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Mechanism of Origin Activation by Monomers of R6K-encoded π Protein

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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 is diminished only slightly by insertion of a full helical turn between two iterons. These studies show also 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

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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 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 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 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

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Abbreviations used: *ori*, origin of replication; Rep, replication initiator; wt, wild type; cam, chloramphenicol; pen, penicillin.

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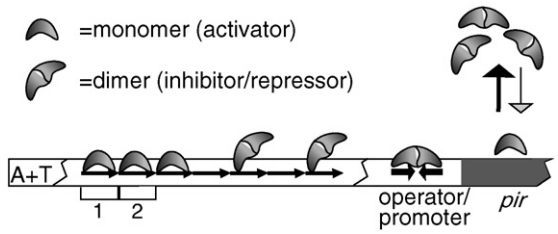

Figure 1. Roles of π monomers and dimers in the regulation of replication from γ *ori*. The seven iterons of γ *ori* are indicated by tandem arrows, while the operator/promoter region is represented by two inverted half arrows. π, encoded by the *pir* gene, can bind to an iteron as a monomer (crescent) or dimer (double crescent), although the predominant form in solution is the 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 contact, while two monomers bound to two tandem iterons are proposed to make a head-to-tail contact. A monomer contacts the iteron with two domains, while a dimer contacts the iteron with only one domain of one of the subunits.

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 offered recently by Kunnimalaiyaan *et al.*; their somewhat surprising data demonstrated that π monomers contact a larger segment of DNA than π dimers (Figure 1).[12] 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 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-iteron 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 two-iteron fragment and find that, unlike π monomers, π dimers bind to adjacent iterons with negligible cooperativity.

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-

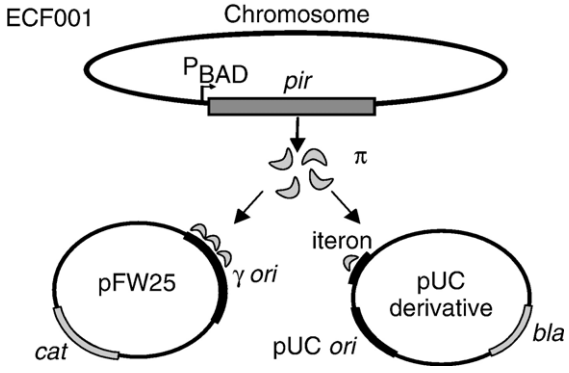

Figure 2. *In vivo* π/iteron binding assay. Plasmids, *oris* and genes are labeled. Expression of *pir* from the P_{BAD} promoter is arabinose-inducible. *cat* encodes chloramphenicol acetyl transferase, conferring resistance to cam. *bla* encodes β-lactamase, conferring resistance to pen. Crescent-shaped symbols represent π monomers.