Discerning the interactions between initiator protein and the origin of replication should provide insights into the mechanism of DNA replication initiation. In the \( \gamma \) origin of plasmid R6K, the Rep protein, \( \pi \), is distinctive in that it can bind the seven 22-bp iterons in two forms; \( \pi \) monomers activate replication, whereas \( \pi \) dimers act as inhibitors. In this work, we used wild type and variants of the \( \pi \) protein with altered monomer/dimer ratios to study iteron/\( \pi \) interactions. High resolution contact mapping was conducted using multiple techniques (missing base contact probing, methylation protection, base modification, and hydroxyl radical footprinting), and the electrophoretic separation of nucleoprotein complexes allowed us to discriminate between contact patterns produced by \( \pi \) monomers and dimers. We also isolated iteron mutants that affected the binding of \( \pi \) monomers (only) or both monomers and dimers. The mutational studies and footprinting analyses revealed that binding DNA, \( \pi \) monomers interact with nucleotides spanning the entire length of the iteron. In contrast, \( \pi \) dimers interact with only the left half of the iteron; however, the retained interactions are strikingly similar to those seen with monomers. These results support a model in which Rep protein dimerization disturbs one of two DNA binding domains important for monomer/iteron interaction; the dimer/iteron interaction utilizes only one DNA binding domain.

Initiation of DNA replication occurs at a specific site called the origin of replication (ori). In one class of replicons, plasmid-encoded replication initiator protein (Rep) binds to tandemly repeated DNA sequences in the ori called iterons. Most iteron-containing plasmids display remarkable similarities among the iteron sequences (1–3). One sequence, 5'-TGAGnG-3', is conserved in several plasmids and shown to be important for Rep binding in vitro as well as for ori function in vivo (Fig. 1 and Refs. 4 and 5). In addition, another sequence cluster that occurs one integral DNA helical turn (10 bp) from the start of the 5'-TGAGnG-3' motif remains highly conserved within iterons of the same replicon (2). Understanding the interactions between Rep proteins and their cognate iterons, and how these nucleoprotein complexes associate with the other components of the initiation apparatus, remain significant objectives in molecular biology.

In \( \gamma \) ori, one of the three origins derived from the antibiotic resistance plasmid R6K, a minimum of five 22-bp iterons (out of seven, total) were shown to be required for ori activation (6). In addition, base substitution in the iteron sequence (G/C 7 to A/T) prevented Rep protein binding to the iteron in vitro and plasmid replication in vivo (4). For plasmid R6K, the cognate Rep protein, called \( \pi \) (7), is encoded by the \( \pi r \) gene. Gel filtration (8, 9) and sedimentation (10) analyses showed that \( \pi \) exists primarily as a dimer in solution. An unusual characteristic of \( \pi \)iteron binding is that when wild type (WT) protein is mixed with a single iteron, also known as a direct repeat, two nucleoprotein complexes are seen in electrophoretic mobility shift assays (EMSA) (e.g. see Refs. 9–13). To identify the components of these complexes, a technique was employed where full-length \( \pi \) and shorter variants were reconstituted into heterodimers prior to DNA binding. Comparative analyses of the resultant banding patterns (in EMSA) suggested that, for WT \( \pi \), the faster migrating complex contains bound monomers of the protein, and the slower migrating complex contains bound dimers (12, 14, 15). Moreover, the binding proficiency of heterodimers in which one \( \pi \) variant lacked the C-terminal DNA binding domain suggested that only one subunit of a \( \pi \) dimer makes specific contact with the iteron DNA (15).

An additional line of evidence for the identification of \( \pi \) monomer-bound and \( \pi \) dimer-bound direct repeat complexes came from treating Rep proteins with subdenaturing levels of agents that can disrupt protein-protein interactions. In EMSA, increasing guanidine HCl concentrations led to a loss of the slower migrating “dimer” band and an increase in the faster migrating, “monomer” band (12, 15). Treatment with guanidine HCl or urea has also been shown to activate a Rep protein (RepA), as has treatment with chaperones (e.g. see Refs. 16 and 17), results implicating monomers as the initiator form of Rep protein (for reviews, see Refs. 18 and 19). Complementing this, hyperreplicative (i.e. copy-up) \( \pi \) variants tend to be more susceptible than WT to guanidine HCl treatment (suggesting dimer instability) and tend to show more monomer complex in EMSA, whereas inactive initiator variants produce predominantly dimer complex (9, 12, 13, 15, 20) (this work). Recently, Bastia and co-workers (9) combined Sephadex G-75 column filtration (for molecular weight determination), guanidine HCl

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The abbreviations used are: WT, wild type; EMSA, electrophoretic mobility shift assay; DMS, dimethyl sulfate.

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challenge, mutant analysis, and EMSA in a single set of experiments. Again, monomer- and dimer-bound nucleoprotein complexes are identified, the former being more prevalent with copy-up variants and the latter more prevalent with WT π protein.

A complete understanding of the pattern of π binding has been complicated by the multiple ways that monomers and dimers of the protein appear to interact with the iteron sequence. There are several possible combinations of nucleoprotein complexes that can occur, and even a probe as simple as dimers of the protein appear to interact with the iteron sequence. The experiments were expected to be composites of various families of nucleoprotein complexes, and the resulting data would likely be too complex to meaningfully address structure/function models of π protein activities (in replication and transcription). By paring the DNA probe(s) down to a single iteron, we hoped to simplify the array of assemblies of π monomers and dimers on γ ori DNA. Resolving the binding characteristics of the monomeric and dimeric forms of π protein is of great significance since the binding (to iterons) of monomers, but not dimers, appears to be required for open complex formation (13).

In the work presented here, we used His-tagged wild type π (His-πWT) and previously characterized variants of the protein with altered monomer/dimer ratios (His-πP106L/F107S (14, 15) and His-πM36A/M38A (13)) to study iteron/π interactions. Each variant differs from the other in several replication-related functional properties such as iteron binding and the ability to facilitate “open complex” formation. His-πP106L/F107S is a copy-up variant; it stimulates increased replication compared with WT π, apparently by elevating the fraction of π monomers relative to the low monomer level observed in the WT protein (14, 15). The variant His-πM36A/M38A fails to support replication, and the highly purified protein does not possess strand-opening activity in vitro (13). The fast migrating (monomer) nucleoprotein complex is absent in EMSA assays when a one-direct repeat probe is mixed with His-πM36A/M38A, but the slower migrating, dimer-bound complex is readily observed (13).

Understanding the process of origin recognition and the architecture of complexes that activate or inhibit replication requires detailed information about iteron/π interaction(s). Here we used several chemical footprinting analyses to characterize the interactions of π monomers and dimers with DNA containing a single iteron. Electrophoretic separation of nucleoprotein complexes allowed us to discriminate between contact patterns produced by each form of the protein. We also isolated base pair substitution mutations that affect the binding of π monomers (only) in addition to mutations that affect both monomers and dimers. Our results indicate that π monomers make much more extensive iteron contacts in comparison with dimers. Monomers interact with the entire iteron, spanning the adjacent major grooves, but dimers interact with only the left half of the iteron. The interactions of π dimers in the left half of the iteron (Fig. 1) are the same as those of π monomers. These results support a model in which Rep protein dimerization disturbs one of two DNA binding domains important for monomer/iteron interaction; the dimer/iteron interaction utilizes only one DNA binding domain.

MATERIALS AND METHODS

Strains and Plasmids—Escherichia coli strain ECF001 contains a chromosomal copy of the pir gene under an arabinose-inducible promoter, ParaA (22). Construction of plasmids pFW25 (23) and pFR1 (20) was previously described. pFL731 is a derivative of pUC9 in which a single iteron is cloned into the HincII site.

Protein Purification—His-πWT, His-πP106L/F107S, and His-πM36A/M38A were purified as described (24).

RESULTS

DNA Preparation—Plasmid pRK1 containing a single iteron was digested with EcoRI/PstI (generating a 91-bp fragment) or SalI/SmaI (generating an 83-bp fragment), and the fragments were purified from a 6% acrylamide gel using a Qiagen gel extraction kit (Qiagen). The fragments were then 3'-end-labeled with [γ-32P]dATP at the EcoRI site or [α-32P]dCTP at the SalI site using Sequenase (U.S. Biochemical Corp.). Unincorporated free nucleotides were removed by passage through a G-50 column (Amersham Biosciences).

Missing Base Interference Footprinting—DNA containing, on average, one missing guanine or adenine (using formic acid; Sigma) or one missing cytosine or thymine (using hydrazine; Sigma) was prepared using published procedures (25). DNA containing, on average, one missing guanine was also prepared using dimethyl sulfate (DMS, Sigma) as described (26). To carry out missing base interference footprinting, 10 ng of DNA was incubated with π protein (His-πWT (1.5 μg), His-πP106L/F107S (1.5 μg), or His-πM36A/M38A (2.5 μg)) in a 25-μl final volume for 15 min at room temperature. The reaction mixture also contained 225 ng of poly(dI-dC) and 2.5 μl of 10× binding buffer (20 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 mM EDTA and 100 mM potassium glutamate). Proteins were diluted in TGE buffer (10 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, and 0.3 mM KC1).

After the binding reaction, iteron-π complexes were separated from unbound DNA by electrophoresis on a 6% acrylamide gel in 0.5× Tris-borate-EDTA buffer. π-bound iteron DNA from monomer or dimer complexes and free DNA were eluted according to the crush and soak method (27), and then strand cleavage was carried out at the abasic sites by incubation in 100 μl of 1 M piperidine at 90 °C for 30 min (25). Fragments were ethanol-precipitated, separated by electrophoresis on denaturing (7 M urea) 9% acrylamide gels, and dried. Gels were scanned by phosphorimaging using a Storm system (Amersham Biosciences). Data points in each lane were normalized to a region of sequence flanking the iteron, which is not affected by π binding, to correct for differences in loading. Normalized profiles were graphed using Sigma Plot (Jandel Scientific). Missing bases that interfered with π binding were underrepresented in the strand cleavage profiles of bound DNA relative to the free DNA or the DNA population that was not incubated with π. Positions where removal of a base completely prevented π binding are identified as strong (or severe) effects, whereas those that partially prevented binding are classified as moderate or weak effects.

Methylation Interference Footprinting—DNA was premethylated with DMS using a published protocol (25). The DNA-protein complexes were formed (with the modified DNA), and the complexes were separated and analyzed as described above for missing base interference footprinting.

Methylation Protection Footprinting—DNA fragments (10 ng) were preincubated with His-πWT (1.2 μg) or variants of π (His-πP106L/F107S (0.9 μg) or His-πM36A/M38A (1.5 μg)) in the binding buffer described above (25-μl final volume) for 15 min at room temperature. Then the DNA was methylated by the addition of 1 μl of DMS followed by incubation at room temperature for 2 min. The complexes were separated on gels and analyzed as described above.

Hydroxyl Radical Footprinting—End-labeled DNA fragment (2 ng) was incubated with 300 ng of protein in a 25-μl final volume. Reactions also contained 65 ng of poly(dI-dC) and binding buffer described above. Cleavage was carried out as previously described (28). After cleavage, the complexes were separated by denaturing (7 M urea) 9% acrylamide gel, and fragments were eluted and analyzed on a denaturing (7 M urea) 9% acrylamide gel.

Incompatibility and EMSA—Plasmid pFL731 was subjected to PCR mutagenesis following a published protocol (29) to create random iteron base pair mutations. The primers used for PCR were 5′-TATAGTCAG-3′/5′-ACGGGATCC-5′ (generating an 83-bp fragment), and the fragments were purified by electrophoresis on a 6% acrylamide gel. PCR fragments were cloned into pUC9 and tested for incompatibility as follows. Iteron-bearing, penicillin-resistant (PenR) derivatives of pUC9 were transformed into E. coli (strain ECMF001) harboring the chloramphenicol-resistant (Cat+) or ori plasmid, pFW25. Transformation mixtures were plated on LB agar supplemented with penicillin (250 μg/ml) and chloramphenicol (15 μg/ml). Plasmids with a mutant iteron (pFL731) causing compatibility were isolated and sequenced (ABI Prism 3100), and the iteron DNA was labeled by PCR using the described primers and [α-32P]dATP. These mutated iteron fragments were used for EMSA (20).
pairs (bp) for \( \pi \) binding was previously demonstrated both genetically and biochemically (4, 10, 30), the relative importance of each bp was not known with regard to binding by \( \pi \) monomers versus dimers, and strand-specific information was similarly lacking. To identify iteron bases that are crucial for \( \pi \) binding, we carried out missing purine and missing pyrimidine interference experiments (Fig. 2, A and B, respectively) with fragments containing a single 22-bp, R6K \( \gamma \) ori iteron (Fig. 1).

Nucleoprotein complexes were formed with His-\( \pi \)P106L/F107S and DNA fragments containing, on average, one missing purine (prepared with formic acid) or one missing pyrimidine (prepared with hydrazine) was incubated with \( \pi \) protein (see "Materials and Methods"). Nucleoprotein complexes containing His-\( \pi \)P106L/F107S monomers (\( M \)) or \( \pi \) dimers (\( D \)) were separated from free DNA (\( F \)) by EMSA on a nondenaturing gel (shown on left). The separated complexes were purified, and iteron DNA was subjected to strand cleavage at the abasic sites and then fractionated on a high resolution denaturing gel (shown on the right). Quantification of monomer-bound (red) and dimer-bound (blue) profiles is shown relative to free DNA (gray) in traces above each set of gel lanes. Sequence positions are numbered as shown in Fig. 1, and some positions are indicated below the gel lanes. Missing bases that interfered with \( \pi \) binding were underrepresented in the strand cleavage profiles of bound DNA relative to free DNA. Missing purine interference footprints (A) and missing pyrimidine interference footprints (B) are shown.

![Diagram](image1.png)

**Fig. 1.** Multiple roles of \( \pi \) protein in the regulation of the \( \gamma \) origin of plasmid R6K. The \( \gamma \) gene encodes \( \pi \) protein, which exists in two forms: monomers (shaded dark gray) and dimers (shaded light gray). The black arrows represent \( \pi \) binding sites. The seven direct repeats of \( \gamma \) ori, also called iterons, are indicated by tandem arrows, whereas a pair of shorter, inverted arrows indicates the inverted repeat in the \( \gamma \) operator/promoter. For the experiments described here, DNA probes contained the seventh iteron, the sequence of which is shown. The highly conserved 5'-TGGAGC-3' motif (19) is in boldface type. \( \pi \) is a multifunctional regulatory protein; monomers activate replication (a), dimers inhibit replication (b), and dimers also bind to the inverted repeat to repress \( \gamma \) transcription (c).

![Diagram](image2.png)

**Fig. 2.** Missing base contact probing with sparingly depurinated and depyrimidated DNA. Iteron DNA was 3'-end-labeled in the top or bottom strands as indicated. DNA containing, on average, one missing purine (prepared with formic acid) or one missing pyrimidine (prepared with hydrazine) was incubated with \( \pi \) protein (see "Materials and Methods"). Nucleoprotein complexes containing His-\( \pi \)P106L/F107S monomers (\( M \)) or \( \pi \) dimers (\( D \)) were separated from free DNA (\( F \)) by EMSA on a nondenaturing gel (shown on left). The separated complexes were purified, and iteron DNA was subjected to strand cleavage at the abasic sites and then fractionated on a high resolution denaturing gel (shown on the right).
show that, in the top strand of the DNA, removal of numerous bases throughout the iteron (A$_{3}$–G$_{7}$, G$_{9}$, and T$_{13}$–G$_{20}$) severely affected binding. Additionally, the removal of most other bases showed weak effects. There were only three bases (A$_{1}$, G$_{11}$, and A$_{22}$) whose absence produced no observable effect on the binding of H$_{9266}$ monomers. In the bottom strand, removal of bases (T$_{8}$–T$_{10}$, T$_{15}$, and A$_{17}$–A$_{21}$) severely affected the binding of H$_{9266}$ monomers. All other bases except T$_{22}$ (no effect) showed moderate or weak effects when removed.

In contrast, for complexes containing H$_{9266}$ dimer, the removal of only a few bases in the left half of the iteron in either the top strand (A$_{3}$–G$_{7}$) or the bottom strand (T$_{8}$–T$_{10}$) severely affected binding. Moderate effects on dimer binding were observed upon the removal of several other bases in top (A$_{1}$, A$_{2}$, and A$_{8}$–A$_{10}$) and bottom (G$_{4}$ and A$_{6}$–C$_{7}$) strands, localized to the left half of the iteron. Additionally, 4 bottom strand bases showed weak but noticeable effects on H$_{9266}$ dimer binding (T$_{1}$–T$_{3}$ and T$_{5}$). Missing bases in the right half of the iteron did not reduce binding. No substantial differences were seen between interference footprints of dimer complexes formed with any of the H$_{9266$ proteins used (His–H$_{9266$ P106L/F107S, and His–H$_{9266$ M36A/M38A; see Supplemental Data). Moreover, depurina-
with DMS (Supplemental Data) yielded similar results to those shown in Fig. 2A (depurination with formic acid).

Analysis of the missing base interference data indicates that the interaction of the π monomer with the iteron is more extensive than the dimer’s interaction. The positions that interfered with π dimer binding represent a subset of the positions that affected monomer binding and occurred only in the left half of the iteron. (Note that data from these and subsequent experiments are also summarized in Fig. 7) Another interesting observation obtained from close inspection of the footprinting gels (Fig. 2) is that, for the dimer-bound complex, an increase in band intensity, relative to the free DNA signal, sometimes occurs in fragments with missing bases in the right half of the iteron (see “Discussion”). Last, we note as a caveat that distortion of the normal structure of the iteron DNA could significantly contribute to the effect of some of the missing bases, since structural changes can occur up to 4 bp from an abasic site (31). Thus, at least a portion of the interference to π binding caused by missing bases could be attributable to effects on a structure important for recognition by π rather than to the loss of direct base contacts.

πIteron Major and Minor Groove Contacts Detected by Methylation Interference and Protection Footprinting—The missing base interference experiments suggested that a surprisingly large number of bases in the iteron are important for π monomer binding, whereas fewer bases are important for the binding of π dimers. However, these results did not distinguish which base surfaces (major groove, minor groove, or both) are contacted or, alternatively, whether the interference signals might derive, in some degree, from indirect effects of missing bases on DNA backbone structure (also see “Discussion”). To determine whether π makes specific base contacts with the N-3 position of adenine in the minor groove and the N-7 position of guanine in the major groove, we conducted methylation interference and protection footprinting experiments using DMS. Methylation interference footprinting with modified DNA and His-πP106L/F107S (Fig. 3A) shows that the modified bases A², A³, G², and G¹⁶ in the top strand and G¹⁹ in the bottom strand strongly interfered with π monomer binding. G¹² in the bottom strand showed partial interference. Adenine residues are less reactive to DMS than guanine residues, and, therefore, the intensities of bands at A residues are lower than those for G; this is true even in the absence of bound protein, which tends to de-emphasize protection signals.

For dimers, in the top strand, modified bases A², A³, and G² strongly interfered with π binding; however, notably, G¹² did not. Similar results were also obtained in methylation protection experiments, and the results when His-πWT was used are shown in Fig. 3B. As seen in the missing base experiments above, methylation interference and protection signals for π monomers were located throughout the iteron, whereas signals for dimers constituted a subset of the monomer signals and were located only in the left half of the iteron.

Probing of Iteron Backbone/π Interactions Using Hydroxyl Radicals—Hydroxyl radicals break the backbone of DNA with little sequence dependence; thus, hydroxyl radical footprinting allows all backbone positions to be monitored for contact with protein (32). Complexes formed with π (His-πP106L/F107S) were treated with hydroxyl radicals (Fig. 4). Iteron/π monomer binding revealed two clusters of protection, 5–7 bp long, on the top strand (A²–A³ and T¹⁸–C¹⁹). In the bottom strand, two clusters of protection, offset by 4 or 5 nucleotides in the 5’ direction from those in the top strand (T¹³–G¹² and T¹⁸–T²⁵) were observed. Similar results were obtained with monomers of His-πWT (see Supplemental Data). A helical presentation of this data indicates that π monomer binds, predominantly, to one face of the double-stranded helix protecting positions along two helical turns (see Fig. 7).

Iteron/π dimer binding revealed, on each strand, one cluster of protection 5–9 bp long (centered at position A⁵ on the top strand and T¹⁰ on the bottom strand) offset by 4 or 5 nucleotides in the 5’ direction on the two strands. The pattern shows that positions protected by the dimer are a subset of positions protected by the monomer and are located only in left half of the iteron. Similar results were obtained with dimer complexes formed with His-πWT or with the variant, His-πM36A/M38A (see Supplemental Data). Enhanced cleavage of the bases in the bottom strand of the iteron (right half) in the presence of dimer suggests possible structural distortion of the DNA upon binding of protein dimers.

Isolation of Iteron Mutants with Reduced Ability to Bind π Protein—When cloned into an otherwise compatible plasmid, iterons inhibit the replication of their plasmid of origin; such DNA sequences are said to cause incompatibility (Inc). We took advantage of iteron-mediated incompatibility (33) to select for iteron mutants (itin) that are π-binding-deficient. The genetic set-up is shown schematically in Fig. 5. To conduct our experiments, we used an E. coli strain (ECF001) that carried an arabinose-inducible pir gene on its chromosome (22); it also harbored the γ ori containing plasmid, pFW25. In this strain, the replication rate of plasmid pFW25 rises with increasing levels of supplied arabinose (and then levels off). Next, we engineered a derivative of the multicopy plasmid, pUC9, to carry a single γ ori in a Pen (pFL731). As expected, pFL731 displayed incompatibility with pFW25 in an arabinose-dependent fashion (data not shown). We pre-
dicted that itn mutants that are markedly deficient for binding would not inhibit replication of plasmid pFW25 (i.e. they would be Inc/H11002). Consistent with this prediction, pFL731, containing a previously characterized itn mutant (G/C to A/T), which completely abolishes binding (see Ref. 4 and Fig. 6A), was compatible with pFW25.

Our ori plasmid and strain combination (pFW25/ECF001) was then used to screen a pool of iteron DNA (cloned into pUC9) that had been randomly mutagenized by PCR. 29 transformants were picked and sequenced, and the binding of itn mutants was examined by EMSA. Based on the differences in the ratios of monomer-bound to dimer-bound nucleoprotein complexes, six mutants were chosen for further analysis (Fig. 6A and B). Quantification of data characterizing the effects of the iteron mutations on itn binding (using 100 ng of protein) is presented in Fig. 6C. One class of itn mutations prevented or reduced the binding of both itn monomers and itn dimers, and these map within the 5'-TGAGnG-3' cluster in the left half of the iteron (T/A6 to C/G; G/C7 to A/T; A/T8 to G/C). Conversely, another class of itn mutations prevented or reduced the binding of monomers and increased the binding of dimers, and these map within a cluster in the right half of the iteron (T/A17 to C/G; A/T18 to G/C; double mutant T/A17 to C/G and insertion of T between G11 and C12; double mutant T/A17 to C/G) (see Fig. 6B and "Discussion"). These results may reflect effects of the iteron mutations on both the affinities of the mutant itns for itn monomers and dimers and the competition for binding between monomers and dimers. In Fig. 6B, with His-πP106L/F107S, more binding of monomers was observed in some mutants (T/A6 to C/G; G/C7 to A/T; A/T8 to G/C; A/T18 to G/C) relative to that seen with WT, presumably due to the elevated level of monomers produced by this variant.

**FIG. 6.** itn mutations affect the binding of π monomers and π dimers. 3'-End-labeled iteron DNA fragments lacking or containing the indicated itn mutations were incubated with increasing amounts of His-πWT (A) or His-πP106L/F107S (B) and subjected to EMSA. In each panel and for each fragment (WT or itn mutants), the left-most lane contains no protein (*), and the next four lanes contain 25, 50, 100, or 200 ng of protein. The positions of free DNA (F) and nucleoprotein complexes containing π monomers (M) or π dimers (D) are indicated. C, wild type and itn mutant sequences (mutations in black and underlined) and their names (left) are shown. The percentage of DNA bound by 100 ng of His-πWT or His-πP106L/F107S monomers (black) and dimers (gray) is shown in histograms. Averages are calculated from three independent experiments.

**DISCUSSION**

Previous studies of iteron/π interactions by footprinting analysis were done using all seven iterons and WT π protein (10) or using π fused with β-galactosidase protein (30). Contact patterns observed on multiple iterons are composites of monomers and dimers, because both forms of π can bind to the iteron. We decided it would be preferable to do footprinting analysis with a single iteron, since it simplifies the array of assemblies of π protein. Furthermore, electrophoretic separation of nucleoprotein complexes allowed us to discriminate between contact patterns produced by π monomers and dimers. The use of π variants (His-πP106L/F107S and His-πM36A/M38A) shifted the monomer/dimer ratio of π molecules, result-
The DNA strands are represented in purple (top) or blue (bottom). Purple and blue spheres represent the backbone contacts (C-4' deoxyribose positions in the top and bottom strands, respectively (43)) for \( \pi \) protein as determined by hydroxyl radical footprinting. Positions that are protected by \( \pi \) from methylation (by DMS) or that interfered with \( \pi \) binding when premethylated are shown as red and yellow spheres for the N-3 position of guanines and N-7 positions of adenines, respectively. Orange base pairs indicate \( \pi \) mutations (T/A6 to C/G, G/C7 to A/T, A/T8 to G/C, T/A17 to C/G, and A/T18 to G/C) that affect the binding of \( \pi \) monomers (only) or both monomers and dimers. The model was created using Insight II software.

Data from footprinting and iteron mutagenesis studies demonstrate that \( \pi \) monomers make more extensive contacts with iteron DNA in comparison to \( \pi \) dimers (Fig. 7). Hydroxyl radical protection footprints indicate that \( \pi \) monomers bind to one face of the DNA helix, protecting the phosphodiester backbone along the two adjacent major grooves and the central minor groove of the iteron (Fig. 7B). \( \pi \) dimers also bind to the same face of the DNA helix but only contact the backbone in the left half of the iteron. Consistent with these data, base surfaces that were protected by \( \pi \) in methylation protection experiments (N-3 positions of adenine in the minor groove and N-7 positions of guanine in the major groove) occur on the same face of the helix as the hydroxyl radical protection signals (Fig. 7B).

The footprint signals and the iteron mutational studies indicate that \( \pi \) monomers and dimers make very similar contacts in the left half of the iteron; this suggests that the same DNA binding domain is responsible for these contacts in monomers and dimers. In contrast, in both missing base interference probing (Fig. 2) and mutational analysis (Fig. 6), alterations in the right half of the iteron reduced the band intensities generated by \( \pi \) monomers but often increased the band intensities generated by \( \pi \) dimers. One possible explanation for these results is that a reduction in competition by monomers resulted in a relative increase in the fraction of dimer-bound complex. Another possibility is that the modifications of the iteron (right half) could have caused a structural distortion in the DNA that favored the binding of \( \pi \) dimers (e.g. Se-Lrp protein contact probing) (34). Furthermore, the enhanced cleavage by hydroxyl radicals in the right half of the iteron in dimer complexes is consistent with the idea that dimer binding may, itself, induce structural distortion in the right half of the iteron.

Monomer-bound complexes of His-\( \pi \)WT and the variant, His-\( \pi \)P106L/F107S, produced similar footprint results. Dimer-bound complexes, obtained with His-\( \pi \)WT and both variants (His-\( \pi \)P106L/F107S and His-\( \pi \)M36A/M38A) produced footprints that, while similar to each other, constituted only a subset of the signals observed in monomers. This shows that the mutations in \( \pi \) affected only the relative abundance of monomer versus dimer in a population. The iteron binding characteristics of the variant proteins were unaffected.

The results of our footprinting studies can be interpreted in terms of structural models of \( \pi \) protein that are based on the
crystallography data from other Rep family proteins. The three-dimensional structure of a full-length, monomeric Rep protein (plasmid F-encoded, RepE54) in complex with its iteron DNA has been determined (35). Data analysis revealed a pseudosymmetric protein comprised of two winged helix domains (WH1 and WH2). Recognition helices from each DNA binding motif bind in adjacent major grooves, whereas a β-hairpin wing from each winged helix domain contacts the flanking minor groove. The structure of the dimeric N-terminal WH1 domain of another Rep family protein, pPS10-encoded RepA (closely related to RepE), suggested that the switch from monomer to dimer might involve the disruption and remodeling of the N- and C termini of WH1 (36, 37). Also, the activation of Rep is coupled to dimer dissociation, converting the dimerization domain into a second origin-binding module. In the RepA dimer, the second DNA binding domain in the N-terminal region of the monomer is thought to be occluded due to dimerization.

Based on the sequence similarities of numerous Rep proteins, it seems likely that the overall structure of these proteins is also similar. The initiator proteins (monomer) of plasmids P1, pSC101, pCU1, pPS10, and R6K have been modeled based on the structure of RepE/iteron (F plasmid) using protein alignment (38). In addition, dimers of plasmids P1, F, and R6K-encoded Rep proteins have been modeled based on RepA (plasmid pPS10) dimer structure. The R6K Rep protein, π, is unique in that, unlike other Rep proteins, the dimer form is capable of binding to the iteron (12, 15).

Our in vitro footprinting results as well as the iteron mutational analysis show that a π monomer contacts the two consecutive major grooves and a flanking minor groove along one face of the DNA helix (Fig. 8). These results are consistent with a model for a π monomer bound to iteron DNA proposed by Sharma et al. (38) on the basis of the RepE/iteron co-crystal structure (35) and the similarity of π to the Rep family protein. Although certain features of the RepE and π interactions with their respective iterons will necessarily differ, some of the backbone and base-specific contacts detected in the π footprints (Fig. 7) appear to involve positions analogous to those identified as RepE/iteron contacts in the co-crystal structure (e.g., protection of R6K iteron position G7 in the conserved 5′-TGAGG-3′ motif; see Figs. 7 and 8).

In contrast to the iteron/π monomer interaction, dimers of π contact only one major groove in the left half of the iteron. Based on the similarities of π protein with RepE and RepA structures, our results suggest that the C-terminal DNA binding domain of both forms of π (monomer and dimer) contacts the left half of the iteron (Fig. 8). The N-terminal DNA binding domain of the π monomer contacts the adjacent major groove (in the right half of the iteron), but upon dimerization this domain becomes occluded. It is possible that the C-terminal DNA binding motif is surface-exposed and available for DNA binding in each subunit of a π dimer. This might facilitate “handcuffing,” a phenomenon that has been proposed to inhibit replication (39, 40) by allowing the C-terminal DNA binding helix from each monomer (in a dimer) to contact parallel DNA helices. Alternatively, the binding of π dimers could foster π-dependent DNA looping between the multiple replication origins of plasmid R6K (41). Multiple origins are not common among prokaryotic replicons, and the unique ability of π dimers to bind iterons may relate to ori selection. Work is under way to study the interactions of π with multiple iterons. π protein also has the ability to autorepress its own transcription by binding to the inverted repeat (42). Only dimers of π bind to the inverted repeat (15). Experiments probing the interactions of π dimers with inverted repeats are also under way.

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Binding Modes of the Initiator and Inhibitor Forms of the Replication Protein π to the \( \gamma \text{ori} \) Iteron of Plasmid R6K
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