Published in final edited form as:

J Mol Biol. 2007 May 11; 368(4): 928–938.

MECHANISM OF ORIGIN ACTIVATION BY MONOMERS OF R6K- ENCODED π PROTEIN

Lisa M. Bowers¹, Ricardo Krüger², and Marcin Filutowicz^{1,*}

¹ Department of Bacteriology, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706, USA

Abstract

One recurring theme in plasmid duplication is the recognition of the origin of replication (*ori*) by specific Rep proteins that bind to DNA sequences called ‘iterons’. For plasmid R6K, this process involves a complex interplay between monomers and dimers of the Rep protein, π , with seven tandem iterons of γ *ori*. Remarkably, both π monomers and π dimers can bind to iterons, a new paradigm in replication control. Dimers, the predominant form in the cell, inhibit replication while monomers facilitate open complex formation and activate the *ori*. Here, we investigate a mechanism by which π monomers out-compete π dimers for iteron binding, and in so doing activate the *ori*. With an *in vivo* plasmid incompatibility assay, we find that π monomers bind cooperatively to two adjacent iterons. Cooperative binding is eliminated by insertion of a half-helical turn between two iterons but only slightly diminished by insertion of a full helical turn between two iterons. These studies also show that π bound to a consensus site promotes occupancy of an adjacent mutated site, another hallmark of cooperative interactions. π monomer/iteron interactions were quantified using a monomer-biased π variant *in vitro* with the same collection of two-iteron constructs. The cooperativity coefficients mirror the plasmid incompatibility results for each construct tested. π dimer/iteron interactions were quantified with a dimer-biased mutant *in vitro* and it was found that π dimers bind with negligible cooperativity to two tandem iterons.

Keywords

cooperativity; plasmid replication; R6K; Rep-iteron interaction; replication control

Introduction

Plasmids are key contributors to virulence, antibiotic resistance and horizontal gene transfer. Thus, unraveling the mechanisms that control the proliferation of plasmids is a matter of practical significance as well as fundamental biological interest. One model for plasmid replication studies is R6K, a self-transmissible *Escherichia coli* plasmid encoding resistance to streptomycin and ampicillin.¹ R6K is a member of a group of plasmids in which replication is controlled by the recognition of an origin of replication (*ori*) by a specific replication initiator (Rep) protein that binds to DNA sequences called iterons.²

Two plasmid-encoded components are necessary for controlled replication of a minimal R6K replicon: γ *ori*, consisting of seven 22 base-pair (bp) iterons, and the *pir* gene, which encodes the Rep protein, π (Figure 1).^{3,4} Like other Rep proteins in this plasmid category, π is primarily

*Corresponding author (M. Filutowicz): Tel. 608-262-6947; Fax. 608-262-9865; E-mail: msfiluto@facstaff.wisc.edu.

²Present address: Universidade Católica de Brasília, Campus II, SGAN 916, Módulo B, W5 Norte, Brasília, Brazil

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

dimeric in solution and strong evidence suggests that dimers inhibit replication while monomers bind the seven iterons of γ *ori* to activate replication.^{5–9} Unlike other Rep proteins, π was long believed to be unique in that the dimeric form is also iteron-binding proficient.^{6–9} Recently, however, dimers of at least two other Rep proteins have also been shown to bind iterons,^{10,11} suggesting that the earlier π studies may have established a significant new paradigm in Rep/iteron binding interactions. This capacity for Rep dimers to compete with monomers for iteron binding adds a new level of complexity to models of plasmid replication control. Thus, we are left with a central question regarding the regulation of replication for R6K and plasmids like it: What mechanism or mechanisms allow π monomers to out-compete dimers for iteron binding?

A partial answer to this question was recently offered by Kunnimalaiyaan *et al.*; their somewhat surprising data demonstrated that π monomers contact a larger segment of DNA than π dimers¹² (Figure 1). An earlier set of qualitative observations hinted at another mechanism by which monomers of π gain an edge over π dimers. In gel shift titrations, π monomers were observed to interact with seven iterons yielding patterns consistent with positively cooperative binding.^{13,14} These inferences were based on the observed steep binding curves that result from site occupancy changing over a relatively small range of protein concentration, a hallmark of cooperative binding *in vitro*.

Because there are reports of strong and specific protein-protein interactions *in vitro* without biological relevance,¹⁵ it is extremely important to support *in vitro* binding data with evidence that the same interactions occur inside the cell. Yet to date, there have been very few demonstrations of the importance of cooperative DNA binding *in vivo* and most have been transcription factors that were assayed with artificial reporter genes.^{16–20} This work first examines whether π monomers bind iterons cooperatively with an *in vivo* π protein titration assay. With this assay, multiple configurations of one and two iterons were tested for their ability to titrate π monomers inside the cell. Second, we quantify cooperative binding of π monomers to the same collection of one- and two-iteron DNA fragments *in vitro*. Until this work, quantitative measurements of π cooperativity could not be made because nucleoprotein complexes containing π dimers could not be distinguished from complexes containing the same number of π molecules bound as monomers. Here, we show both *in vivo* and *in vitro* that π monomers demonstrate three common characteristics of proteins that bind cooperatively: π monomers bind to adjacent iterons in a greater-than-additive fashion, a π monomer bound to a strong consensus site helps recruit a π monomer to an adjacent mutated site, and binding of π monomers to iterons is sensitive to the spacing between iterons and to their relative helical orientation. Finally, we assess the binding of a dimer-biased π variant to a 2-iteron fragment and find that unlike π monomers, π dimers bind with negligible cooperativity to adjacent iterons.

Results

Do cooperative π interactions occur *in vivo*?

The assay used to evaluate π monomer binding *in vivo* was based on a phenomenon called “plasmid incompatibility”, which is generally described as the failure of two co-resident plasmids to be stably inherited, often due to the sharing of one or more elements of the plasmid replication system.²¹ For example, when iterons are cloned into an otherwise compatible plasmid, they inhibit replication of a γ *ori* plasmid.²² The number of iterons and the amount of π monomers in the cell both affect the degree of incompatibility.²²

In the plasmid incompatibility assay depicted in Figure 2, two plasmids compete for limited π monomers in the cell. First, a chloramphenicol (cam) resistant γ *ori* plasmid, pFW25,²³ was established in the *E. coli* host strain, ECF001.²⁴ Replication of pFW25 was dependent on

monomers of π produced from the chromosome of ECF001, where *pir* expression was under control of the arabinose-inducible P_{BAD} promoter. ECF001 harboring pFW25 was then transformed with a series of iteron-containing pUC9 derivative plasmids that were high copy number and conferred penicillin (pen) resistance. Since arabinose was not included in the plates used for transformation, *pir* expression was uninduced and π levels were limiting; thus, the iterons cloned into pUC9 affected replication of pFW25 by competing for π monomers in the cell.

The various configurations of iterons cloned into pUC9 were designed to test if cooperative interactions occur between π monomers bound to iterons. If certain configurations of iterons bound π monomers with greater cooperativity, they would have a higher degree of incompatibility with pFW25 and result in fewer colonies resistant to both cam and pen. Constructs were created with two iterons separated by 0 bp (wild-type (wt) arrangement), 5bp (half-helical turn), and 10 bp (full helical turn) (See Materials and Methods for iteron sequences). If cooperative contacts occur, the changes in site-spacing and helical orientation were expected to disrupt or enhance the protein-protein interactions that stabilize DNA-bound monomers.²⁵

Results of the plasmid incompatibility assay are depicted in Figure 3. Transformation with a pUC9 derivative containing a single iteron resulted in half the number of colonies as transformation with pUC9 alone on media with cam + pen. This means that even a single iteron on a high-copy pUC9 derivative can titrate π away from the γ *ori* plasmid and reduce or inhibit its replication. Transformation with the pUC9 derivative containing two iterons separated by 5 bp resulted in half the number of colonies as the 1-iteron fragment. This means that doubling the number of iterons but orienting them on opposite faces of the DNA resulted in an additive increase in π binding to the pUC9 derivative (decrease in colonies resistant to cam + pen). However, the pUC9 derivative containing the 2-iteron fragment with no space between the iterons (wt configuration) was completely incompatible with pFW25. This greater-than-additive increase in π binding is indicative of cooperativity. Adding 10 bp between the two iterons resulted in an intermediate number of colonies, suggesting that helical orientation is more important than distance for cooperative interactions.

Increasing the concentration of π did not alleviate the incompatibility of the two plasmids because the wt protein is dimer-biased. This result was expected as it is well documented that overproduction of π inhibits replication of γ *ori* plasmids.⁵ In contrast, when the experiment was carried out with an isogenic strain (ECF003)²⁴ that produces a monomer-biased π variant, π •P106L^ΔF107S,^{8,26} incompatibility was not observed with any of the above constructs, presumably because π monomers were not limited (L.B., M.F., data not shown). Western blots of both host strains showed that the amount of π produced in uninduced cells is barely detectable and the presence of the different pUC9 derivatives had no influence on the amount of π in induced or uninduced cells (L.B., M.F., data not shown).

π bound to a consensus site promotes π binding to a mutated site *in vivo*

If π binds cooperatively, π bound to a strong consensus site should promote occupancy of an adjacent mutated site at subsaturating protein concentrations. To test this hypothesis, the above plasmid incompatibility assay was carried out with constructs containing one wild type (wt) iteron and one iteron with a well-characterized mutation of the G/C⁷ bp to A/T (represented as *), which nearly abolishes π binding *in vitro* and *in vivo*.^{12,27} Because π binds weakly to the 1-iteron* fragment, it is not surprising that transformation with the pUC9 derivative containing 1-iteron* resulted in a similar number of colonies on cam + pen as transformation with pUC9. If π bound to iterons independently (without cooperativity), transformation with the 2-iteron* probe would be expected to yield the same results as the 1-iteron probe because the mutated iteron alone binds π so weakly. However, fewer colonies survived with the 2-

iteron* probe than the 1-iteron probe; therefore, we can infer that π bound to the wt iteron facilitates π binding to the mutated iteron (cooperativity). Introducing a half-helical turn between the wt and mutated iterons eliminated this cooperative interaction and resulted in binding similar to the 1-iteron fragment. Finally, a 10-bp space between the wt and mutated iterons resulted in the same number of colonies as the construct with no space, again consistent with the idea that helical orientation is more important than distance for cooperative π interactions.

***In vitro* binding properties of π •wt, π •P106L^{F107S} and π •M36A^{M38A} to a DNA fragment containing 2 iterons**

To support the indirect observations that π binds cooperatively to iterons in the cell, π -iteron interactions were also examined *in vitro*. Gel shift titrations with π and a DNA fragment containing two iterons yield complicated binding patterns because both monomers and dimers are iteron-binding proficient. Results are further complicated because two different types of complexes have the same molecular weight - one DNA fragment with two iterons can bind to either two π monomers or one π dimer. Thus, it is necessary to distinguish each possible complex in order to ensure that the correct complexes are quantified.

To separate the two different complexes with the same molecular weight, large DNA fragments with 60 bp flanking the two iterons were used. It was previously estimated that one dimer causes an apparent bending of the DNA by approximately 50° while two head-to-tail monomers cause an apparent bending of the same DNA by approximately 75°. ²⁸ Thus, the large flanking sequence allowed these two complexes to be separated electrophoretically based on differential apparent bending (Figure 4). To distinguish dimer-bound complexes from complexes with two monomers, we utilized well-characterized π variants biased toward monomer or dimer binding. It was previously reported that π •P106L^{F107S} binds to iterons primarily as a monomer ^{8,12} while π •M36A^{M38A} binds predominantly as a dimer. ^{8,29} Thus, the identities of the complexes were inferred based on their positions in the gel and their differential concentrations with each of the π variants (Figure 4).

It was evident from these gel shift titrations that only a minimal fraction of π •wt binds as two monomers to a two-iteron DNA fragment *in vitro*. This is understandable due to previous reports that chaperones are required for π •wt to monomerize upon iteron binding. ³⁰ Therefore, in the absence of chaperones, an increase in π •wt concentration results in an increase in dimer binding. This is why a large shift to the two-monomer complex does not occur as protein concentration increases *in vitro*. For this reason, the monomer-biased variant, π •P106L^{F107S}, was used for all *in vitro* quantifications of DNA binding. This variant has been shown to be predisposed to iteron DNA ligand-induced monomerization. ⁹ With this π variant, a steep binding curve was observed with the fraction of one monomer shifting to two monomers over a relatively small change in protein concentration, an indication of cooperative binding. π •M36A^{M38A} was used in all experiments as a size marker for the 1 dimer DNA complex.

Quantification of cooperative π monomer binding to two thermodynamically identical iterons

Cooperativity can be quantitatively assessed based on k_{12} , a constant obtained from binding equations derived by the statistical mechanical approach ³¹ and fitted to data from titrations of protein with DNA. Values of $k_{12} > 1$ are considered to be cooperative. The same DNA sequences used in the plasmid incompatibility assays with 0, 5, and 10 bp between two iterons (Figure 5(a)) were also used to quantify cooperative interactions between two π monomers *in vitro*.

Figure 5(b) shows a representative gel shift titration of π •P106L^ΔF107S added to each of the above probes. The fraction of total DNA that was free, bound to one monomer, and bound to two monomers was quantified and plotted as a function of π concentration (Figure 5(c)). To calculate the cooperativity coefficient, k_{12} , data in Figure 5(c) were subjected to a least-squares linear regression analysis using Eq. (1c) (See Materials and Methods for all formulas). This analysis provided the macroscopic binding constants, K_1 and K_2 , which were used to calculate k_{12} using Eq. (2): $k_{12}=4K_2/K_1^2$.

During the analysis of this complex system, a few challenges were encountered. First, the total concentration of π monomers could not be determined because π is dimeric in solution and presumably monomerizes upon binding to DNA.⁹ Therefore, the value used for π concentration was the total amount of π added to the sample. Second, increasing the π concentration beyond 800 ng per sample caused aggregation; therefore, protein binding to 100% of the DNA was not achieved. Finally, dimer binding could not be completely eliminated, even with the monomer-biased variant. Importantly, each of these stipulations is expected to result in an underestimation of the cooperativity coefficient. Furthermore, all of the DNA fragments were subjected to the same conditions, enabling a sound assessment of the relative cooperativity coefficients for each of the iteron spacings.

With these caveats in mind, the data in Table 1 show that π monomers bind the 2-iteron construct with a cooperativity coefficient of 210. Separation of the two binding sites by 10 bp (a full helical turn) decreased the k_{12} slightly to 156 and separation by 5 bp (a half-helical turn) essentially abolished cooperativity ($k_{12}=1.8$). These results support the hypothesis that π monomers bind to γ *ori* with positive cooperativity and the distance and orientation between adjacent binding sites influence these cooperative interactions.

Estimation of cooperative π monomer binding to two heterogeneous binding sites

As with the *in vivo* assay, we hypothesized that mutating one of the two iterons, causing a decrease of the natural affinity of π for that site, would enhance the apparent cooperativity because π bound to the consensus site would promote occupancy of the adjacent mutated site. This hypothesis was tested with another set of 2-iteron probes containing one wt iteron and one iteron with the G/C⁷>A/T mutation (represented as *).

First, the relative difference in binding affinity for the 1-iteron and 1-iteron* fragments was calculated by performing gel shift titrations with π •P106L^ΔF107S (Figure 6). The binding affinity of each fragment was determined by subjecting the plotted data to a least-squares linear regression analysis using Eq. (3). The binding affinity of the 1-iteron fragment (k_1) was $1.9 \pm 0.1 \times 10^7 \text{ M}^{-1}$ and the 1-iteron* fragment (k_2) was $1.0 \pm 0.2 \times 10^6 \text{ M}^{-1}$. Thus, binding affinities for the two iterons were substantially different ($k_1/k_2=19$).

Next, gel shift titrations were performed with heterogeneous 2-iteron probes with 0, 5, and 10 bp separating the wt and the mutated iterons (Figure 7(a) and (b)). The fraction of total DNA that was free, bound to one monomer, and bound to two monomers was quantified and plotted as a function of π concentration (Figure 6(c)). The macroscopic binding constants, K_1 and K_2 , were determined by subjecting data in Figure 6(c) to a least-squares linear regression analysis using Eq. (1c). When the affinities of the two binding sites are substantially different, Eq. (4) holds true: $k_{12} \approx (K_2^*h)/K_1^2$ (derived in Materials and Methods). As mentioned above, we found h , defined as k_1/k_2 , to have a value of 19 for these two iterons.

The data in Table 1 suggest that the same trends hold true for heterogeneous and homogeneous binding sites: π binds with greater cooperativity to the 2-iteron* probe than the 2-iteron*+10 probe and binds with little cooperativity to the 2-iteron*+5 probe. If the assumptions inherent in Eq. (4) are valid, the estimated cooperativity involved in π binding to two heterogeneous

iterons is greater than the estimated cooperativity involved in π binding two wt iterons for each of the three spacings tested (Table 1).

Quantification of cooperative π dimer binding to two thermodynamically identical iterons

The dimer-biased π variant, π •M36A⁺M38A was used to quantify π dimer binding to a 2-iteron probe. This variant was previously characterized to bind only as a dimer to iterons both *in vivo* and *in vitro*.^{8,29,32} Figure 8(a) shows a representative gel shift titration of π •M36A⁺M38A added to the 2-iteron fragment. The fraction of total DNA that was free, bound to one dimer, and bound to two dimers was quantified and plotted as a function of π concentration (Figure 8(b)). To calculate the cooperativity coefficient, k_{12} , data in Figure 8(b) were subjected to a least-squares linear regression analysis using Eq. (1c). This analysis provided the macroscopic binding constants, K_1 and K_2 , which were used to calculate k_{12} using Eq. (2). The results in Table 1 show that dimers of π •M36A⁺M38A bind to two tandem iterons independently or with negligible cooperativity.

Discussion

There is an ongoing debate about whether cooperative interactions can be inferred from gel shift titrations. Some argue that differential stabilities of protein/DNA complexes during electrophoresis can significantly distort interpretations of protein binding.^{33,34} Furthermore, *in vitro* binding assays often neglect the influence of known and unknown factors including DNA architecture, chaperones, and other host-encoded binding proteins such as DnaA, IHF, and Fis.^{35–37} Even with this level of uncertainty, there have been very few studies of cooperative binding *in vivo*. Thus, this work is distinguished by the development of the plasmid incompatibility assay to monitor cooperative Rep-iteron interactions in the context of the cell and the corroboration of the *in vivo* data by quantitative binding studies *in vitro*.

One might expect plasmid compatibility to be an “all-or-nothing” phenomenon. However, a stronger titration of π by iterons cloned into the pUC9 derivative is believed to result in a lower average copy number of the γ *ori* plasmid. Because cam resistance conferred by the γ *ori* plasmid is gene dosage dependent, some cells in the population will immediately have insufficient copies of the *cat* gene to confer resistance. However, other cells in the population may survive several rounds of cell division before the copy number is too low to confer resistance. This results in a repeatable and significant difference in plasmid compatibility with constructs that differ only by the number of bases separating two iterons. Clearly, orientation of the two iterons on opposite faces of the helix results in a dramatic decrease in the amount of π binding by constructs with two homogeneous or two heterogeneous iterons.

At first glance, it may seem that there are two competing explanations for the plasmid incompatibility data: first, the stated explanation that iterons on the pUC9 derivative titrate π monomers away from the γ *ori* plasmid and second, the iterons on the pUC9 derivative handcuff with γ *ori* of the resident plasmid. Handcuffing has been proposed as a mechanism that limits the copy number of R6K and other plasmids,^{38,39} whereby Rep dimers link two *oris* with each subunit of the dimer binding to one *ori*.²⁹ A closer examination of the data supports the monomer titration explanation over handcuffing. First, handcuffing occurs at high concentrations of π protein but π was limited in this experiment. Second, handcuffing is caused by dimers, which have been shown to bind very poorly to two adjacent iterons (Figure 8).²⁸ Therefore, if this assay measured handcuffing, the number of colonies containing the 1-iteron construct would resemble the number of colonies containing the 2-iteron construct, which is not the case. Third, two dimers can bind the 2-iteron+10 probe with equal or greater affinity than the 2-iteron probe (Figure 3). Therefore, if this assay measured handcuffing, the number of colonies containing the 2-iteron+10 construct should equal or be fewer than the number of colonies containing the 2-iteron construct. Again, the data do not support this possibility.

The plasmid incompatibility assay is important because it is a functional assay performed in the context of the cell but the *in vitro* quantification data is necessary to understand the degree of cooperativity compared to other systems. A scan of the literature shows that k_{12} values can be as high as 2000 for the most-cooperative interactions²⁵ but values are more often reported in the range of 10–200.^{40,41} Thus, quantification of the gel shift titration data suggests that cooperative interactions between π monomers are relatively robust compared to most systems. In direct contrast, the dimer-biased π variant binds two tandem iterons with negligible cooperativity, suggesting a mechanism by which monomers out-compete dimers for iteron binding.

This demonstration of strong cooperative binding of π monomers to two iterons may be an integral new insight into the mechanism of plasmid copy regulation. First, it offers an explanation as to how γ *ori* is saturated with π monomers when dimers abound. For instance, cooperative interactions may prevent or stall monomers from disassociating from iterons or rearranging back into dimers, increasing the opportunity to recruit more monomers to adjacent iterons. Second, the observation that cooperativity may be enhanced when one binding site is weaker than the adjacent site is quite relevant to our system because the seven iterons have different degrees of sequence divergence. The middle iteron is most divergent with 5 differences from the consensus sequence.³ Initial studies show that the more divergent iterons have a lower affinity for π (L.B., S. Rakowski, and M.F., unpublished data); therefore, cooperativity may be especially useful in filling the middle iterons with monomers, enabling saturation of the *ori*.

Finally, γ *ori* is only one example of an ever-expanding group of iteron-containing plasmids that depend on interactions between a replication protein and the reiterated DNA binding sequences.² It seems likely that if cooperative interactions are involved in initiation from γ *ori*, they may also be involved in similar systems. Many plasmids in this group, including R6K, are self-mobilizable and there are times in the life-cycle of these plasmids when the Rep protein concentration would be very low, such as after the plasmid is transferred by conjugation to a cell without Rep. In these situations, cooperative interactions between Rep monomers may be especially helpful in initiating replication. If this is true, it may help explain the evolution of cooperative protein/protein and protein/DNA interactions involved in plasmid replication initiation.

Materials and Methods

Bacterial growth

Bacteria were grown aerobically at 37° C in LB. ECF001²⁴ was grown with amp at 25 µg/mL. ECF001 containing pFW25²³ was grown with cam at 15 µg/mL. ECF001 containing pUC9 derivatives was grown with pen at 750 µg/mL.

Oligos

Oligos were purchased from either IDT (Coralville, IA) or the UW Biotechnology Center (Madison, WI). The top strand is indicated below in the 5'-3' direction. The bottom strand is complimentary to the top strand. Bold letters indicate the G/C⁷>A/T mutations.

1-iteron: AAACATGAGAGCTTAGTACGTT; 1-iteron*:
AAACATAAGAGCTTAGTACGTT; 2-iteron:
AAACATGAGAGCTTAGTACGTGAAACATGAGAGCTTAGTACGTT; 2-iteron*:
AAACATGAGAGCTTAGTACGTGAAACATAAGAGCTTAGTACGTT Sequence
separating the two iterons in the 2-iteron+5 and 2-iteron*+5 constructs: TTAAC Sequence
separating the two iterons in the 2-iteron+10 and 2-iteron*+10 constructs: TTAAC

Construction of plasmids

Each double-strand oligo (above) was blunt-end cloned into the *HincII* site of pUC9 (Promega, Madison, WI) for the *in vivo* binding assays and the *HpaI* site of pBend5⁴² for the gel shift titrations. Standard cloning techniques were followed⁴³ and new clones were verified by sequencing.

In vivo incompatibility assay

The incompatibility assay is depicted in Figure 2. Standard preparation of calcium chloride competent cells was followed for host strain ECF001+pFW25 with the culture grown in LB +cam+0.02% arabinose. 100 ng of each pUC9 derivative was transformed into the host strain and LB+0.02% arabinose was added to a final volume of 1 mL for an outgrowth period of 45 minutes. 100 μ L of a 1:10 dilution of each transformation mixture was spread onto LB+cam +pen plates. Plates were incubated for 24 hours at 37° C.

Protein Purification

His- π •WT, His- π •P106L[^]F107S, and His- π •M36A[^]M38A were purified as described.^{44,45}

DNA probe preparation

pBend5 derivative plasmids were digested with *EcoRV* and the iteron-containing fragments were purified from a 6% polyacrylamide gel by a gel extraction kit (Qiagen, Valencia, CA). The 1-iteron fragments were 149 bp each and the 2-iteron fragments were 171 - 181 bp, depending on the sequence between the iterons. All DNA fragments were end-labeled with [γ ³²P]ATP using polynucleotide kinase.

Gel shift titrations

80 pg labeled iteron-containing probe was mixed with 65 ng polydI:dC and binding buffer (2 mM Tris-HCl (pH 7.5), 0.6 mM MgCl₂, 0.1 mM EDTA, 10 mM potassium glutamate) in a 14 μ L reaction. Then, 1 μ L protein in TGE buffer (10 mM Tris-HCl (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 0.3 M KCl) was added. The protein-DNA equilibrium mixtures were incubated at room temperature (27° C) for 15 minutes then 3 μ L loading dye was added (20% glycerol, 0.5X TBE buffer⁴³, bromphenol blue). The samples were loaded onto 6% polyacrylamide gels (37:1 acrylamide:bisacrylamide and 0.5X TBE buffer) that had been pre-electrophoresed for 1 hour at 150V. The samples were run at a constant voltage of 180V for 90 minutes. Dried gels were imaged with a Typhoon[™] phosphorimager (GE Healthcare).

Estimation of cooperativity coefficient for two homogeneous binding sites

All experimental data from the gel shift titrations were fitted to the following equations using KaleidaGraph software (Reading, PA). The following equations were based on the statistical mechanical approach^{31,34} but because π binding to 100% of the DNA could not be achieved due to protein/DNA aggregation at high protein concentrations, these formulas were modified to account for the baseline and maximum fraction for a given titration.⁴⁶

$$\theta_0 = P_0 + (P_{\max} - P_0) \cdot 1 / Z \quad (\text{Eq. 1a})$$

$$\theta_1 = P_0 + (P_{\max} - P_0) \cdot K_1 L / Z \quad (\text{Eq. 1b})$$

$$\theta_2 = P_0 + (P_{\max} - P_0) \cdot K_2 L^2 / Z \quad (\text{Eq. 1c})$$

θ_0 , θ_1 , and θ_2 are fractions of free DNA, single monomer complex, and two monomer complexes, respectively. P_0 and $P_{\max}$ are the baseline and maximum fraction for a given

titration. Z is the binding polynomial and is equal to $1 + K_1L + K_2L^2$. L is protein concentration, $K_1=(k_1 + k_2)$ and $K_2=(k_1k_2k_{12})$. k_1 and k_2 are the binding affinity constants for the first iteron and the second iteron of the 2-iteron complex, and k_{12} is the cooperativity coefficient describing the interaction of protein molecules occupying both sites.

Once K_1 and K_2 are obtained from the least squares linear regression analysis, k_{12} can be derived by a few simple rearrangements. When the two binding sites are identical ($k_1=k_2$), then $K_2=k_1^2k_{12}$ and a rearrangement of this equation gives $k_{12}=K_2/k_1^2$. Also when the two binding affinities are identical, $K_1=2*k_1$. Squaring this equation gives $K_1^2=4*k_1^2$ or $k_1^2=K_1^2/4$. Substituting this equation into the k_{12} equation results in:

$$k_{12} = (4K_2) / K_1^2 \quad (\text{Eq. 2})$$

Estimation of cooperativity coefficient for two heterogeneous binding sites

When the affinities of two binding sites are heterogeneous, k_{12} can be estimated if one binding site has a substantially weaker affinity than the other ($k_1 \gg k_2$) and if the heterogeneity factor (h) is known ($h = k_1/k_2$). To determine h , k_1 and k_2 must be estimated individually. The interaction between π and a single iteron can be described by the Langmuir isotherm:⁴⁷

$$Y = k_1 L / (1 + k_1 L) \quad (\text{Eq. 3})$$

Where Y is the fraction of single monomer complex, L is protein concentration, and k_1 is the binding affinity constant.

Once h is known and K_1 and K_2 are obtained from the least squares linear regression analysis using Eq. 1c, k_{12} of two heterogeneous binding sites can be estimated by a few simple rearrangements. As defined above, $K_2 = k_1 k_2 k_{12}$ and after rearranging, $k_{12} = K_2 / (k_1 k_2)$. If the heterogeneity factor (h) is known ($h = k_1/k_2$), then $k_2 = k_1/h$. Thus, k_1/h can be substituted for k_2 to give $k_{12} \approx K_2 * h / k_1^2$. Also as defined above, $K_1 = (k_1 + k_2)$. Therefore, in situations where $k_1 \gg k_2$, $K_1 \approx k_1$ so $k_1^2 \approx K_1^2$. Finally, K_1^2 can be substituted for k_1^2 , which results in:

$$k_{12} \approx K_2 * h / K_1^2 \quad (\text{Eq. 4})$$

Acknowledgements

We are particularly indebted to Sheryl Rakowski and Selvi Kunnimalayaan for many helpful discussions and Richard Burgess and Tom Record for comments on the manuscript. This work was supported by NIH grant GM40314 and USDA/HATCH grant WIS04952 to M.F.. R.K. was supported by CAPES/Brasília/Brazil.

References

1. Kontomichalou P, Mitani M, Clowes RC. Circular R-factor molecules controlling penicillinase synthesis, replicating in *Escherichia coli* under relaxed or stringent control. J Bacteriol 1970;104:34–44. [PubMed: 4919751]
2. Krüger, R.; Rakowski, SA.; Filutowicz, M. Participating elements in the replication of iteron-containing plasmids (ICPs). In: Funnell, BE.; Phillips, GJ., editors. Plasmid Biology. ASM Press; Washington, DC: 2004.
3. Stalker DM, Kolter R, Helinski DR. Nucleotide sequence of the region of an origin of replication of the antibiotic resistance plasmid R6K. Proc Natl Acad Sci U S A 1979;76:1150–4. [PubMed: 375227]
4. Chen D, Feng J, Krüger R, Urh M, Inman RB, Filutowicz M. Replication of R6K γ origin *in vitro*: discrete start sites for DNA synthesis dependent on π and its copy-up variants. J Mol Biol 1998;282:775–87. [PubMed: 9743626]
5. Filutowicz M, McEachern MJ, Helinski DR. Positive and negative roles of an initiator protein at an origin of replication. Proc Natl Acad Sci U S A 1986;83:9645–9. [PubMed: 3540947]

6. Urh M, Wu J, Forest K, Inman RB, Filutowicz M. Assemblies of replication initiator protein on symmetric and asymmetric DNA sequences depend on multiple protein oligomerization surfaces. *J Mol Biol* 1998;283:619–31. [PubMed: 9784371]
7. Wu J, Sektas M, Chen D, Filutowicz M. Two forms of replication initiator protein: positive and negative controls. *Proc Natl Acad Sci U S A* 1997;94:13967–72. [PubMed: 9391136]
8. Krüger R, Konieczny I, Filutowicz M. Monomer/dimer ratios of replication protein modulate the DNA strand- opening in a replication origin. *J Mol Biol* 2001;306:945–55. [PubMed: 11237610]
9. Abhyankar MM, Reddy JM, Sharma R, Bullesbach E, Bastia D. Biochemical investigations of control of replication initiation of plasmid R6K. *J Biol Chem* 2004;279:6711–9. [PubMed: 14665626]
10. Das N, Chatteraj DK. Origin pairing ('handcuffing') and unpairing in the control of P1 plasmid replication. *Mol Microbiol* 2004;54:836–49. [PubMed: 15491371]
11. Giraldo R, Andreu JM, Díaz-Orejas R. Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. *Embo J* 1998;17:4511–26. [PubMed: 9687517]
12. Kunnimalaiyaan S, Krüger R, Ross W, Rakowski SA, Filutowicz M. Binding modes of the initiator and inhibitor forms of the replication protein π to the γ *ori* iteron of plasmid R6K. *J Biol Chem* 2004;279:41058–66. [PubMed: 15247259]
13. Urh M, York D, Filutowicz M. Buffer composition mediates a switch between cooperative and independent binding of an initiator protein to DNA. *Gene* 1995;164:1–7. [PubMed: 7590295]
14. Filutowicz M, York D, Levchenko I. Cooperative binding of initiator protein to replication origin conferred by single amino acid substitution. *Nucleic Acids Res* 1994;22:4211–5. [PubMed: 7937147]
15. Lazarides E, Lindberg U. Actin is the naturally occurring inhibitor of deoxyribonuclease I. *Proc Natl Acad Sci U S A* 1974;71:4742–6. [PubMed: 4140510]
16. Astromoff A, Ptashne M. A variant of lambda repressor with an altered pattern of cooperative binding to DNA sites. *Proc Natl Acad Sci U S A* 1995;92:8110–4. [PubMed: 7667253]
17. Barbaric S, Munsterkotter M, Goding C, Horz W. Cooperative Pho2-Pho4 interactions at the PHO5 promoter are critical for binding of Pho4 to UASp1 and for efficient transactivation by Pho4 at UASp2. *Mol Cell Biol* 1998;18:2629–39. [PubMed: 9566882]
18. Hansen FG, Christensen BB, Nielsen CB, Atlung T. Insights into the quality of DnaA boxes and their cooperativity. *J Mol Biol* 2006;355:85–95. [PubMed: 16298387]
19. Vashee S, Kodadek T. The activation domain of GAL4 protein mediates cooperative promoter binding with general transcription factors in vivo. *Proc Natl Acad Sci U S A* 1995;92:10683–7. [PubMed: 7479865]
20. Burz DS, Rivera-Pomar R, Jackle H, Hanes SD. Cooperative DNA-binding by Bicoid provides a mechanism for threshold-dependent gene activation in the *Drosophila* embryo. *Embo J* 1998;17:5998–6009. [PubMed: 9774343]
21. Novick RP. Plasmid incompatibility. *Microbiol Rev* 1987;51:381–95. [PubMed: 3325793]
22. Stalker, DM.; Shafferman, A.; Tolun, A.; Kolter, R.; Yang, S.; Helinski, DR. Direct repeats of nucleotide sequences are involved in plasmid replication and incompatibility. In: Ray, DS., editor. *The Initiation of DNA Replication*. XXII. Academic Press; New York: 1981. p. 113-124.
23. Wu F, Wu J, Ehley J, Filutowicz M. Preponderance of Fis-binding sites in the R6K γ origin and the curious effect of the penicillin resistance marker on replication of this origin in the absence of Fis [published erratum appears in *J Bacteriol* 1997 Apr;179(7):2464]. *J Bacteriol* 1996;178:4965–74. [PubMed: 8759862]
24. Pluciennik A, Iyer RR, Napierala M, Larson JE, Filutowicz M, Wells RD. Long CTG*CAG repeats from myotonic dystrophy are preferred sites for intermolecular recombination. *J Biol Chem* 2002;277:34074–86. [PubMed: 12087090]
25. Mao C, Carlson NG, Little JW. Cooperative DNA-protein interactions. Effects of changing the spacing between adjacent binding sites. *J Mol Biol* 1994;235:532–44. [PubMed: 8289280]
26. Krüger R, Filutowicz M. Dimers of π protein bind the A+T-rich region of the R6K γ origin near the leading-strand synthesis start sites: regulatory implications. *J Bacteriol* 2000;182:2461–7. [PubMed: 10762246]
27. McEachern MJ, Filutowicz M, Helinski DR. Mutations in direct repeat sequences and in a conserved sequence adjacent to the repeats result in a defective replication origin in plasmid R6K. *Proc Natl Acad Sci U S A* 1985;82:1480–4. [PubMed: 3883361]

28. Krüger R, Rakowski SA, Filutowicz M. Isomerization and apparent DNA bending by π , the replication protein of plasmid R6K. *Biochem Biophys Res Commun* 2004;313:834–40. [PubMed: 14706617]
29. Kunnimalaiyaan S, Inman RB, Rakowski SA, Filutowicz M. Role of π dimers in coupling (“handcuffing”) of plasmid R6K’s γ *ori* iterons. *J Bacteriol* 2005;187:3779–85. [PubMed: 15901701]
30. Zzaman S, Reddy JM, Bastia D. The DnaK-DnaJ-GrpE chaperone system activates inert wild type π initiator protein of R6K into a form active in replication initiation. *J Biol Chem* 2004;279:50886–94. [PubMed: 15485812]
31. Brenowitz M, Senear DF, Shea MA, Ackers GK. Footprint” titrations yield valid thermodynamic isotherms. *Proc Natl Acad Sci U S A* 1986;83:8462–6. [PubMed: 3464963]
32. Peng Y, Rakowski SA, Filutowicz M. Small deletion variants of the replication protein, π , and their potential for over-replication-based antimicrobial activity. *FEMS Microbiol Lett* 2006;261:245–52. [PubMed: 16907728]
33. Kleinschmidt C, Tovar K, Hillen W. Computer simulations and experimental studies of gel mobility patterns for weak and strong non-cooperative protein binding to two targets on the same DNA: application to binding of tet repressor variants to multiple and single tet operator sites. *Nucleic Acids Res* 1991;19:1021–8. [PubMed: 2020542]
34. Senear DF, Brenowitz M. Determination of binding constants for cooperative site-specific protein-DNA interactions using the gel mobility-shift assay. *J Biol Chem* 1991;266:13661–71. [PubMed: 1856200]
35. Zzaman S, Reddy JM, Bastia D. The DnaK-DnaJ-GrpE chaperone system activates inert wild type π initiator protein of R6K into a form active in replication initiation. *J Biol Chem* 2004;279:50886–94. [PubMed: 15485812]
36. Lu YB, Datta HJ, Bastia D. Mechanistic studies of initiator-initiator interaction and replication initiation. *Embo J* 1998;17:5192–200. [PubMed: 9724655]
37. Wu F, Levchenko I, Filutowicz M. A DNA segment conferring stable maintenance on R6K γ -origin core replicons. *J Bacteriol* 1995;177:6338–45. [PubMed: 7592407]
38. McEachern MJ, Bott MA, Tooker PA, Helinski DR. Negative control of plasmid R6K replication: possible role of intermolecular coupling of replication origins. *Proc Natl Acad Sci U S A* 1989;86:7942–6. [PubMed: 2682632]
39. Park K, Han E, Paulsson J, Chatteraj DK. Origin pairing (‘handcuffing’) as a mode of negative control of P1 plasmid copy number. *Embo J* 2001;20:7323–32. [PubMed: 11743008]
40. Majka J, Zakrzewska-Czerwinska J, Messer W. Sequence recognition, cooperative interaction, and dimerization of the initiator protein DnaA of *Streptomyces*. *J Biol Chem* 2001;276:6243–52. [PubMed: 11073953]
41. Chen S, Iannolo M, Calvo JM. Cooperative binding of the leucine-responsive regulatory protein (Lrp) to DNA. *J Mol Biol* 2005;345:251–64. [PubMed: 15571719]
42. Kim J, Zwieb C, Wu C, Adhya S. Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene* 1989;85:15–23. [PubMed: 2533576]
43. Sambrook, J.; Russell, DW. *Molecular cloning: a laboratory manual*. 3. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, N.Y.: 2001.
44. Wu J, Filutowicz M. Hexahistidine (His₆)-tag dependent protein dimerization: A cautionary tale. *Acta Biochimica Polonica* 1999;46:591–599. [PubMed: 10698267]
45. Krüger R, Filutowicz M. Characterization of His-tagged, R6K-encoded π protein variants. *Plasmid* 2003;50:80–5. [PubMed: 12826061]
46. Gavigan SA, Nguyen T, Nguyen N, Senear DF. Role of multiple CytR binding sites on cooperativity, competition, and induction at the *Escherichia coli* *udp* promoter. *J Biol Chem* 1999;274:16010–9. [PubMed: 10347150]
47. Brenowitz M, Senear DF, Shea MA, Ackers GK. Quantitative DNase footprint titration: a method for studying protein-DNA interactions. *Methods Enzymol* 1986;130:132–81. [PubMed: 3773731]

Abbreviations

ori

origin of replication

Rep	replication initiator
bp	base pair
wt	wild type
cam	chloramphenicol
pen	penicillin

Figure 1.

Roles of π monomers and dimers in the regulation of replication from γ *ori*. The seven iterons of γ *ori* are indicated by tandem arrows while the operator/promoter region is represented by two inverted half arrows. Small vertical arrows indicate the start sites for leading strand synthesis. π , encoded by the *pir* gene, can bind to an iteron as a monomer (crescent) or dimer (double crescent) although the predominant form in solution is dimer. π binds to the operator/promoter only as a dimer.⁶ Shading indicates that the two monomer subunits of a dimer make head-to-head contacts while two monomers bound to two tandem iterons are proposed to make head-to-tail contacts. A monomer contacts the iteron with two domains while a dimer contacts the iteron with only one domain of one of the subunits.

Figure 2.

In vivo binding assay. Plasmids, *oris* and genes are labeled. Expression of *pir* from the P_{BAD} promoter is arabinose-inducible. *cat* encodes chloramphenicol acetyl transferase, conferring resistance to cam. *bla* encodes β -lactamase, conferring resistance to pen. Crescent-shaped symbols represent π monomers.

Figure 3.

π binds cooperatively to two iterons *in vivo*. Experiments were conducted per Figure 2 and Materials and methods. The numbers of transformants on plates with cam+pen were expressed as ratios against the pUC9 control (no iteron). → represent iterons and * represents the G/C⁷>A/T mutation. '5' and '10' represent the number of base pairs between the two iterons. Decreasing ratios suggest increased titration of π by the incoming iteron-containing pUC9 derivative. The data represent the average of three independent experiments.

Figure 4.

π •wt, π •P106L^{F107S} and π •M36A^{M38A} have different binding patterns with a 2-iteron probe. Lane 1 is DNA only. Gray and white triangles represent increasing concentrations of π •wt and π •P106L^{F107S}, respectively, starting with 6.25 ng and doubling for each lane. The black square represents 200 ng of π •M36A^{M38A}. Black crescents represent π monomers and gray double crescents represent π dimers.

Figure 5.

π binds cooperatively to two thermodynamically identical iterons *in vitro*. Cooperative interactions are influenced by helical rotation and distance between the two iterons. (a) A depiction of each iteron-containing probe, not including flanking DNA. '5' and '10' refer to the number of bp between iterons. '1' and '2' refer to the first and second iterons of γ *ori*, respectively. (b) Gel shift titrations of purified π with the corresponding probes in (a). Lane 1 is DNA only. White triangles represent increasing concentrations of π •P106L^{F107S}, starting with 6.25 ng and doubling for each lane. The black square represents 200 ng of π •M36A^{M38A}. Black crescents represent π monomers and gray double crescents represent π dimers. (c) Quantification of gel shift titration data with π •P106L^{F107S}. The fraction of the total radioactivity as free DNA (circles), DNA containing a single π monomer (squares), and DNA containing two π monomers (diamonds) was determined. Continuous, dashed, and dotted lines correspond to the best fit of the data for equations 1a–1c, respectively. The protein concentrations in (b) are a subset of those plotted in (c).

Figure 6.

The binding affinity of the 1-iteron and 1-iteron* probes. (a) A depiction of each iteron-containing probe, not including flanking DNA. * indicates the G/C⁷>A/T mutation. (b) Gel shift titrations of the binding of purified π to the corresponding probes in (a). Lane 1 is DNA only. White triangles represent increasing concentrations of π P106L^{F107S}, starting with 6.25 ng and doubling for each lane. The black square represents 200 ng of π M36A^{M38A}. Black crescents represent π monomers and gray double crescents represent π dimers. (c) Quantification of gel shift titration data with π P106L^{F107S}. The fraction of the total radioactivity as free DNA (circles) and DNA containing a single π monomer (squares) was determined. Continuous and dashed lines correspond to the best fit of the data for Eq. (3). The protein concentrations in (b) are a subset of those plotted in (c).

Figure 7.

π binds cooperatively to two heterogeneous iterons *in vitro*. Cooperative interactions are influenced by helical rotation and distance between the two iterons. (a) A depiction of each iteron-containing probe, not including flanking DNA. '5' and '10' refer to the number of bp between iterons. '1' and '2' refer to the first and second iterons of γ *ori*, respectively. * represents the G/C⁷>A/T mutation in iteron #2. (b) Gel shift titrations of purified π with the corresponding probes in (a). Lane 1 is DNA only. White triangles represent increasing concentrations of π ●P106L^F107S, starting with 6.25 ng and doubling for each lane. The black square represents 200 ng of π ●M36A^M38A. Black crescents represent π monomers and gray double crescents represent π dimers. (c) Quantification of gel shift titration data with π ●P106L^F107S. The fraction of the total radioactivity as free DNA (circles), DNA containing a single π monomer (squares), and DNA containing two π monomers (diamonds) was determined. Continuous, dashed, and dotted lines correspond to the best fit of the data for equations 1a–1c, respectively. The protein concentrations in (b) are a subset of those plotted in (c).

Figure 8.

π M36A^M38A binds without cooperativity to a two-iteron probe *in vitro*. (a) Gel shift titrations of purified π with the 2-iteron probe. Lane 1 is DNA only. The black triangle represents increasing concentrations of the dimer-biased π variant π •M36A^M38A: 12.5 ng, 25 ng, 50 ng, 100 ng, 150 ng, 200 ng, and 250 ng. Gray and white squares represent 200 ng of π •wt and π •P106L^F107S, respectively. Black crescents represent π monomers and gray double crescents represent π dimers. (b) Quantification of gel shift titration data with π •M36A^M38A. The fraction of the total radioactivity as free DNA (circles), DNA containing a single π dimer (squares), and DNA containing two π dimers (diamonds) was determined. Continuous, dashed, and dotted lines correspond to the best fit of the data for equations 1a–1c, respectively.

Table I

Cooperative π /iteron interactions are influenced by distance, helical rotation, and affinity of the two iterons as well as monomer/dimer bias

Protein	DNA probe	$K_1 (\times 10^5) (M^{-1})$	$K_2 (\times 10^{13}) (M^{-2})$	k_{12}
π •P106L ^Δ F107S (monomer-biased)	2-iteron	8.90 ± 8.00	4.13 ± 0.63	209
	2-iteron+5	156.18 ± 41.32	11.31 ± 2.24	2
	2-iteron+10	7.32 ± 4.37	2.09 ± 0.24	156
	2-iteron*	9.37 ± 3.56	1.60 ± 0.15	346
	2-iteron*+5	23.60 ± 11.87	0.86 ± 0.24	29
	2-iteron*+10	5.02 ± 2.81	0.37 ± 0.06	282
π •M36A ^Δ M38A (dimer-biased)	2-iteron	16.99 ± 0.82	0.37 ± 0.04	5