats present in the extra cluster. As few as six extra repeats can result in a completely nonfunctional γ origin. However, this inactive γ origin plasmid containing the six extra repeats is functional when placed in a strain that underproduces the wild-type π protein or when placed in the presence of any of several copy-up mutant π proteins. On the basis of these observations, we propose that the nucleoprotein structures formed by the binding of π protein to the seven 22-bp direct repeats at the γ origin are capable of coupling with each other *in vivo* and that replication initiation is prevented at such coupled origins. In support of this model of replication control, we demonstrate by electron microscopy analysis that the π protein has the ability to associate two DNA molecules containing γ origin sequences and also show that π enhances the DNA ligase-catalyzed multimerization of a DNA fragment containing the γ origin.**

Plasmid R6K is a 38-kilobase-pair (kb) self-transmissible *Escherichia coli* plasmid that can replicate from any of three origins, termed α, β, and γ, to a characteristic copy number of 15–20 per chromosome (1, 2). Replication from any one of these origins requires the presence in cis of an approximately 300-base-pair (bp) region from the centrally located γ origin as well as the R6K initiator protein, known as π, which can be provided in trans (3–5). The γ origin and the π protein, encoded by a gene (*pir*) located between the γ and β origins, together make up a minimal functional R6K replicon (6). The π protein binds to the seven 22-bp direct repeats (iterons) within the essential γ origin region and to an eighth direct repeat plus a pair of smaller inverted repeats that partially overlap the promoter of its own gene (7, 8). Binding of π to the γ origin and to the promoter region has been shown to be essential for origin activity and for π autoregulation, respectively (9–11). While necessary for initiation, the π protein has also been shown to inhibit R6K replication when overproduced (12, 13). The isolation of recessive π mutants that produce a greatly elevated copy number of an R6K replicon without changing the intracellular π level indicates that involvement in the negative regulation of replication is a normal function of the π protein *in vivo* (8, 14).

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Like the π protein, the direct repeats of the γ origin are also known to be capable of inhibiting R6K replication. When inserted into cloning vectors such as pBR322, the R6K γ origin, but not the α and β origins, interferes with the replication of R6K-derived replicons in *E. coli* cells (4, 15). The 22-bp direct repeats of the γ origin are necessary for this incompatibility; as the number of repeats is decreased, the degree of replication inhibition also decreases (15). On the basis of observations presented in this study we propose that the negative regulation of replication by π protein and the direct repeats is due to the ability of the π protein to mediate the association of repeat clusters at the origins of replication of plasmid molecules, resulting in intermolecularly coupled plasmids that are blocked in their ability to initiate replication.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids. The *E. coli* strains used in this study were C600 (*thr, leu, thi, supE46, lacY, tonA*) and C2110 (*polA1, his, rha*). The following plasmids have been previously described: pRK419 (16); pMM3, pMM4 (9); pPR1, pPR1Δ6, pPR1Δ6R1, pPR1Δ6R3, pPR1Δ10, pPR1Δ14 (13); and pMF34 (8). Plasmids pMM18 and pMM20 were constructed by removing a 132-bp *Sna*BI fragment from pMM3γ117 (9) and inserting it into the *Hin*cII site of pUC7. Plasmid pMM18 contains a single copy of this fragment and pMM20 contains two tandem copies of this fragment. Plasmid pPR1Δ22 is a BAL-31 deletion derivative of pPR1 that still contains the *pir* promoter and the nearby eighth direct repeat. The six derivatives of pPR1Δ22 containing mutant *pir* genes were constructed by the replacement of a 307-bp *Eco*RI*–*Bgl* II fragment (base pairs 497–804; ref. 17) with the equivalent fragment isolated from the original plasmid source of each *pir* mutant.

Electron Microscopy of π·DNA Complexes. The binding reactions were set up with 200 ng of DNA and 200 ng of purified π protein in a buffer consisting of 20 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 100 mM KCl, and 5 mM $MgCl_2$. The final reaction volumes were 10 μl. The reaction mixtures were incubated for 15 min at 33°C. Glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) was added to a final concentration of 0.1% and the reactions continued for 15 min at 33°C. The fixed samples were then diluted 1:50 in water and applied to carbon-coated microscopy grids rendered hydrophilic by treatment with alcian blue (18). The grids were then rotary-shadowed with tungsten wire and examined by using a Philips EM300 electron microscope. Reactions involving RNA polymerase holoenzyme were set up as described for π protein reactions except that RNA polymerase (gift from G. A. Kassavetis and E. P. Geiduschek; University of California, San Diego) was added at a concentration of 2 μg per 10-μl reaction volume.

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RESULTS

Origin Mutations That Weaken Incompatibility Also Contain Changes Known to Weaken π Binding. As a first step to determine the mechanism by which the 22-bp repeats of the γ origin interfere with R6K replication, we isolated γ origin mutants that display a decreased ability to block replication. A previously described sodium bisulfite-induced mutant bank of the γ origin was used as a source of the incompatibility-deficient mutants (9). This mutant bank was constructed by using the plasmid pMM3, which contains most of pBR322, including its origin of replication and the penicillin resistance determinant, and an ≈700-bp segment of R6K including the functional R6K γ origin region. When present in *polA*$^+$ cells, where it is maintained at the relatively high copy number characteristic of the pBR322 origin, pMM3 expresses a sufficient level of incompatibility to completely exclude the mini-R6K replicon pRK419. By selecting for pMM3 clones that could be comaintained with pRK419, it was possible to isolate a large number of mutants from the mutant bank that displayed weakened incompatibility.

These mutants, designated Inc$^{+/-}$, contained mutations in the direct repeats of the origin that are expected to reduce the ability of π to bind to the origin. A G-to-A transition at the ninth position in a repeat has previously been shown to reduce the ability of π to bind to that repeat (9). A G-to-A transition at the ninth position of any but the fourth repeat also results in the creation of a *Hin*dIII restriction site. Therefore, by digesting DNA from a pool of Inc$^{+/-}$ mutants with *Hin*dIII it was possible to readily monitor the occurrence of this type of mutation that weakens π binding. As seen in Fig. 1, a large majority of Inc$^{+/-}$ mutants contain new *Hin*dIII sites (lane I), a percentage considerably higher than that of the mutant bank as a whole (lanes 0, 5, 10, 20, 40, and 90). DNase protection and gel retardation experiments have

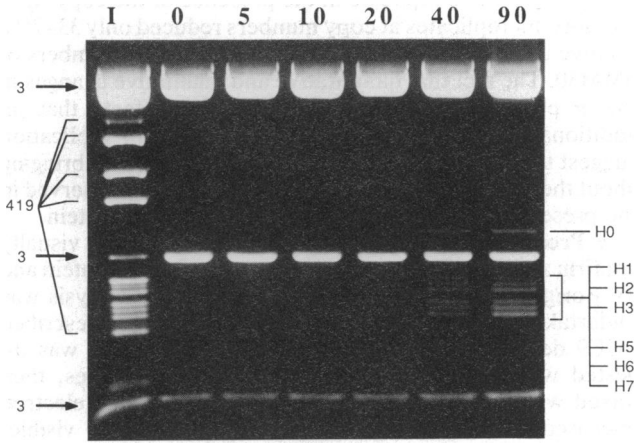

FIG. 1. Detection of extra *Hin*dIII sites in total sodium bisulfite-treated pMM3 DNA and in Inc$^{+/-}$ pMM3 DNA. A pool of Inc$^{+/-}$ pMM3 DNA was generated by transforming C600(pRK419) with pMM3 DNA from the 20- and 40-min time points of the bisulfite mutant bank (9), mixing together the resulting hundreds of penicillin-resistant, kanamycin-resistant colonies, and isolating the plasmid DNA from the mixture. The DNA from this Inc$^{+/-}$ mutant pool (labeled I) was then cleaved with a combination of *Hin*dIII and *Eco*RI and subjected to electrophoresis on a 1.2% agarose gel alongside similarly digested DNA from six time points (given in minutes above each lane) of the bisulfite bank. The fragments produced by wild-type pMM3 are indicated by 3. Fragments labeled H1, H2, H3, H5, H6, and H7 arise as a result of transitions that create *Hin*dIII restriction sites as described in the text, the number referring to the repeat in which the new *Hin*dIII site resides. The fragment labeled H0 occurs as a result of one of two possible C-to-T changes that eliminate the one natural *Hin*dIII site at position 1 (17), and 419 refers to fragments of the plasmid pRK419.

directly demonstrated that individual Inc$^{+/-}$ mutants contain repeats that are less able to bind π protein (data not shown).

Increasing the Level of π Does Not Suppress Incompatibility. One possible mechanism to account for the interference with R6K replication by high intracellular levels of repeats is by binding to the π protein. The following experiment was carried out to determine whether or not increasing the intracellular π would result in a decrease in the incompatibility expressed by plasmid pMM3 against derivatives of R6K. C600 (*polA*$^+$) cells containing the mini-R6K replicon pRK419 alone or in combination with any of several RK2 replicon plasmids that overproduce π to various levels were transformed with plasmid pMM3 to determine if pRK419 and pMM3 could be comaintained. The RK2 replicon plasmids produced a level of π that was equivalent to the wild-type level (pMM4), or 2.25-fold (pPR1Δ6R1), 4.5-fold (pPR1Δ6R3), or 9.0-fold (pPR1Δ6) above normal (13). Since the extra plasmids replicate by means of an RK2 replicon they are fully compatible with pMM3 and pRK419. It was observed that the higher levels of π produced by the RK2 derivatives do not suppress the Inc$^+$ phenotype of pMM3. These data suggest that R6K incompatibility cannot be overcome by increased levels of the initiator protein and together with the fact that the π concentration is not the rate-limiting factor for γ origin replication (13) argue strongly against a simple titration model of replication control.

Addition of a Second Repeat Cluster in Cis Near to the γ Origin Can Markedly Inhibit Replication. The fact that a γ origin repeat cluster inserted into another plasmid can inhibit the replication of an R6K replicon implies that the repeats can play a trans-acting regulatory role. As an initial test of a possible physical association between repeat clusters, we constructed a series of R6K derivatives that contain a second cluster of repeats in cis. If physical association of repeat clusters occurs and can inhibit replication, the presence of two clusters linked in close proximity to each other might inactivate the origin or lead to a severe reduction in plasmid copy number.

To obtain such plasmids, the *Eco*RI fragments containing 6 or 12 γ origin direct repeats, derived from pMM18 and pMM20, respectively, were cloned in either of the two *Eco*RI sites flanking the R6K sequences of pMM3. In addition to obtaining both orientations of each fragment at each location, we also obtained clones containing double inserts at one site or the other. These derivatives were isolated from a *polA*$^+$ *E. coli* strain and then used to transform the *polA*$^-$ strain C2110 carrying pMM4 (an RK2 replicon plasmid encoding wild-type π) to test how well the γ origin of each derivative replicates. In a *polA*$^-$ background the pBR322 origin is unable to function, so that replication of pMM3 and its extra-repeat derivatives is dependent upon a functional R6K γ origin. The copy number of the γ origin plasmid was estimated by plating each transformation onto LB plates containing different concentrations of penicillin G. The level of penicillin resistance is known to be proportional to the gene dosage of the β-lactamase gene that is present on pMM3 and can be used to estimate plasmid copy number (19). That the level of penicillin resistance reflects copy number of the pMM3 derivatives has been confirmed by measuring the level of plasmid DNA in cell extracts (data not shown). The first conclusion that can be derived from the penicillin resistance results (Table 1) is that the extra repeats have little or no effect when replication occurs in the *polA*$^+$ strain C600 in the presence or absence of π protein [results with C600 and C600(pMM4)]. However, when replication occurs by means of the R6K γ origin [C2110(pMM4)], all the derivatives containing extra repeats display a markedly reduced copy number compared to pMM3 (a minimum of 3-fold down). A second point is that increasing the number of additional repeats from 6 to 12 at a given site and in a given orientation

Table 1. Relative copy numbers of extra-repeat derivatives of pMM3 at three levels of π

Plasmid	Location of extra repeats*	Orientation of extra repeats†	Number of extra repeats	MIC of penicillin G, μg/ml			
				C600 (*polA*$^+$)	C2110 (*polA*$^-$)		
				pMM4‡ (100%)	pMM4 (100%)	pPR1Δ14 (≈1–2%)	pPR1Δ10 (≈0.5%)
None	—	—	—	20	60	60	60
pMM3	—	—	—	1200	1200	400	200
pMM30	Site 1	Direct	6	1200	400	200	150
pMM31	Site 1	Inverted	6	1000	60	150	80
pMM32	Site 2	Direct	6	1000	400	200	150
pMM33	Site 2	Inverted	6	1200	300	200	100
pMM34	Site 1	Direct	12	1200	200	150	80
pMM35	Site 1	Inverted	12	1000	60	80	60
pMM36	Site 2	Direct	12	1200	200	150	80
pMM37	Site 2	Inverted	12	1200	100	100	60
pMM38	Site 2	Direct	6+6§	1000	200	150	80
pMM39	Site 1	Inverted	12+12	1000	60	60	60
pMM40	Site 2	Inverted	12+12	1200	60	60	60

Relative copy numbers of pMM3 and derivatives of pMM3 containing extra γ origin iterons were estimated by the minimum inhibitory concentration (MIC) of penicillin G. Each plasmid was used to transform strain C2110 (*polA*$^-$) containing one of three plasmids that produce different intracellular levels of π protein (given in parentheses as a percentage relative to the wild-type level) and plated on LB plates containing different concentrations of penicillin G. A 95% or greater reduction in the number of colonies (relative to fully permissive penicillin levels) was considered inhibitory.

*The locations of the insertions of the extra-repeat clusters were at the two *Eco*RI sites of pMM3 (9). Site 1 is designated as the site nearest the gene encoding β-lactamase.

†Orientation is given relative to the γ origin repeats of pMM3.

‡A similar level of resistance was observed for pMM3 and its derivatives in C600 (*polA*$^+$) in the absence of pMM4.

§6+6 differs from 12 in having a portion of the pUC7 polylinker sequence between the two sets of six iterons. In each of the three double inserts shown, the orientation of both clusters is the same.

further decreases the copy number of the replicon. Third, the position and orientation of a given fragment can influence the copy number considerably. The most striking example of this can be seen by comparing the copy numbers of pMM30 and pMM31. These two plasmids represent the two orientations of the six-repeat fragment inserted at site 1. Whereas pMM30 replicates at a copy number as high as any of the extra-repeat derivatives, pMM31 is completely inactive as a γ origin. These results indicate that the degree of inhibition stemming from the repeats is influenced by more than simply their dosage within the cell and are consistent with the possibility that physical association between repeat clusters is involved in the inhibitory properties of the repeats.

Suppression of the Reduced Replication Ability of Extra-Repeat Plasmids. In addition to examining the copy number of the extra-repeat derivatives of pMM3 in a strain producing a normal level of π protein, we have also examined their ability to replicate in backgrounds where the π level is reduced 50- to 200-fold below normal, levels known to be permissive for γ origin replication (13). As shown in Table 1, for pMM3 in strain C2110 a decrease in the π level to ≈1–2% and ≈0.5% of normal reduces penicillin resistance to approximately one-third normal and one-sixth normal, respectively. Although in general the extra-repeat derivatives of pMM3 decrease in copy number with decreasing π levels, the orientation of the extra-repeat cluster appears to have less of an effect on copy number at lower concentrations of π. The most extreme example of this is pMM31, which is completely nonfunctional at the normal π level but functional at greatly decreased π levels.

An even more striking suppression of replication inhibition is observed when the pMM31 construct is placed in cells producing a copy-up mutant π protein. pMM3 and the extra-repeat derivatives pMM30 and pMM31 were used to transform strain C2110 (*polA*$^-$) containing a compatible plasmid (RK2 replicon) that provided either wild-type or any one of six high-copy mutant π proteins [encoded by *pir1*, *pir13*, *pir104*, *pir116*, *pir200*, and *pir405*cos (20)]. The plasmids encoding the π protein contain the *pir* gene under control of its natural promoter and differ from one another only by the presence of the mutations in the *pir* gene (20). On the basis of the level of penicillin resistance, it was found that pMM31 not only is able to replicate in the presence of the copy-up π mutants but replicates at copy numbers reduced only 33–75% relative to pMM3 and close or equal to the copy numbers of pMM30. The fact that quantitative and qualitative changes in the π protein can significantly alter the effects that an additional repeat cluster can have on γ origin replication suggest that the π protein plays an important role in bringing about the position and orientation effects that are observed in the presence of normal amounts of wild-type π protein.

π Protein Can Couple γ Origin Sequences. To visually confirm and characterize the interactions of the π protein and its γ origin binding sites, electron microscopic analysis was undertaken. When plasmid pMF34, a previously described pUC9 derivative containing the R6K γ origin (8), was digested with any one of several restriction enzymes, then mixed with the π protein and observed under the electron microscope, protein-bound fragments were clearly visible. Depending upon the particular experiment, 21–88% of the fragments containing the γ origin had protein bound to them at the position expected for binding to the γ origin repeats (Fig. 2 *A* and *C*). No π binding was observed on DNA fragments that did not contain the γ origin repeats. In addition to observing individual γ origin fragments bound by π, we found that most (364 of 405 molecules) of the γ origin fragments with π protein bound to them were present as paired structures joined together by protein (Fig. 2 *B*, *D*, and *E*). The region of pairing corresponded to the locations of the direct repeats on the fragments. In a parallel experiment using RNA polymerase and a restriction fragment containing a promoter, doublets were not detected among 449 DNA·protein complexes observed.

The coupling ability of π is also apparent from the results of the addition of purified π protein to a reaction mixture containing T4 ligase and R6K repeat-containing fragments.

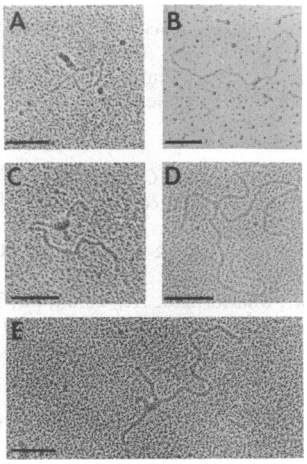

FIG. 2. Electron micrographs of $\pi\cdot\gamma$ origin complexes. Nucleoprotein complexes between repeat-containing fragments and wild-type π were formed as described in *Experimental Procedures*. The fragments used are as follows: a 1149-bp *Taq* I fragment from pMF34 with the repeats located at one end (*A, B*); a 1967-bp *Bgl* I fragment with the repeats located 266 bp from one end (*C, D*); and a mixture of the 1967-bp *Bgl* I fragment and a 691-bp *Pvu* II fragment from pMF34 with the repeats located 241 bp from one end (*E*). Single π-bound molecules are shown in *A* and *C*. Paired molecules are shown in *B, D,* and *E*. The bars equal 0.1 μm. In separate experiments with π-bound, repeat-containing restriction fragments, approximately 96% of *Hae* II molecules (133 fragments examined), 85% of *Taq* I molecules (163 fragments examined), and 90% of *Bgl* I molecules (109 fragments examined) were observed to be paired, with the remainder present as single molecules.

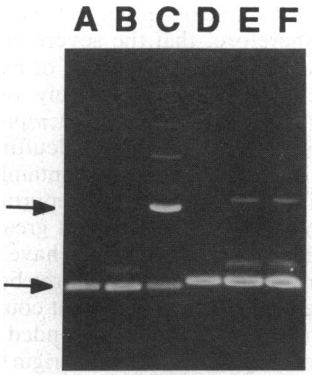

FIG. 3. Enhancement by π protein of ligase-catalyzed multimer formation of repeat-containing fragments. Lanes A–C show a purified 691-bp *Pvu* II restriction fragment (1 μg) containing the 22-bp repeats of the γ origin from plasmid R6K with no protein (lane A), after ligase treatment (lane B), and after ligase treatment in the presence of π protein (lane C). Lanes D–F show a purified 714-bp *Pvu*II restriction fragment (1 μg) from plasmid RK2 containing the set of five 17-bp repeats of the RK2 origin (21), with no protein (lane D), after ligase treatment (lane E), and after ligase treatment in the presence of π protein (lane F). The arrows indicate the positions of monomer (lower) and linear dimer (upper) forms of the *Pvu* II fragments. The faint band above the monomer linear form is covalently closed circular monomer. Ligations were carried out in a 500-μl reaction mixture containing 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 30 mM NaCl, 1 mM ATP, 20 mM dithiothreitol, gelatin at 100 μg/ml, 10 units of T4 ligase, and, where indicated, 1 μg of purified π protein. Reactions were carried out at 30°C for 30 min, and the products were extracted once with phenol/chloroform (50:50, vol/vol) and once with chloroform and precipitated with ethanol before loading on a 1.0% agarose gel. Electrophoresis was carried out at room temperature for 2 hr at 9 V/cm and the gel was stained with ethidium bromide.

As shown in Fig. 3, the addition of π protein substantially increases the formation by T4 DNA ligase of higher multimer forms (mostly dimers) of a blunt-ended 691-bp *Pvu* II restriction fragment containing the 22-bp repeats of the R6K origin. We interpret these results to mean that π, through its ability to couple two DNA fragments, increases the localized concentration of free DNA ends, thereby promoting efficient fragment ligation to form multimers. Under the same conditions this increased formation of multimers is not observed when purified π protein is added to a 714-bp *Pvu* II fragment containing a functional set of five 17-bp direct repeats of the origin region of the heterologous plasmid RK2. The electron microscopy and ligation data provide evidence that the pairing of γ origin fragments by π protein is in fact a specific property of $\pi\cdot\gamma$ origin complexes and lend considerable credibility to the possibility that repeat-mediated inhibition of replication is due to physical association between repeat clusters.

DISCUSSION

The goal of the work described here was to gain further insight into the mechanism by which plasmid R6K regulates its replication. The analysis of γ origin mutants that display a decreased ability to express R6K incompatibility indicated that these $Inc^{+/-}$ mutants are highly enriched for the presence of mutations known to weaken the ability of π to bind to the affected repeat (8). This suggests that the ability of high levels of repeats to interfere with R6K replication is a function of the repeats' ability to bind π protein.

In addition, we observed that different placements and orientations of a cluster of six extra repeats in a γ origin replicon can result in widely varying degrees of replication inhibition, ranging from a fewfold decrease in copy number to a complete blockage of replication. This observation is not easily reconcilable with any of the titration models (15, 22–24) proposed for plasmid replication control. These data, as well as the other properties of these extra-repeat γ origin derivatives, are better explained by a mechanism of regulation of initiation of plasmid replication that we term the "handcuffing" model (25). This model proposes that an R6K plasmid molecule with π bound to the direct repeats of the γ origin is replication-proficient, but that the coupling of two such molecules by π–π interactions blocks initiation of replication of each of the participating plasmids. Such replicon association, or "handcuffing," would be expected to be favored at high copy number and disfavored at low copy number, thereby providing a means for the plasmid to adjust to fluctuations in copy number and maintain a constant rate of initiation of replication.

Coupling between repeat clusters might also be favored by physically linking two repeat clusters, such as occurs when a second repeat cluster is cloned in close proximity in cis to the native γ origin. For example, the extreme replication inhibition that is seen with the extra-repeat derivative pMM31 may be due to the cis association of the two repeat clusters present on the plasmid. The different degrees of replication inhibition observed with the different extra-repeat derivatives would be expected according to the handcuffing model, since the ability to undergo cis association might be highly dependent upon such factors as the relative orientation of the two repeat clusters and the distance between the clusters. The fact that plasmid pMM31, and to a lesser extent pMM35, become functional in the presence of a reduced π level might be expected according to the handcuffing model. Since the cis association of π-repeat complexes requires that both repeat clusters have π bound to them, it is possible that in the presence of a low π level the origin cluster of repeats binds enough π for initiation of replication but not enough for cis association. Copy-up π mutants would be expected to have altered protein–protein interactions and be deficient at

coupling π-repeat complexes either in trans or in cis. It was not surprising, therefore, that the severe effect on γ origin replication caused by a nearby cluster of extra repeats was observed to be partially or completely overcome in the presence of any of six copy-up mutant π proteins.

An important prediction of the handcuffing model is that the addition of π protein to DNA containing the γ origin should result in the formation of nucleoprotein pairs. Electron microscopy analysis revealed that greater than 80% of the γ origin fragments that were seen to have π protein bound to them were present as paired structures bound together in the region of the repeats, indicating that coupling can occur. The effect of π on the ligation of blunt-ended DNA fragments carrying the direct repeats of the R6K origin (Fig. 3) provides further evidence for the specific coupling activity of the π protein. Earlier reports that π can bring together two γ origin regions as assayed either by an enhancement of topoisomerase-induced plasmid concatemerization (26) or by electron microscopy (27) are also supportive of the handcuffing model as presented here. Pal and Chattoraj (28) have independently proposed a model for the control of plasmid P1 replication that includes a mechanism similar to the handcuffing model.

There are two related facts concerning the π protein that are not readily explained by the simple handcuffing model presented above. One is the ability of the π protein to inhibit R6K replication when it is overproduced in the absence of a source of excess repeats (13). A second is that overproduction of the amino-terminal third of the π protein (the domain that is affected by the copy-up mutations) inhibits R6K replication in spite of the fact that this truncated protein cannot bind DNA (A. Greener, M. Filutowicz, and D.H., unpublished data). This indicates that the ability to bind DNA directly is not absolutely essential for the negative role of π. To accommodate these facts, we propose that the inhibition of R6K replication can also result from an accumulation of a second "layer" of π protein via π–π, rather than π–DNA, interactions at the γ origin. It is also conceivable that the inhibitory action of the repeats themselves might stem from their ability to facilitate the addition of this second π layer, as would occur if two π·origin complexes associated together. Origin coupling might therefore be thought of as a facilitation of a process that π alone can accomplish when present at high enough levels.

In addition to a potential role in the negative regulation of R6K replication, the bridging of two regions of DNA by π protein may also play a role in the activation in cis of R6K replication. It has recently been shown that the π protein can bring about the looping together of a γ origin with β origin sequences (27). Aggregation between the γ origin and the β or α origin region mediated by the π protein could easily be envisioned as the mechanism by which events occurring at the γ origin lead to replication initiation at the distant α and β origins. The regulation of R6K replication might then be viewed as a competition between inhibitory γ–γ aggregation and stimulatory γ–α and γ–β interactions. Comparing the ability of wild-type π and the mutant π proteins to bring about trans or cis coupling between their different DNA-binding sites would further test the handcuffing model.

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