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Mutational and *in vivo* methylation analysis of F-factor PifC protein binding to the *pif* operator and the region containing the primary origin of mini-F replication

(*in vivo* footprinting/gene regulation/DNA–protein interaction/plasmid replication/*oriV1*)

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ABSTRACT **We have used *in vivo* methods to identify multiple DNA-binding sites for the negatively autoregulated mini-F replication factor PifC. Sequence analysis of *pif* operator constitutive mutants, isolated as insensitive to repression by PifC, establishes the structure of *pifO*. This site contains a 17-base-pair (bp) region of dyad symmetry with 7-bp perfect inverted repeats separated by 3 bp. *In vivo* DNA methylation studies with dimethyl sulfate show that the reactivity of five of six guanine residues in the *pifO* region is altered in the presence of PifC protein. In addition, there are several sites of PifC-dependent methylation enhancement and protection upstream of *pifO* within repeated sequences bearing homology to *pifO*. The significance of the repeated PifC binding sequences and their relationship to the primary origin of mini-F replication (*oriV1*) are discussed.**

It has recently been shown that replication originating from the mini-F primary origin (*oriV1*) requires the product of the *pifC* gene (1). We have demonstrated that the PifC protein negatively regulates expression of the F factor *pif* operon, which contains the *pifC*, *pifA*, and *pifB* genes (2, 3). The *pifA* and *pifB* gene products inhibit development of bacteriophage T7 (4–6), and the *pifC* product, in addition to its role as an autoregulated replication factor, inhibits conjugal transfer of the broad host-range plasmid RP4 (7).

In view of its roles in replication and regulation of gene expression, we wished to characterize the interaction of the PifC protein with DNA target sites in the *pif* regulatory region and at *oriV1*. *pifO*, the site required in *cis* for autoregulation, leads to derepression of the F-factor *pif* operon when present in *trans* on high copy-number plasmids (3). In addition, *pifO* is located in the region within which bidirectional replication fork initiation has been mapped by electron microscopy (8). We now report several nucleotide sequence alterations that result in *cis*-dominant *pifO*^{c} mutations.

To study interactions between PifC protein and its DNA target sites *in vivo*, we have developed a procedure, similar to that described by Nick and Gilbert (9) and Giniger *et al.* (10), that uses dimethylsulfate as a chemical probe to analyze DNA–protein interactions. Using the *in vivo* footprinting technique we have detected PifC-dependent alterations in the methylation pattern of the *pifO* region. Several additional binding sites have been detected upstream of *pifO* and occur at repeated sequences that bear homology to *pifO*.

MATERIALS AND METHODS

Strains, Plasmids, and Media. The *Escherichia coli* K-12 strain used was RV200 (F$^-$ Δ*lacX74 rpsL200*). Solid and liquid growth media (ML broth) have been described (2, 5). The F-factor DNA sequences present in plasmids pEB100 (E. Buchert, personal communication) and pMBO80 (11) are shown in Fig. 1. pEB100 consists of the 3.9-kilobase (kb) *Eco*RI fragment from pGS103 (6) cloned into the *Eco*RI site of pBR322. pMBO80 contains the 570-base-pair (bp) *Bam*HI/*Pvu* II fragment from pGS103 cloned between the *Bam*HI and *Pvu* II sites of pBR322. pEB100Cam5 (E. Buchert, personal communication) is a pEB100 derivative containing the *supF* suppressible *pifC5(Am)* amber mutation, which maps at ≈42.0 kb. pEB100Cam5 is completely PifC$^-$ in the Su$^-$ strain RV200.

DNA Manipulations. Isolation of plasmid DNA, use of restriction endonucleases, DNA ligation, and transformation procedures have been described (2).

Sequence Analysis of *pifO*^{c} Mutations. The *pifO36*, *pifO53*, *pifO55*, *pifO56*, *pifO57*, *pifO59*, *pifO62*, *pifO63*, and *pifO70* mutations were isolated on F'C521 (2) and recombined onto plasmids pVU14 (3) or pMBO80 as described (3). The nucleotide sequences of the mutant *pifO* regions were determined by the method of Maxam and Gilbert (12). The top-strand sequence was obtained by 5'-end-labeling at the *Mlu* I site (42.56 kb; Fig. 1) with polynucleotide kinase (New England Nuclear) and [γ-^{32}P]ATP, and the bottom strand sequence was obtained by 3'-end-labeling at the same site using DNA polymerase I/Klenow fragment (International Biotechnologies) and [α-^{32}P]dCTP (12).

***In Vivo* Methylation Analysis.** RV200 carrying pMBO80, pEB100, or pEB100Cam5 was grown overnight at 37°C in ML broth containing ampicillin at 50 μg/ml (Sigma), diluted 1:250 into 25 ml of ML broth, and incubated at 37°C with vigorous shaking. Twenty milliliters of logarithmic-phase culture (OD_{600} = 0.6) was withdrawn and the cells were pelleted in a Sorvall SS-34 rotor at 10,000 rpm for 10 min at room temperature. Cells were resuspended in 1 ml of 50 mM sodium cacodylate, pH 8.0/1 mM EDTA and incubated at 20°C for 5 min, at which time dimethyl sulfate was added to a final concentration of 0.5%. Cells were briefly mixed in a Vortex and incubation at 20°C was continued for 2 min. The methylation reaction was terminated by transferring the dimethyl sulfate-treated cells into 30 ml of ice-cold MPBS (150 mM NaCl/4 mM Na_2HPO_4/2 mM KH_2PO_4). Cells were pelleted (10,000 rpm, 10 min, 4°C), washed with 10 ml MPBS on ice, resuspended in 100 μl of 15% sucrose/50 mM Tris·HCl, pH 8.0, and transferred into 1.5-ml microcentrifuge tubes. Cells were lysed by addition of 25 μl of lysozyme (5 mg/ml in 0.25 M Tris·HCl, pH 8.0), incubation at room temperature for 5 min, followed by addition of 50 μl of 0.25 M EDTA (pH 8.0), further incubation for 5 min, and addition of 40 μl of TLM (0.3% Triton X-100/200 mM EDTA/150 mM Tris·HCl, pH 8.0). Incubation at room temperature was continued until cell lysis was evident (≈10 min). Samples were centrifuged in a microcentrifuge for 15 min and the

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

pellets were removed. Heat-inactivated DNase-free RNase (1 μl; 10 mg/ml) was added followed by incubation at room temperature for 10 min. Protein was extracted with 200 μl of phenol/$CHCl_3$ (1:1, vol/vol). Samples were mixed in a Vortex, centrifuged for 10 min, and the aqueous layers were extracted with $CHCl_3$. DNA was precipitated by addition of cold ethanol and pelleted by centrifugation for 10 min. DNA pellets were rinsed with 1 ml of cold 70% ethanol and dried under vacuum in a Speed Vac concentrator. Samples were then resuspended in 50 μl of 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

Methylated DNA (50 μl) was digested with appropriate restriction enzymes (see *Results*) and 3'-end-labeled with DNA polymerase I/Klenow fragment and [α-^{32}P]dNTP as described (12). End-labeled fragments were isolated from 5% polyacrylamide gels (12), ethanol-precipitated twice from 0.3 M sodium acetate, rinsed with 70% ethanol, and dried under vacuum. DNA was cleaved at sites of guanine methylation by resuspending pellets in 100 μl of 1 M piperidine and incubating at 90°C for 30 min. Piperidine was removed by lyophilization, followed by two rounds of resuspension in 100 μl of H_2O and further lyophilization. Dry pellets were resuspended in sequencing dye (80% deionized formamide/10 mM NaOH/1 mM EDTA/0.1% xylene cyanol/0.1% bromphenol blue), heated for 3 min at 90°C, cooled on ice, and electrophoresed on thin 8% polyacrylamide sequencing gels (12).

RESULTS

Nucleotide Sequence of *cis*-Dominant Pif Regulatory Mutations. The presence of the *pifC* gene in *trans* to a *pifC–lacZ* fusion carried on F'lac or bacteriophage λ derivatives results in repression of fusion expression and a Lac$^-$ phenotype (2, 3). Spontaneous independent *cis*-dominant operator constitutive *pifO* mutations that result in decreased inhibition of *pif* operon expression in the presence of the *pifC* product have been isolated (3). We have located nine of these mutations, by recombinational analysis, to the 570-bp *Bam*HI/*Pvu* II fragment present on pMBO80 (Fig. 1). Analysis of the nucleotide sequence of this fragment (17) showed a single open reading frame capable of coding for the PifC protein with a region of dyad symmetry preceding the putative translation initiation codon and ribosome-binding site.

The nucleotide sequence of each of the *pifO* mutations was determined by the protocol of Maxam and Gilbert (12). As shown in Fig. 2, each of the operator constitutive mutants had single base substitutions or deletions located within a 17-bp region, with 2-fold rotational symmetry, containing perfectly matched 7-bp inverted repeats. Eight of nine *pifO* mutations are located in the right-hand repeat, and five of these mutations result from identical A-to-G transitions in the top strand at position 658 (see Fig. 2 for explanation of nucleotide coordinates). This set of mutations is particularly interesting because the resulting sequence, CC(T/A)GG (position 655–659), is recognized by the *dcm* methylase of *E. coli* K-12, which methylates position C-5 of the internal cytosine residue (18, 19). Since 5-methylcytosine reacts very slowly with hydrazine (20), the corresponding bands are absent in sequencing ladders obtained by the chemical protocol. The identity of the mutations at position 658 was verified by sequencing both strands.

***In vivo* Methylation Analysis of *pifO*.** Reaction of dimethyl sulfate with duplex DNA, both *in vitro* and *in vivo*, results in preferential methylation at the N-7 position of guanine, which lies in the major groove (21). The presence of DNA-binding proteins can alter the relative reactivity of purine residues that are involved in specific DNA–protein interactions. We have developed a simple method for the analysis of DNA–protein interactions as they occur *in vivo*, by using partial methylation by dimethyl sulfate. Our procedure is similar to that reported by Nick and Gilbert (9) and Giniger *et al.* (10) except that sites of methylation are visualized without the need for genomic Southern transfer and hybridization with radiolabeled probe.

Cells containing plasmid pMBO80 (*pifO*$^+$ Δ*pifC*), pEB100 (*pifO*$^+$ *pifC*$^+$), or pEB100Cam5 [*pifO*$^+$ *pifC5(Am)*; see Fig. 1] were gently harvested during exponential growth, concentrated, and treated with dimethyl sulfate for 2 min at a final concentration of 0.5%. Plasmid DNA was isolated (see *Materials and Methods*) and digested with the appropriate restriction enzymes. Although treatment with dimethyl sulfate increases the susceptibility of DNA to breakage, plasmid-specific restriction fragments were easily seen against a background of chromosomal fragments. Cohesive ends generated by restriction enzyme treatment were 3'-end-labeled using DNA polymerase I (Klenow fragment) and α-labeled deoxynucleoside triphosphate. The 430-bp *Mlu* I/*Bam*HI fragment (positions 562–992) and the 306-bp *Mlu* I/*Nco* I fragment (positions 562–868) were isolated. Partially methylated purified end-labeled fragments were cleaved at sites of guanine methylation by heating in the presence of 1 M piperidine, and the denatured products were analyzed by electrophoresis on a sequencing gel.

The effect of PifC protein on the *in vivo* methylation pattern of both strands of the *pif* operator is shown in Fig. 3 (bottom strand labeled at the *Mlu* I site at position 561) and Fig. 4 (top strand labeled at the *Nco* I site at position 869). The extent of methylation of five of the six guanine residues in *pifO* is affected by the presence of functional PifC protein. Enhancement is seen at positions 651 (bottom strand) and 659 (top strand), with a remarkably strong enhancement at position 656 (bottom strand), which lies in between the inverted

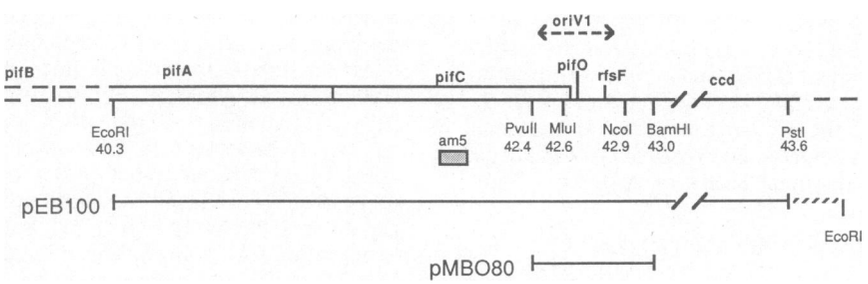

FIG. 1. Structure of pBR322 derivatives carrying sequences from the F-factor *pif–oriV1* region. Restriction sites have been assigned F-factor kilobase coordinates as described (2, 3). This figure is drawn to scale and interruptions are indicated by slashed lines. The top line represents F-factor DNA sequences. The F sequences retained by plasmids pEB100 and pMBO80 are indicated by solid lines. pEB100 contains a 0.46-kb *Pst* I/*Eco*RI linker derived from the F-factor *Eco*RI fragment f6, located to the left of the *Pst* I site at 43.6 kb. The coding sequences for the *pifC*, *pifA*, and *pifB* gene products (2) are shown as open rectangles. *pifO*, *oriV1*, and *rfsF* are described in the text. The *ccd* locus (also termed *lynA* and *let*) causes inhibition of cell division when the copy number of mini-F drops to ≈1 per cell (13–15) and is capable of generating an SOS signal (16). The approximate location of the *pifC5(Am)* mutation present on pEB100Cam5 is indicated (E. Buchert, personal communication).

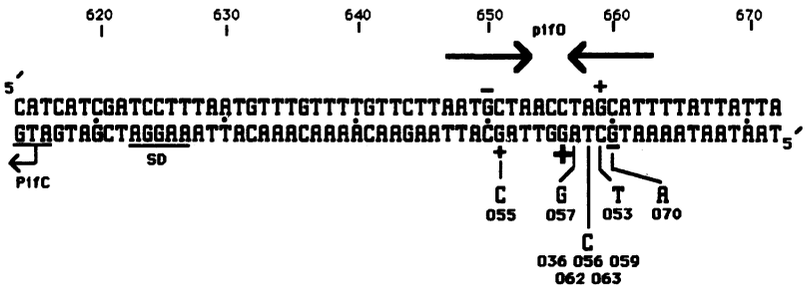

FIG. 2. Mutational and *in vivo* methylation analysis of the *pif* regulatory region. Nucleotide coordinates are derived from F-factor kilobase coordinates such that position 620, for example, corresponds to 42.620 kb on the F-factor map (see Fig. 1). The putative PifC translation initiation signals are indicated and were deduced from the nucleotide sequence of the *pif* region from 42.100–42.997 kb (17). Nucleotide-sequence alterations in the bottom strand that result in spontaneous *pifO*^{c}-regulatory mutations are shown along with the allele designation. The perfect 7-bp inverted repeats contained within the *pifO* region are indicated by arrows. Sites of *in vivo* methylation protection and enhancement observed in the presence of PifC protein are designated by − and +, respectively, and are determined from results shown in Figs. 3 and 4. SD, Shine–Dalgarno sequence.

repeats. Protection is seen at positions 650 (top strand) and 660 (bottom strand). These effects are observed in an Su$^-$ host with plasmid pEB100 but not with pEB100Cam5, which carries an amber mutation in *pifC*, or with pMBO80, which is deleted for most of the PifC coding sequences. Figs. 3 and 4 also show that the Maxam and Gilbert guanine tracts obtained *in vitro* with linear deproteinized DNA are nearly identical to those obtained *in vivo* in the absence of functional PifC protein. Sites of protection and enhancement are summarized in Figs. 2 and 5.

It is likely that protection against methylation can result from steric hindrance. It also seems possible that DNA–protein interactions that alter either DNA conformation or the nucleophilicity of the N-7 position of guanine could result in increased or decreased rates of methylation. The major enhancement at position 656, which occurs on the back face of the DNA helix with respect to PifC binding, could be due to a localized alteration in DNA structure, although the adjacent guanine residue at position 655 is not appreciably enhanced.

***In vivo* Methylation Analysis Reveals PifC-Dependent Alterations Upstream of the *pif* Operator.** The presence of PifC protein results in methylation enhancement and protection at several guanine residues that are located upstream of the *pif* regulatory region. As shown in Figs. 3 and 4, protection is observed at nine sites and enhancement is seen at three upstream guanine residues between positions 673 and 750.

The sites of PifC-dependent methylation enhancement and protection are summarized in Fig. 5. Each of the methylation alterations occurs at or near sites showing homology to the 7-bp sequence that is repeated in a symmetric manner at *pifO* (AATGCTA). These repeated sequences are asymmetric and are present in both orientations. Since the *pifC* product is required for replication of mini-F at *oriV1* (1), the upstream PifC binding sites may be involved in mini-F replication or its control (see *Discussion*).

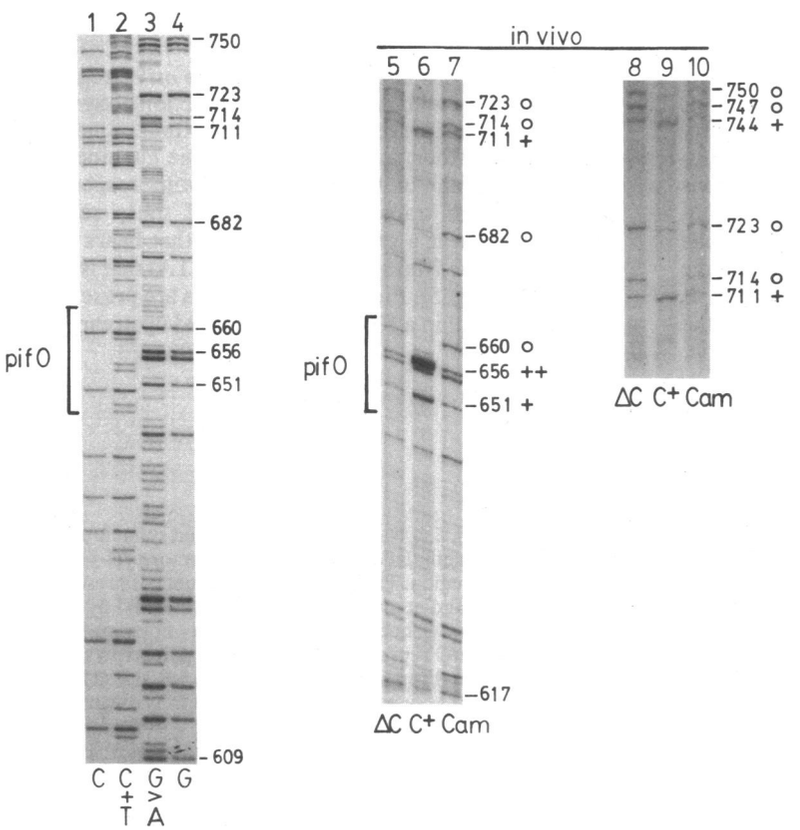

FIG. 3. *In vitro* sequence and *in vivo* methylation analysis of the effect of PifC protein on the methylation pattern of the bottom strand of the *pifO–oriV1* region. Lanes 1–4 show the products of the C, C+T, G>A, and G reactions obtained by using the *in vitro* chemical sequencing protocol of Maxam and Gilbert (12) on the *Mlu* I (42.6 kb)/*Bam*HI (43.0 kb) fragment 3′-end-labeled at the *Mlu* I site. This sequence corresponds to the bottom strand shown in Figs. 2 and 5, and the nucleotide coordinates are described in the legend to Fig. 2. *pifO* designates the location of the inverted repeats shown in Fig. 2. Lanes 5–10 show the *in vivo* guanine footprint obtained from the same end-labeled fragment as shown in lanes 1–4. Lanes 5 and 8, pMBO80 (Δ*pifC*); lanes 6 and 9, pEB100 (*pifC*$^+$); lanes 7 and 10, pEB100Cam5 [*pifC5(Am)*]. The samples shown in lanes 5, 6, and 7 are identical to those in lanes 8, 9, and 10, except that the latter samples were electrophoresed for a longer period of time. + and ++ represent enhanced methylation and o indicates decreased methylation (protection) in the presence of functional PifC (lanes 6 and 9) as compared to the extent of methylation seen in the absence of functional PifC (lanes 5 and 7, and lanes 8 and 10). Approximately equal amounts of radioactivity were loaded in lanes 5–10.

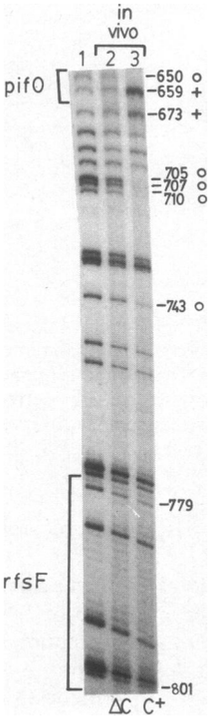

FIG. 4. *In vivo* methylation analysis of the effect of PifC protein on the methylation pattern of the top strand of the *pifO–oriV1* region. Lane 1 shows the *in vitro* methylation pattern of the *Mlu* I (42.6 kb)/*Nco* I (42.9 kb) fragment 3′-end-labeled at the *Nco* I site and sequenced using the guanine reaction described by Maxam and Gilbert (12). The guanine residues are from the top strand as shown in Figs. 2 and 5. Lanes 2 and 3 show the *in vivo* guanine methylation pattern of the top strand of the same fragment shown in lane 1, obtained from plasmids pMBO80 (Δ*pifC*) and pEB100 (*pifC*$^+$), respectively. Nucleotide coordinates are described in the legend to Fig. 2, and *pifO* is defined in the legend to Fig. 3. + and ○ indicate methylation enhancement and protection observed in the presence of PifC (lane 3) compared to absence of functional PifC (lane 2). *rfsF* designates the location of the inverted repeats involved in origin-associated site-specific recombination (11). Approximately equal amounts of radioactivity were loaded in lanes 1–3.

Fig. 4 also demonstrates that there are no PifC-associated alterations in the top strand between positions 751 and 805. We have observed no effects in the top strand from position 806 to 863, or in the bottom strand from position 894 to 981 (data not shown).

DISCUSSION

Single nucleotide substitutions and deletions that result in continued expression of the F factor *pif* operon in the presence of PifC repressor have been isolated (3). These mutations occurred at five positions within a 17-bp region containing perfectly matched 7-bp inverted repeats (Fig. 2). The structure of *pifO* is therefore typical of many prokaryotic DNA–protein binding sequences and suggests that PifC protein binds to this site as a multimer with 2-fold symmetry (see ref. 22 for a review).

Five of the nine independently isolated *pifO*^{c} mutations arise from identical substitutions at position 658. The resulting mutant sequence is recognized and methylated by the *dcm* methylase. The mutations at position 658 therefore have two consequences, an A·T base pair at position 658 is replaced by a guanine·5-methylcytosine base pair, and the cytosine residue at position 656 is methylated. We are currently examining the effect of *dcm* methylation on the function of this class of *pifO* mutants.

In vivo methylation analysis shows that the reactivities of the N-7 positions of five of the six guanine residues in *pifO* are affected by the presence of PifC (Fig. 3 and 4). Sites of methylation enhancement and protection within the inverted repeats of *pifO* are symmetric and indicate that binding to each repeat occurs in a similar manner with contacts in adjacent major grooves on the same face of the helix. The marked enhancement observed at position 656, at a guanine residue in the central portion of the *pif* operator, occurs in the major groove on the opposite face of the helix.

Although *in vivo* methylation results indicate that several alterations are dependent on the presence of PifC, they do not provide direct proof that PifC actually binds to these sites. Alternative explanations for these results would include the possibility that PifC protein is required for binding of an unidentified DNA-binding protein but does not bind to DNA itself. We have no genetic evidence for the involvement of additional *trans*-acting factors in the regulation of *pif* expression. Proof that the sites of *in vivo* methylation enhancement and protection result from binding of PifC to DNA will require *in vitro* footprinting with purified PifC protein.

Several proteins that are involved in the initiation of plasmid DNA replication have been shown to be negatively autoregulated (see ref. 23 for a review). For the F-factor derivative mini-F and the autonomously replicating prophage P1, genetic evidence suggests that the autoregulated *repE* (mini-F) and *repA* (P1) gene products bind to sites that function in *trans* as incompatibility loci and may serve to adjust the intracellular concentration of these proteins (24–28). The mini-F *incB* and *incC* loci are composed of a series of directly repeated 19-bp and 22-bp sequences, respectively (29, 30), which contain an 8-bp sequence that is homologous to one arm of the putative operator sequence of the *repE* gene. In addition, the location of the *incB* repeats coincide with the location of the unidirectional secondary origin of mini-F replication (*oriV2*) as determined by electron microscopy (31) and deletion analysis of *oriV2*-dependent replicons (29).

The interactions between PifC and the *oriV1* region share several features with the proposed interaction between RepE and *oriV2*. In the presence of PifC, the extent of methylation is increased at four, and decreased at nine guanine residues located upstream of the *pifO* region with respect to the direction of *pif* transcription. Each of these residues is located at or near a repeated sequence that bears 70–100% homology to the 7-bp sequence (AATGCTA) that is repeated within *pifO* (Fig. 5). Eight of these repeats are present in both orientations within a 100-bp region located upstream of *pifO*.

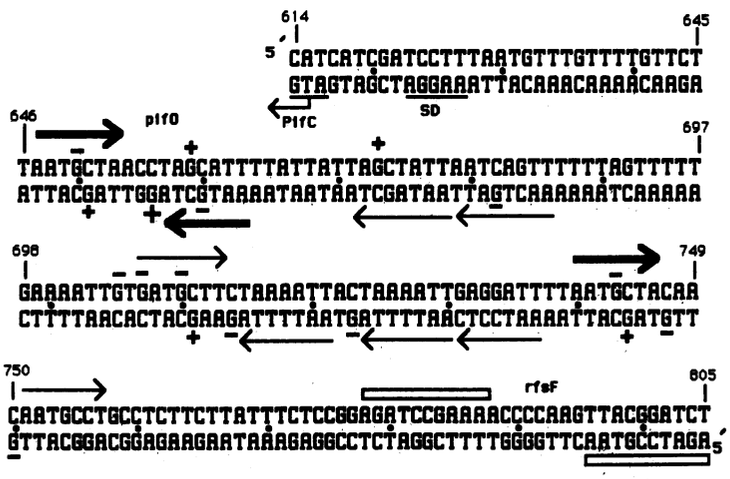

FIG. 5. Nucleotide sequence and summary of *in vivo* methylation analysis of the F-factor *pifO–oriV1–rfsF* region. The 7-bp repeated sequences present in *pifO* and at an upstream site are indicated by wide arrows. Sequences that are partially homologous to the *pifO* repeats are indicated by thin arrows. The inverted repeats comprising the *rfsF* site (11) are shown as open rectangles. Sites of *in vivo* methylation protection and enhancement observed in the presence of PifC protein are designated by − and +, respectively. Nucleotide coordinates are described in the legend to Fig. 2. The location of *oriV1* is discussed in the text.

Some of the PifC-dependent methylation effects at these repeats are similar to those seen at *pifO* (i.e., protection at positions 682, 710, and 743; enhancement at positions 673, 711, and 744). However, the absence of internal methylation alterations and the presence of adjacent protection at other repeats is in contrast to the effect of PifC on *pifO* methylation. It therefore appears that PifC is capable of both symmetric binding to the *pif* operator as well as binding to the asymmetric upstream repeats, although different forms of the protein (monomeric vs. multimeric) may be involved.

Although the *cis* requirements for *oriV1* function have not been precisely determined, electron microscope studies place the origin at ≈42.6 kb (ref. 8; see legend to Fig. 2 for explanation of F-factor kilobase coordinates). Deletion of the sequences between 42.8 kb and 43.0 kb eliminates origin function (1), and sequences upstream of 43.0 kb are required in *cis* for replication (19). *pifO* and the upstream PifC binding sites are located within the *oriV1* region, between coordinates 42.646 and 42.757 kb. In addition, this region contains a 44-bp sequence (positions 661–704 in Fig. 5) that is 86% A+T-rich, a feature that is commonly found at or near replication origins (29).

The function of the PifC binding sequences located upstream of *pifO* is not known. One or more of these sequences may constitute the site at which PifC binding occurs during the initiation of replication from *oriV1*. The fact that several binding sites are present might indicate a requirement for cooperative binding or that one or more of the repeats functions to titrate PifC protein, thereby adjusting its intracellular concentration. Similar titration models have been proposed for the mini-F *oriV2* replicon (24, 26) and for the regulation of P1 prophage replication (32).

Both of the above models require that the titrating sequences do not derepress the autoregulated replication initiator. The upstream PifC binding sequences do not efficiently derepress the *pif* operon in *trans*. *oriV1* regions containing single mutations in *pifO* (see Fig. 2), when present on pSC101 or pBR322 replicons, have little effect on F'lac *pif* expression when compared to the *trans* effect of the wild-type operator (3).

The *in vivo* methylation experiments were performed with both *pifC* and *pifO* present on high copy-number pBR322 derivatives, whereas mini-F is normally present at 1–2 copies per cell. In the multicopy situation, the intracellular concentration of PifC would be increased, but this increase would be disproportionately lower than the increased gene dosage (33). The ratio of PifC monomers to *pifO* sites or upstream binding sites, relative to mini-F, would therefore be expected to be decreased as a result of high copy-number and autoregulation.

The F factor is capable of mediating site-specific *recA*-independent recombination between two *oriV1* regions at a specific site termed *rfsF* (refs. 11 and 34; Fig. 5). Our genetic analysis indicates that PifC is not required for this reaction and we find no difference in the methylation pattern of *rfsF* in the presence or absence of PifC. The relationship between the *rfsF* site and *oriV1* remains to be determined.

The *in vivo* DNA footprinting technique described in this paper is a general method that should be applicable to many prokaryotic and eukaryotic systems. The technique requires that the DNA sequence of interest is contained, either naturally or by means of recombinant DNA techniques, on an extrachromosomal element that can be isolated free from chromosomal and other extraneous DNA. In addition, the DNA binding protein(s) of interest must be present in sufficient quantity to ensure that a significant portion of the DNA-binding sequences are complexed with protein at any given time. The major advantages of our technique are the simplicity of the steps involved and the high level of resolution that can be obtained.

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