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Initiator protein π can bind independently to two domains of the γ origin core of plasmid R6K: the direct repeats and the A+T-rich segment

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ABSTRACT

The π protein of plasmid R6K functions in both replication and transcription. π autoregulates its own synthesis and is required for replication of the R6K γ origin. π performs these functions by binding to specific DNA sites arranged as pairs of 6–10 bp inverted repeats (IRs) or as a cluster of seven tandem 22 bp direct repeats (DRs) which lack symmetry. The sites share the TGAGRG nucleotide motif (where R is A or G). The DRs and IRs flank the central A+T-rich segment of the γ origin. In this work we carried out DNase I and hydroxyl radical protection experiments on various deletion derivatives of the γ origin complexed with π protein. These experiments revealed binding of π to a novel site embedded within the A+T-rich segment. This interaction manifests primarily by the appearance of the enhanced scissions of DNA by DNase I and hydroxyl radicals. π interaction with the A+T-rich site is independent of π binding to the DRs and IRs. We propose that π protein can recognize distinct families of DNA sequences in the γ origin.

INTRODUCTION

For many replicons, initiation of DNA replication depends on replicon-specific initiator proteins. These proteins localize the origin and allow an ordered series of protein–protein interactions, culminating in the development of the replication fork. Initiator proteins also frequently function as transcription factors negatively regulating expression of their own genes. Localization of the origin and repression of transcription depend on sequence-specific binding of the initiator to DNA. These sequences are frequently called iterons.

This laboratory studies a basic replicon derived from plasmid R6K because it is stably maintained at a defined copy number in logarithmically growing cultures (1). Hence, it has all the features needed to elucidate the various aspects of regulated initiation of replication and plasmid stability (1,2). As shown in Figure 1, such a replicon is composed of a *cis*-acting γ origin sequence and π protein encoded by the R6K *pir* gene which can function either in *cis* or in *trans* (3,4). The π protein is bifunctional in replication; it activates the origin at low a level and inhibits replication at a high level (5,6). A balance between these two activities is believed to influence the frequency of initiation of DNA replication and consequently the plasmid copy number. The mechanisms allowing π protein to either activate or inhibit replication are not known. In addition to its function in replication, π acts as a transcription factor negatively regulating the expression of its own gene, *pir* (7–9). The π possesses sequence-specific DNA binding activity; it recognizes sites of different sequence composition and geometry. However, all sequences known to bind π share the TGAGRG nucleotide motif (where R is A or G) (2,10).

As demonstrated by several different assays, π protein binds the seven 22 bp direct repeats (DRs) in the γ origin (8,11–13). More importantly, a direct relationship between γ origin function and π protein binding to the DRs has been also demonstrated (14). It was found that G to A transitions at the first and second Gs in the TGAGRG motif of either the 1st or 6th DR resulted in the inability of π to bind to the mutated repeat *in vitro* and the concomitant loss of γ origin activity *in vivo* (14). We have recently shown that these same Gs are protected by π protein against methylation *in vivo* (15). A positive role for the seven 22 bp DRs in replication was also demonstrated with deletion mutants of the γ origin that precisely removed some of the DRs (16). Although a γ origin lacking one or two DRs remains functional, deletion of three or more of them inactivated the origin (16).

Yet another π binding site has been recently discovered in the enhancer segment of the γ origin (1). The enhancer π site contains a pair of inverted half repeats (IRs). Mutational analysis revealed that each half site is required for π binding, but the site has no known function (1). Another IR forms a core of the *pir* gene operator to which π binds, thereby regulating its own expression (13). Interestingly, the pattern of protection and cleavage of IRs in the enhancer and *pir* gene operator are very similar (1,8,9,13); thus π probably binds both pairs of IRs similarly.

A+T-rich segments are common among many origins replicating via the Cairns mode (17,18). Their thermodynamical instability is expected to aid in the development of the replication fork. It is of fundamental interest, therefore, to determine which proteins can bind to such segments and, by doing so, may perhaps alter their stability. DNase I and hydroxyl radical footprinting data presented in this paper, clearly show that π protein can bind to the A+T-rich segment independently on binding to any other site within the boundaries of γ origin (IRs and DRs). The protected region contains a very poor match to the known consensus DNA sequences of π binding sites. Therefore, we propose that π binds to the two families of DNA sequence.

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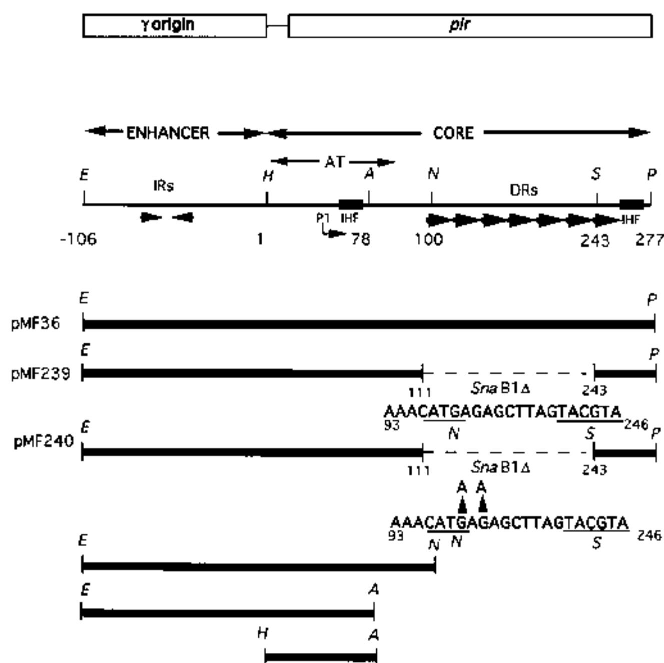

Figure 1. The γ origin replicon of plasmid R6K. (**Top**) The two components required for replication are indicated, namely the *cis*-acting γ origin and *pir* gene which encodes π protein. The thin line between the γ origin and *pir* gene segments indicates that the *pir* gene can be provided in *cis* or in *trans*. (Middle) The following segments/binding sites of the γ origin are indicated: double-headed arrows demarcate the core, enhancer and A+T-rich segments; seven tandem arrowheads indicate 22 bp DRs, head-to-head arrowheads represent IR in the enhancer; IHF (*ihf*1 and *ihf*2 sites); and P1 (silent promoter). Restriction sites that pertain to this study are abbreviated: *A*, *Ase*I; *E*, *Eco*RI (artificial site); *H*, *Hin*dIII, *N*, *Nla*III; *P*, *Pst*I; *S*, *Sna*B1. (**Bottom**) Portions of the γ origin contained in various γ origin derivatives. Dotted lines indicate deletion of six DRs in pMF239 and pMF240. The nucleotide sequence of the single DR is shown. Mutations in pMF240 are indicated. Coordinates correspond to DNA sequence obtained by Stalker *et al*. (23).

MATERIALS AND METHODS

DNA templates

The construction of plasmids pMF36 (which carries seven 22 bp DRs), and pMF239 (which carries one of these 22 bp DRs) has been described (5,19). The pMF240 (which carries one mutated 22 bp DR; Fig. 1) was made using a mutant which has three base changes; two indicated in Figure 1 at 7th, 9th, a third at 21st position (not indicated) in the first 22 bp DR creates *Sna*B1 site. Steps for constructing pMF240 involving a deletion of *Sna*B1 fragment containing six DRs were identical to those described for the construction of pMF239 except mutant γ117 instead of γ134 was used (19,20). For footprinting analysis, these plasmids were 3′- or 5′-end-labeled at the artificial *Eco*RI site (coordinate –106), the naturally occurring *Hin*dIII site (coordinate +1) or *Ase*I site (coordinate +78) as described (11), followed by digestion with another restriction enzyme indicated in the figure legend.

DNase I and hydroxyl radical protection assays

DNase I footprinting was performed as described (11,13). Samples (5–10 ng) of ^{32}P-end-labeled DNA were incubated at 25°C for 10 min in 20 µl of the following buffer (20 mM Tris–HCl pH 7.5, 6 mM $MgCl_2$, 1 mM EDTA, 166 mM K-glutamate) containing varying amounts of purified π protein. Samples were treated with 1 U DNase I (Boehringer Mannheim), freshly diluted from stock with the reaction buffer supplemented with 50 mg BSA/ml. Digestion was allowed to proceed for 30 s at 25°C. The reaction was stopped by the addition of 50 µl 20 mM Tris–HCl pH 7.5, 20 mM EDTA, 0.1% SDS. Samples were treated with phenol and DNA was ethanol precipitated. DNase I cleavage products were analyzed on 7 M urea–8% polyacrylamide gels.

Hydroxyl radical footprinting was performed according to the published protocol (21). A 50 µl reaction mix contained the same reaction buffer which was used for DNase I footprinting (see above). To each reaction sample 0.6 µl 100 mM sodium ascorbate (Sigma) was added, followed by 4 µl 0.15% (v/v) H_2O_2 (J. T. Baker Inc.) and 6 µl of a solution of freshly mixed 50 mM $Fe(NH_4)_2(SO_4)_2$ (Sigma) and 100 mM Na_2EDTA (Sigma). Cleavage was allowed to proceed for 2 min at 37°C, after which 80 µl 20 mM thiourea (Sigma) and 60 µl 0.3 M NaCl were added to quench the reaction. The samples were then precipitated with ethanol using 1 µg glycogen (Boehringer Mannheim) as carrier. Cleavage products were analyzed as described for DNase I footprinting.

Purification of π protein

The π protein was purified according to the previously published procedure (11). Briefly, *Escherichia coli* frozen cell paste, containing overproduced π protein from vector pPT39 (11) was thawed (4°C) and suspension adjusted to 100 mM KCl, 5 mM DTT and 200 µg/ml lysozyme. The lysis procedure was as described (11). The lysate was centrifuged at 30 000 *g* for 30 min and the supernatant was loaded onto a heparin–Sepharose column (0.9 × 5 cm), equilibrated with the buffer containing 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 1 mM DTT with 100 mM KCl. Proteins were eluted with 75 ml of a linear gradient of KCl (0.1–1.0 M). Fractions containing π protein, identified by SDS–PAGE, were pooled and loaded onto a hydroxylapatite column (0.9 × 3.0 cm) equilibrated with the buffer containing 20 mM KPO_4 pH 7.0, 50 mM KCl and 1 mM DTT. Proteins were eluted with 30 ml of a linear KPO_4 gradient (0.02–0.50 M). Fractions containing the π protein, which eluted at ~0.4 M KPO_4, were aliquoted and stored frozen at –70°C. Purified π protein was at least 95% pure as determined by Comassie blue staining of polyacrylamide gels obtained by of SDS–PAGE analysis. In some experiments we used π protein extracted from exclusion bodies and purified by an alternative protocol which was described elsewhere (22).

RESULTS

Background information and rationale

It has been originally observed in DNase I footprinting assays that the area of strong protection at coordinates +93 to +246 and periodic enhancements, each in seven DRs, are produced by π binding to a DNA fragment containing the entire γ origin (11). Moreover, another 'footprint' noted in the presence of π seems to lie outside the DRs (11). This second footprint has been localized within the segment adjacent to DRs that is A+T-rich; the enhanced DNase I cleavage sites at coordinates +15 and +51 form the boundary of the protected area. The altered susceptibility of the A+T rich segment to DNase I cleavage in the presence of π can be caused by one of the several factors or their combination: (i) π protein may bind to this site independently of binding to the

other sites. (ii) π binding to the A+T-rich segment may require prior occupancy of DRs and/or IRs (positive cooperativity). (iii) π binding to the seven DRs and/or IRs may induce a change in the conformation of the A+T-rich segment altering its susceptibility to the DNase I cleavage. Because previous experiments showing enhancements in the A+T-rich region used DNA fragments carrying other π sites, we could not distinguish between these possibilities (1,11). We describe in this paper the exploration of these various possibilities.

π protein alters the pattern of DNase cleavage in the A+T-rich segment independently of binding to the DRs and IRs

Our reference sample was prepared with the *Eco*RI–*Pst*I fragment obtained from plasmid pMF36. This plasmid contains the entire γ origin sequence [nucleotides (nt) –106 to +280; see Fig. 1]. As shown in Figure 2, there are several prominent features in the resulting digestion patterns. First, the area containing cluster of seven 22 bp DRs is protected. A specific phosphodiester bond (the first A in the TGAGRG motif) is hypersensitive to DNase I cleavage in each DRs unit forming a characteristic ladder of enhancements. Secondly, the two positions (+15 and +51) in the A+T-rich segment are hypersensitive to cleavage. Thirdly, in the enhancer segment, protection is evident from nt –71 to –46, with enhancement at positions –46 and –68. This region contains IRs motif (Figs 1 and 7). Binding of π to this site has been recently described and it is known that mutating one or both halves of the IRs abolished π binding (1).

Several strategies, described below, were employed to determine if π binding to the A+T-rich segment is dependent on or independent of binding to the DRs in the core and IRs in the enhancer. First, we tested whether the entire DRs cluster is needed for π binding to the A+T-rich segment. In these experiments we used two constructs in which six out of seven DR units are deleted. A single remaining DR unit in these constructs is either wt (pMF239) or mutated (pMF240); see Materials and Methods and Figure 1. Studies with mutant γ-117 indicated that transition mutations at the G_7 and G_9 positions of the 1st DR prevent π binding (14). As shown in Figure 3, even when all but one of the DRs are deleted, π still binds to the A+T-rich region. Furthermore, the characteristic enhancements at coordinates +15 and +51 can be seen with both DNA fragments, regardless of whether π binds (pMF239) or does not bind (pMF240) to the single DRs unit. These results suggest that the enhancements at nt +15 and +51 are produced in a manner independent of π binding to DRs.

To confirm this conclusion, we next carried out footprinting assays with fragments containing only 7 of the 22 bp DR (*Eco*RI–*Nla*III fragment; nt –106 to +101), or lacking any remnants of the DR unit (*Eco*RI–*Ase*I fragment; nt –106 to + 78). Such fragments were obtained from plasmid pMF239 (see Fig. 1). With each of these two fragments the enhancements at the nt +15 and +51 can be clearly seen (Fig. 3). Therefore, we conclude that these enhancements are produced by π binding, and that this binding is independent of binding of π to DRs.

Although the last two templates tested lacked the DRs, they still contained IRs in the enhancer (Fig. 1). Therefore, it was possible that π interaction with the A+T-rich segment depends on the presence of this site. Such a possibility was tested in a series of experiments with a fragment lacking the enhancer. A suitable fragment was obtained by cleaving pMF239 plasmid DNA with

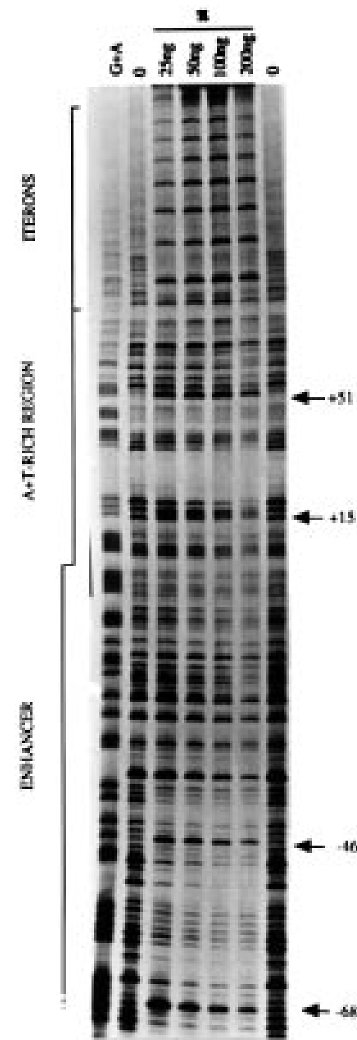

Figure 2. DNase I footprint of π binding to the entire γ origin. The *Eco*RI–*Pst*I γ origin fragment of plasmid pMF36 (shown in Fig. 1) was labeled at the *Eco*RI site and recut with *Pst*I. The enhancer, A+T-rich region and DRs (iterons) are bracketed. Sites of enhanced DNase I cleavage in the presence of π in the enhancer (–46 and –68) and A+T-rich region (+15 and +51) are indicated by arrows.

the *Ase*I and *Hin*dIII; such a cleavage liberates a fragment containing exclusively the A+T-rich segment of the γ origin (nt +1 to + 78; Fig. 1). In this case, by differential end-labeling, we examined DNase I cleavage pattern on both strands of DNA. π-dependent DNase I cleavage enhancements at positions +15, +35 and +51 are seen in the top strand and in positions + 34 and +37 in bottom strand (Fig. 4). The nt +16, +18 and +20 on the bottom strand appear weakly protected against DNase I cleavage. All these data together indicate that π can bind to the A+T-rich region in the absence of any other segments of the γ origin.

π protein alters hydroxyl radical footprinting of the A+T-rich segment

Considering the generally low susceptibility of A+T-rich segments to DNase I, our analysis turned out to be surprisingly productive. However, we wished to obtain independent conformation of π binding to the A+T-rich site by using a reagent that is less selective

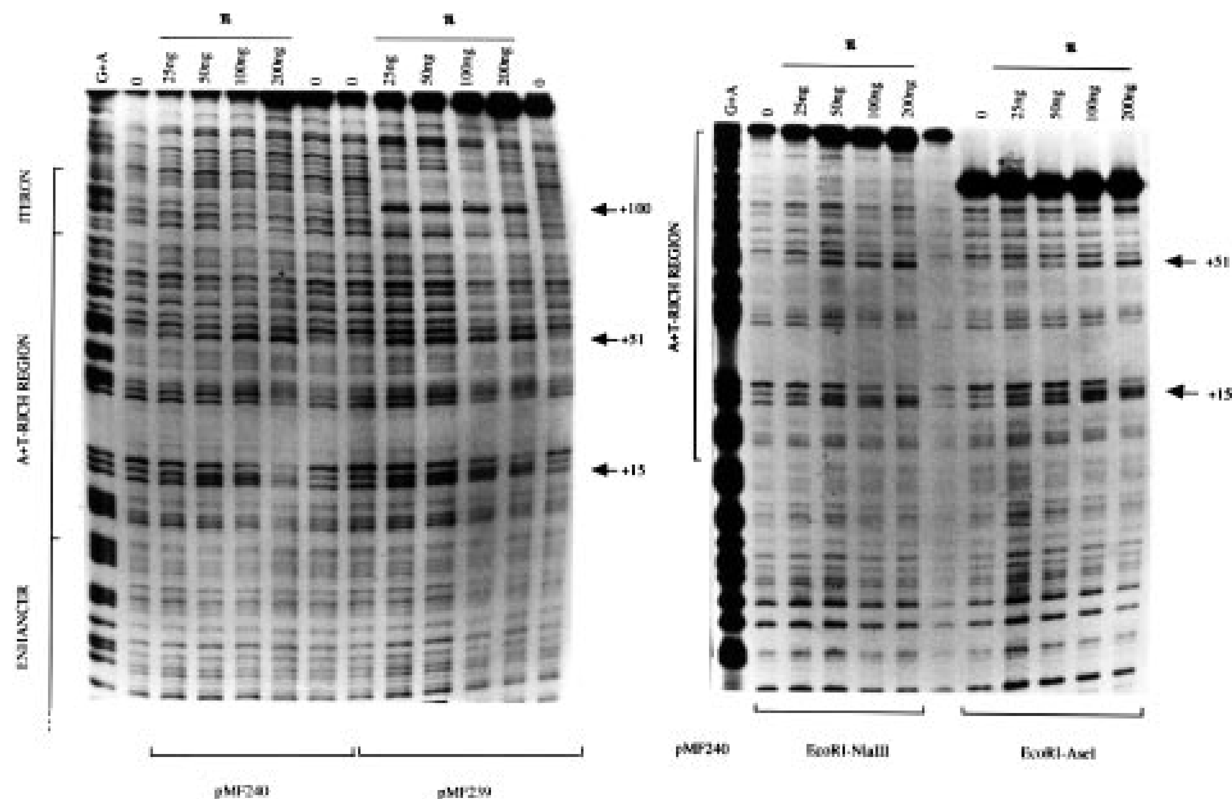

Figure 3. (**Left**). DNase I footprint of π binding to the deletion derivatives of the γ origin (pMF239 and pMF240; see Fig. 1). The *Eco*RI–*Pst*I γ origin fragment of each plasmid was labeled at the *Eco*RI site and recut with *Pst*I. The enhancer, A+T-rich region and a single DR unit (iteron) present on these fragments are indicated. The enhanced cleavage sites in the A+T-rich segment (+15 and +51) and in the wild-type DR (+100) are indicated by arrows. (**Right**). DNase I footprint of π binding to the fragments labeled at *Eco*RI and recut with either *Nla*III or *Ase*I generated from plasmid pMF240 (see Fig. 1). The enhanced cleavage sites in the A+T-rich segment (+15 and +51) are indicated by arrows.

and smaller than DNase I. This was achieved by choosing hydroxyl radicals that attack the deoxyribose sugars arrayed along the surface of DNA (21). This reagent yields greater resolution of the DNA binding reactions than DNase I footprinting which, under most circumstances, merely demarcates the DNA sequence that is bound by a protein.

Binding assays were carried out with the DNA fragment produced by the *Ase*I–*Hin*dIII cleavage. The representative autoradiographs and its densitometric scanning are shown in Figures 5 and 6 respectively. It is clear that π alters the susceptibility of specific backbones to free radicals; the enhancements are seen at nt +15, +19, +20, +26, +30, +34, +35, +51 and +54 in the top strand and at nt +12, +14 - +16, +23, +31, +33 and +34 in the bottom strand. Moreover, weak protection by π can be seen between the following coordinates: 16–18, 21–25, 27–29 and 31–33. These data provide independent evidence for π binding to the A+T-rich segment in the absence of other π binding sites.

DISCUSSION

The interaction of π with the γ origin can be monitored by areas of protection from and enhancements of DNase I cleavage (11,12). We have originally reported that such signals span a large area of the γ origin core encompassing the cluster of seven DRs and the adjacent A+T-rich segment (1,11). Our present investigations of π interactions with the latter segment have been facilitated by the general interest in elucidating the function of the A+T-rich segments that are common to many origins replicating via Cairns mode (17,18).

In this work we employed the wild-type γ origin and various deletion derivatives, dissecting these templates with combinations of restriction enzymes. Such a series of fragments was employed in sensitive footprinting assays. DNase I and hydroxyl radical footprinting data provide compelling evidence for π binding to a site in the A+T-rich γ origin segment. At least *in vitro*, this binding is independent of π binding to previously characterized sites in the core (DRs) and enhancer segments of the γ origin (IRs). It is noteworthy that π interactions with the A+T-rich segment are manifested primarily by the appearance of the enhanced cleavages by DNase I and hydroxyl radicals. This is an unusual feature, particularly for the latter reagent. The enhanced DNase I and hydroxyl radicals cleavages suggest that π binding causes conformational changes in the minor groove of the A+T-rich segment (33). The best of our knowledge enhanced strand scission of specific deoxyriboses by hydroxyl radicals has been observed only in footprints generated by the binding of a reconstituted RNA polymerase (containing the α235 mutant subunits) to the *lac*UV5 promoter (W. Ross, personal communication). The mechanism of the enhanced susceptibility of DNA to the specific cleavage reagents is not known.

The A+T-rich segment contains at least two known sites for the DNA binding proteins: the consensus –35 and –10 hexamers [P1 promoter (23) and Fig. 1] and *ihf*1 site can bind *in vitro* purified

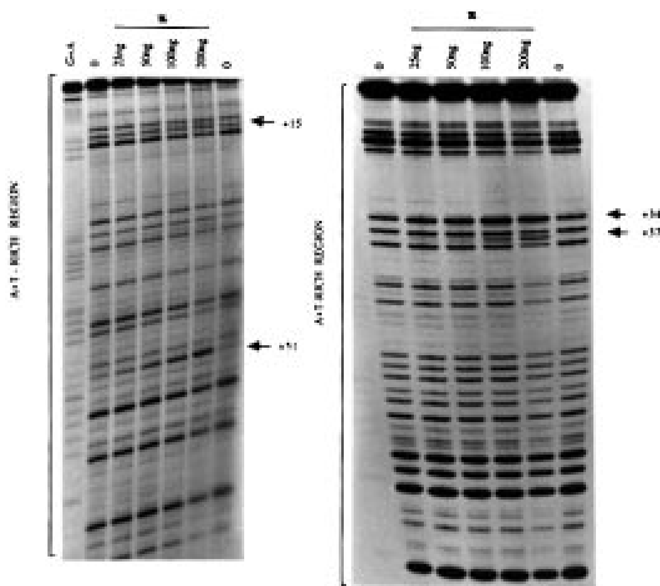

Figure 4. DNase I footprint of π binding to the *Hin*dIII–*Ase*I fragment from plasmid pMF239 (see Fig. 1) labeled at 3′-OH *Hin*dIII site (left) or 3′-OH *Ase*I site (right). The enhanced cleavage sites (+15 and +51 for *Hin*dIII-labeled end) and (+34 and +37 for *Ase*I-labeled end) are indicated by arrows.

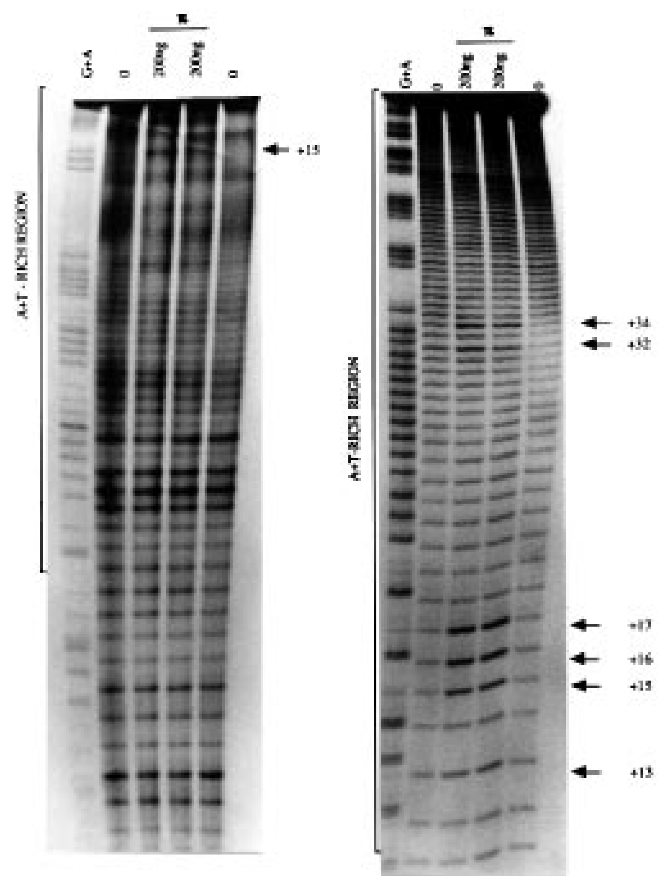

Figure 5. Hydroxyl radical footprint of π binding to the *Hin*dIII–*Ase*I fragment from plasmid pMF239 (see Fig. 1) labeled at 3′-OH *Hin*dIII site (left) or 3′-OH *Ase*I site (right). Some enhanced cleavage sites are indicated by horizontal arrows (see Fig. 6 for densitometric scans).

RNA polymerase (M.F., data not shown) and IHF protein (19,24,25) respectively. The smallest A+T-rich segment tested here and shown to bind π contains the P1 promoter and *ihf*1. However, several facts argue against the possibility that binding of either of these two proteins could be responsible for the footprinting signals we report here. (i) The footprinting pattern produced by IHF and/or RNA polymerase binding are very clearly defined and the cleavage patterns differ from the pattern observed in the presence of highly purified π protein (19,24,25 and data not shown). (ii) Both RNA polymerase and IHF protein interactions with the A+T-rich segment can be monitored by the gel retardation assay, whereas π binding cannot (19,24,25 and data not shown). (iii) The enhancements in the DRs and A+T-rich segment are produced at similar concentration of π (even though those at positions +15 and +51 are weaker). (iv) Similar footprinting data were obtained in the presence of π protein that was extracted from exclusion bodies and purified by an alternative protocol (22 and data not shown). The aforementioned observations argue against the possibility that the footprint in the A+T-rich segment is produced by binding of RNA polymerase, IHF or a contaminating protein. It should be also mentioned that the entire A+T-rich origin segment or the part of it to which π binds cannot be deleted/substituted without the loss of the γ origin function (16,26). This observation remains to be explored to elucidate whether π binding to the A+T-rich segment is essential for the γ origin activity and whether it facilitates activator and/or inhibitor function of π. However, our own data suggest that π may inhibit replication through binding to the A+T-rich segment of the γ origin. The following facts appear to support this notion. (i) IHF protein permits γ origin replication at otherwise inhibitory π levels (6). (ii) IHF reverses the inhibitory effect of π by binding to *ihf*1 site in the A+T-rich segment of the γ origin and not by binding to the other site (*ihf*2) that lies to the left the DRs cluster (32 and Fig. 1). (iii) IHF is not required for *in vivo* and *in vitro* replication dependent on mutant variants of π protein that inhibit replication less than wt π (6,32). (iv) In the neocarzinostatin protection studies, the IHF protein can bind to the *ihf*1 site only when added first; when π protein was added first IHF could not bind to *ihf*1 site (24). Thus, the IHF binding to the *ihf*1 site is occluded by π protein. This latter result is also consistent with the ability of π protein to bind the A+T-rich segment. Additional experimentation will be required to support the aforementioned model.

What might be the DNA sequence in the A+T-rich segment to which π binds? To answer this question, we first briefly review some facts about other well characterized sequences to which π binds (Fig. 7). It was found that enhanced DNase I cleavage sites occur in each of seven 22 bp DRs at A_8. Secondly, double mutant γ-117 at positions G_7, G_9 abolishes π binding to a DR unit when it is either isolated or flanked by wt copies of DRs (14 and Fig. 3). G_7 and G_9 are protected against methylation both *in vitro* and *in vivo* (15,27). Because these protected/mutated bases are within the TGAGRG motif present in both DRs and IRs it is reasonable to assume that a single domain of π protein confers binding to these sites. Although the remaining sequences flanking the TGAGRG core are required for the π protein binding to the 22 bp DRs (Fig. 3 and data not shown), it is not known which of those

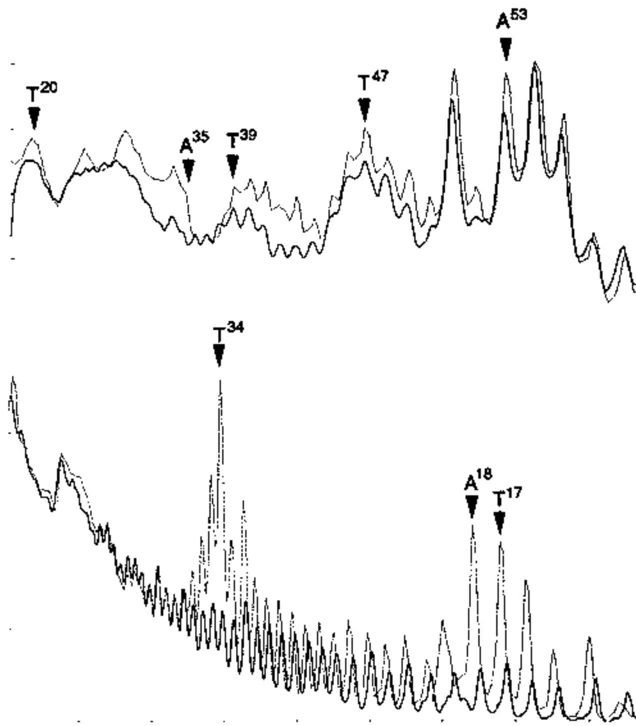

Figure 6. Densitometric scans of the hydroxyl radical footprints shown in Figure 5 for the fragment labeled at 3′-OH *Hin*dIII (**Top**) and 3′-OH *Ase*I (**Bottom**). In both panels the profiles of cleavage products of naked DNA (heavier lines) are shown together with those of π–DNA complex (lighter lines). The most prominent enhanced cleavage sites are indicated.

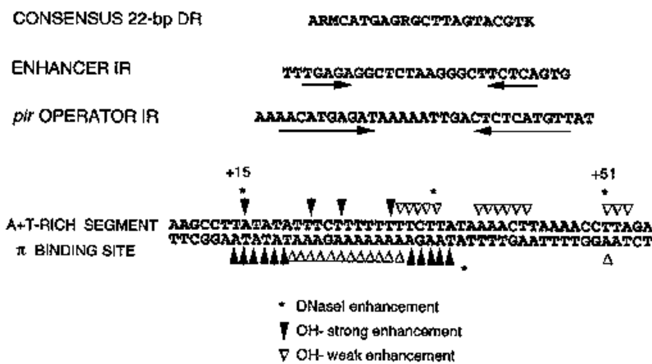

Figure 7. (**Top**) Consensus binding sites for π protein in the γ origin replicon. (**Bottom**) Summary of the DNase I and hydroxyl radical cleavages in the A+T-rich segment of the γ origin.

bp are essential. However, 18 out of 22 bp are fully conserved among all 22 bp repeats (Fig. 7). Six of those 18 bp are not shared with the IRs in the *pir* gene operator or with the IRs in the enhancer. At least for the IRs in the enhancer, the major *cis*-acting stabilizing factor appears to be a second half of IRs. This conclusion is supported by a recent finding that mutating one half-site from TGAGAA to GGGGAA, while leaving the other half site intact, severely decreased the affinity of wt π protein for both halves of the enhancer IRs (1). Since π binds to sites arranged as IRs (symmetric site) or DRs (asymmetric sites), we have proposed that π protomers must be able to assume different conformations to recognize two types of information, sequence and geometry of two types of binding sites (2,13). This hypothesis is strongly supported by the properties of π mutant (πS87N) that can bind DRs but cannot bind IRs (13). However, the πS87N can bind, although somewhat weaker than wt π, to the A+T-rich in the presence of the enhancer and DRs (data not shown).

We searched for a similarity between the consensus sequence of the 22 bp DRs unit or IRs unit and the A+T-rich segment to which π binds. The best match found (13 out of 18 bp, does not include nt G_7 whose role in binding of π to a 22 bp DRs unit has been established. Moreover, we could not detect any significant similarity between the two known pairs of IRs and A+T-rich segment. For this reason it is tantalizing to speculate that π may possess domains recognizing distinct families of DNA sequences. Another possibility is that the overall A+T richness may relax the stringency of the sequence to which π binds. This seems unlikely since the aforementioned degenerated DR sequence is far from the enhancement produced by π binding at the nt +15.

It should be mentioned that the ability of proteins to bind two families of DNA sequence is not unusual: a bipartite organization was originally discovered for the Int and A proteins controlling site-specific recombination of λ and Mu phages respectively (28,29). The DNA boxes and 13mers present in A+T-rich segment of the *oriC* of *E.coli* seem to independently bind purified DnaA protein, even though there is no sequence similarity between these targets (30). Genetic and biochemical data indicate that the Rep IB protein of R1162 plasmid can bind the origin's repeats as well as the A+T-rich segment nearby (31).

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