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Autorepressor properties of the π-initiation protein encoded by plasmid R6K

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ABSTRACT

A DNA fusion containing the promoter of the *pir* gene of plasmid R6K that encodes for the π-initiation protein and the β-galactosidase gene of *Escherichia coli* (*lacZ*) is described. The synthesis of β-galactosidase promoted by this *pir-lac* fusion was almost completely inhibited when an R6K sequence containing the *pir* gene was provided *in trans* in *E. coli*. Transcription *in vitro* from the *pir* promoter but not the *trp* promoter of *E. coli*, was inhibited by purified π protein indicating that the π protein alone is responsible for repression of its own gene and that the effect is promoter specific. The DNA-protein interaction sites in the *pir* regulatory region have been determined for the π protein and *E. coli* RNA polymerase using the DNase I protection method. The binding sites for these two proteins overlap for three helical turns. Competition DNA binding experiments show that the π protein will displace bound RNA polymerase. From these studies we conclude that repression of the *pir* gene is accomplished by binding of the π protein and this association blocks access of RNA polymerase to the *pir* promoter region.

INTRODUCTION

Replication functions of plasmid R6K are clustered within a 4 kilobase (kb) segment (1,2,3,4). This region contains three origins of replication designated α, β and γ and the structural gene *pir* that encodes the π protein (5). The π protein can act *in trans* to support replication *in vivo* of a γ-origin containing plasmid (1). Using an *in vitro* replication system, it has been found that the π protein acts before or during a transcriptional event preceding initiation of R6K replication (6).

Nucleotide sequence analysis of the R6K origin region has revealed the presence of eight 22 base pairs (bp) direct repeats (7). Seven of the repeats are arranged in tandem within the γ-origin, while the eighth repeat resides near the putative promoter of the *pir* gene located in HindIII fragment 9 (8). It has been proposed that binding of the π protein to the γ-origin and to the site near the *pir* promoter is required for initiation of plasmid R6K replication and autoregulation of the *pir* gene, respectively (1).

Shafferman *et al.*, (9) reported construction of a recombinant plasmid

that contained the lacZ gene fused to the HindIII fragment 9 of plasmid R6K. The β-galactosidase synthesis promoted by the pir_p in this fragment was shown to be repressed by a small derivative of R6K, plasmid pRK419 (9). In the same study, R6K HindIII fragments 9 and 15 (which provide a functional π protein), when inserted into the Escherichia coli chromosome via a Charon 3 phage, were also capable of repressing β-galactosidase production directed by the pir_p. Recently, it has been shown that a purified hybrid protein of π and β-galactosidase binds to the seven repeats within the γ-origin and to the eighth repeat near the putative pir promoter (10).

In this work we report that a segment of R6K DNA containing essentially only the structural gene for the π protein is capable of repressing the pir_p in vivo. In addition, purified π protein is shown to specifically repress in vitro the pir_p. Utilizing the DNaseI footprint technique, we also demonstrated that the π protein and RNA polymerase bind to the operator-promoter region of the pir gene and that the binding sites overlap for three helical turns. Furthermore, purified π protein will disrupt the RNA polymerase pir_p complex. Although the molecular details of autoregulation remain to be determined, these observations are consistent with autorepression of the pir gene by virtue of competition between the π protein and RNA polymerase for binding to the pir_p.

MATERIALS AND METHODS

Plasmids and bacterial strains:

The E. coli strains MC1000 and MB2 were used for β-galactosidase assays and purification of the π protein respectively. Construction of plasmids pMF45, pGD1, and pGD2 are described in the Results section. The plasmid pRK419, a small derivative of R6K containing β and γ origins of replication was described previously (1). Laboratory constructs pRK665 and pRK690 contain BglII fragments 1 and 2 of the plasmid pRK419 (the pir structural gene) and HaeII fragment specifying γ origin functions cloned into pBR322 derivatives, respectively (11).

Assay of β-galactosidase activity:

Assays were carried out according to Miller (12) with bacterial cultures grown in M9 medium supplemented with appropriate antibiotics.

DNA manipulations:

Clonal analysis of plasmid DNA and the isolation of plasmid DNA were performed as described previously (1). Restriction enzymes BglII, EcoRI, HincII and FnuDII were obtained from New England Biolabs and used according to the

manufacturer's protocols. DNA fragments were isolated from 0.7% agarose gels run with Tris-acetate buffer as follows. Agarose slices containing the DNA fragment were crushed by passing through a syringe (needle size 18 g), resuspended with 3 volumes of buffer containing 10 mM Tris-HCl pH 8.0, 1 M NaCl, 0.1 mM EDTA-Na_2, and frozen at -70°C. Samples were thawed at room-temperature, and the agarose was removed by centrifugation at 10,000 rpm. The supernatant was extracted with phenol and the DNA was precipitated with isopropanol, washed with ethanol, dried under vacuum, resuspended in DSB buffer (6 mM Tris-HCl pH 8.0, 6 mM NaCl, 0.1 mM Na_2-EDTA) and ligated with T4 ligase (New England Biolabs).

Purification of the π protein:

To obtain large quantities of π protein for purification, a FnuDII-EcoRI-linkered fragment containing the pir gene was inserted downstream of the λ P_R promoter. The π protein was purified from extracts made from cells of strain MB2 harboring this recombinant plasmid, designated pPT39. Construction of this plasmid and the scheme of purification will be described elsewhere.

In vitro transcription:

In vitro transcription experiments were carried out according to Lee and Yanofsky (13). A 25 µl reaction volume contained 40 mM Tris-acetate pH 8.0, 120 mM KCl, 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, 4 mM spermidine, 0.7 µg of RNA polymerase (Boehringer-Mannheim), 2.7 mMATP, 0.7 mM CTP, 1.1 mM GTP, 1.4 mM UTP, 10 µCi of α(^{32}P) CTP and 16 nM of DNA template. Where appropriate, π protein was added 10 min prior to RNA polymerase addition and incubated at 37°C. Transcription was carried out for 15 min at 37°C and the reaction terminated by addition of 0.1% SDS, 7 M urea, 0.01% bromophenol blue and 0.01% xylene cyanol. The entire sample was fractionated by electrophoresis on a 6% polyacrylamide gel containing 7 M urea.

Labeling and isolation of DNA fragments:

The plasmid pRK690 was used as a source of the restriction fragment containing regulatory sequences of the pir gene. The DNA was labeled at the 5' end with the polynucleotide kinase (Bethesda Research Laboratories) and γ(^{32}P) ATP. Labeled fragments were separated on polyacrylamide gels and DNA was eluted from appropriate slices of polyacrylamide by diffusion.

DNaseI protection:

Experiments were carried out according to method of Galas and Schmitz (14). End-labeled DNA (approximately 10 ng) was incubated at 37°C for 15 min in 23 µl buffer E (20 mM Tris-HCl pH 7.5, 6 mM $MgCl_2$, 1% ethylene glycol, 5% glycerol, 1 mM Na_2-EDTA and 55 mM KCl) containing indicated amounts of π pro-

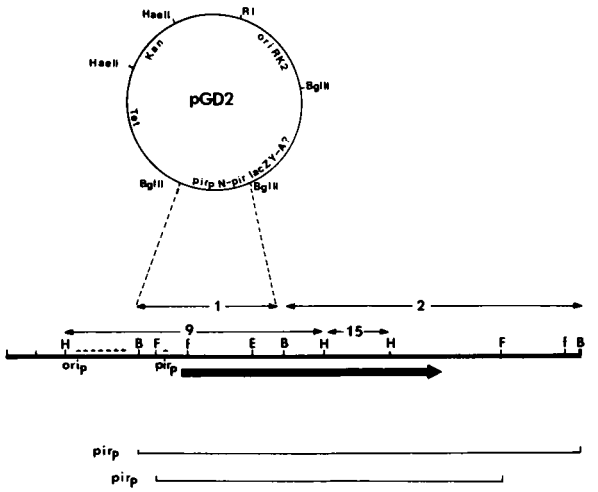
tein and/or RNA polymerase (Boehringer-Mannheim). Samples were cooled to room-temperature and treated with 10 ng of DNaseI freshly diluted from an 1 mg per mL stock solution with buffer E containing 50 µg per ml of BSA and 0.1 mM $CaCl_2$ and the digestion was allowed to proceed at room-temperature for 6-8 min. The reaction was stopped by the addition of 50 µl of 20 mM Tris-HCl, pH 7.5, 20 mM Na_2-EDTA, 0.1% $NaDodSO_4$, 50 µg of sheared calf thymus DNA per ml and treatment at 100°C for 3 min. Samples were phenol extracted and DNA was precipitated with isopropanol. DNaseI cleavage products were separated on 12% or 8% polyacrylamide gels containing 7 M urea.

RESULTS

Repression of pir gene expression in vivo by the π protein

The plasmid pRK775 containing HindIII fragment 9 inserted upstream of the β-galactosidase gene (which lacks its own promoter) has been described previously (9). Promotion of β-galactosidase expression by HindIII fragment 9 and repression of enzyme synthesis by R6K sequences containing the *pir* gene provided *in trans* indicate that the promoter resides within the cloned fragment 9 and suggests that its activity is inhibited by the product of the *pir* gene. More recent studies have shown that in addition to the pir_p, HindIII fragment 9 contains a second promoter that promotes transcription toward the pir_p and has been found to lie within the γ-origin segment (15) (Fig. 1). To more rigorously test the effect of the π protein on its own promoter (pir_p), we constructed recombinant plasmids pGD1 (Tet^r) and pGD2 (Tet^r, Kan^r) that contain the BglII fragment 1 of plasmid pRK419 inserted upstream of the β-galactosidase gene (Fig. 1). A plasmid construct that lacks the BglII fragment containing the *pir* promoter does not promote expression of the *lacZ* gene (data not shown).

Autogenous regulation of the *pir* gene was proposed previously based on the studies in which the source of the π structural gene was the plasmid pRK419 or a segment of R6K containing HindIII fragments 9 and 15 inserted into the *E. coli* chromosome *via* Charon 3 phage (λ-*pir*) (9). In either case the *trans*-acting segment contained in addition to the *pir* gene extra sequences of R6K acting *in trans* including the *incA* and *incB* regions (S. Yang, A. Shafferman and D. R. Helinski, unpublished; S. Yang, D. R. Helinski, unpublished). Moreover, the λ-*pir* construct specifies a π protein that is altered at the C-terminus (8). In order to further delineate the R6K segment that is capable of repressing the *pir* promoter, we measured β-galactosidase activity in strain MC1000 harboring plasmid pGD2 alone or harboring pGD2 together with plasmids



pir Source	β-gal Activity	%
none	395	100
none (pBR322)	389	98
pRK665	12	3
pMF45	14	4

Figure 1. Restriction map of plasmid pGD2 and expression of β-galactosidase under control of the pir promoter in the presence of pir sequences provided in trans. Plasmid pRK775 (9) was digested partially with BglII and resulting cleavage products were cloned into the unique BglII site of plasmid pRK248 (23). Clones containing BglII fragment 1 of plasmid pRK419 and the BglII lacZY-A? cartridge fragment were selected. One of the β-galactosidase positive clones was designated pGD1. This plasmid was partially digested with HaeII and subsequently ligated with a purified HaeII fragment specifying Kan^R. Transformants resistant to kanamycin were selected and one of them designated pGD2. Restriction analysis confirmed that in all other respects plasmids pGD1 and pGD2 are identical. Relevant restriction sites of an R6K replicon containing the γ-origin pir gene and the β-origin are indicated. B, E, F, f, and H refer to BglII, EcoRI*, FnuDII, HinfI and HindIII, respectively. The positions of BglII fragments 1 and 2 and HindIII fragments 9 and 15 are indicated. The eight 22 bp repeats are indicated by arrows and the pir coding region by a thick arrow. pir_p refers to the pir gene promoter. β-galactosidase activity was determined as described in Materials and Methods. The plasmids pRK665 and pMF45 are described in the text.

pRK665 or pMF45. The plasmid pRK665 contains BglII fragments 1 and 2 of plasmid pRK419 cloned into a derivative of pBR322 (11). Plasmid pMF45 contains the FnuDII fragment encoding the entire pir gene from pRK419 inserted as an EcoRI linkered fragment into the unique EcoRI site of plasmid pBR322. As shown in Figure 1, strain MC1000 containing plasmid pGD2 alone or with pBR322 produces 386-395 units of β-galactosidase. Greater than 95% repression of β-galactosidase activity was observed when plasmids pRK665 or pMF45 were also present, indicating that the segment of R6K containing the pir gene encodes a product which is capable of repressing transcription from the pir_p.

Purified π protein represses transcription from the pir promoter in vitro

To determine whether or not the π protein alone is responsible for

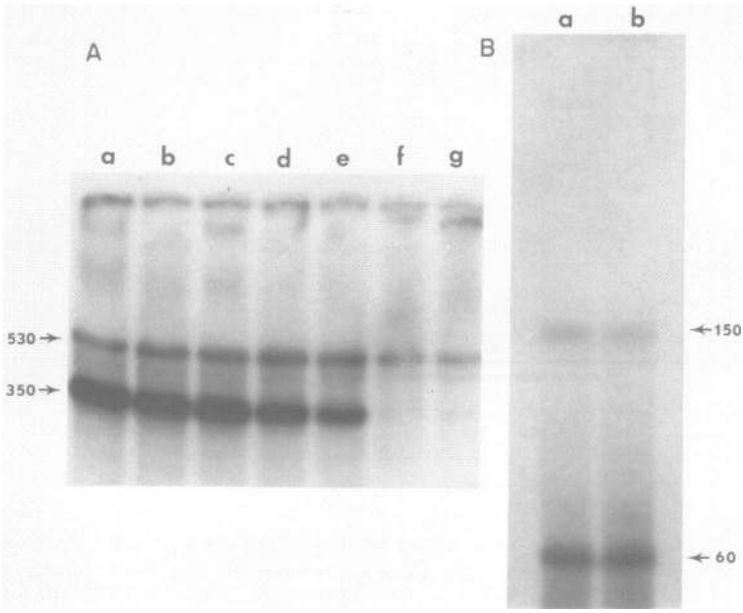


Fig. 2. In vitro transcription of a restriction fragment containing the pir promoter (A) and the trp promoter (B). Transcription reactions were carried out as described in Materials and Methods and the transcripts were analyzed on 6% polyacrylamide, 8M urea containing gels in TBE buffer. The BglII fragment 1 of plasmid pRK419 (shown in Fig. 1) was transcribed without additions (lane a), 0.9 ng (lane b), 2.8 ng (lane c), 8.3 ng (lane d), 25 ng (lane e) 74 ng (lane f) and 222 ng (lane g) of purified π protein. The HindIII fragment (150 bp) of plasmid pAD9 (kindly provided by A. Das and C, Yanofsky) was transcribed without (lane a) and with 222 ng (lane b) of the π protein.

repression of the pir_p, in vitro transcription studies were carried out using purified π protein and BglII fragment 1 of plasmid pRK419 that contains pir_p (Fig. 1). The results are shown in Fig. 2A. Transcription in vitro yields two RNA runoff products of approximately 527 and 350 bases corresponding to a full length and transcript initiated from the pir_p promoter, respectively (lane a). When transcription was carried out in the presence of various concentrations of π protein the synthesis of pir-RNA decreased with increasing concentration of the protein (lanes b to f). Full inhibition of the transcript formation was seen in the presence of 100 ng of π protein added (lane g). This titration experiment indicated that total repression occurred when the equivalent of approximately 20 π protein monomer were added per DNA template. In a similar experiment (Fig. 2B) with DNA containing the trp-leader, no repression of transcription was observed. These results indicate that π

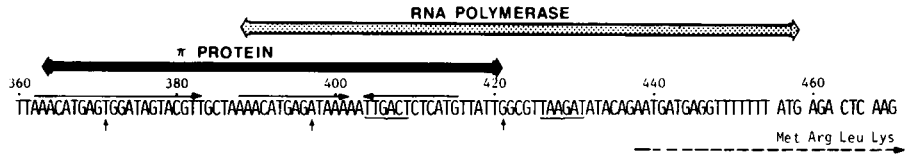


Fig. 3. π protein and RNA polymerase binding sites in the promoter-operator region of the pir gene. The coordinates are taken from Stalker et al. (8). The -35 and -10 regions are underlined. The horizontal arrows indicate the eighth direct and the inverted repeats which show partial homology with the repeat of 22 bp. The vertical arrows indicate positions of enhanced DNaseI cleavage frequencies observed in the presence of π protein. The dotted line refers to the 5'-end of pir-mRNA determined by S1 protection mapping procedure (Filutowicz, data not shown).

protein solely is responsible for inhibition of transcription initiating from the pir promoter and that the effect is promoter-specific.

Binding sites for π protein and RNA polymerase in the pir regulatory region

Binding sites for the π protein and RNA polymerase in the pir regulatory region were determined by using the DNase footprint technique (14). Results for the sense strand are summarized in Figure 3 and autoradiograms are reproduced in Figure 4. A DNase footprint of the BglII-EcoRI fragment of plasmid pRK690 (5'-end labeled at the BglII site; coordinate 277) without (Fig. 4A1) or with (Fig. 4A2) 20 ng of the π protein indicate that π protein binds to a segment of DNA between coordinates 363-421. In addition to protection from DNase cleavage, enhanced cleavage by DNase was found at bases 371, 397 and 421. At a ten-fold lower concentration of π (Fig. 4A3) poor protection is seen between residues 397-421, although bases 371, 397 and 421 remain almost equally more susceptible to DNase cleavage as in the presence of a ten-fold higher concentration of π protein. The binding sites for RNA polymerase were determined under the same conditions as those for the binding of π protein. Full protection of a 58 base segment between coordinates 389 and 457 delineated the binding site for RNA polymerase in the pir regulatory region. This segment includes a consensus RNA polymerase binding site (-35), Pribnow box (-10) and a Shine-Dalgarno sequence (SD) (Fig. 3). In addition, preliminary mapping analysis in vivo of the 5' end of pir mRNA by the S1 digestion method (16) indicates that the transcript begins with the complementary one of the two adenine residues (coordinates 438-439, Fig. 3). It is important to point out that the segment of 32 bases between coordinates 389 and 421 is bound by both π protein and RNA polymerase.

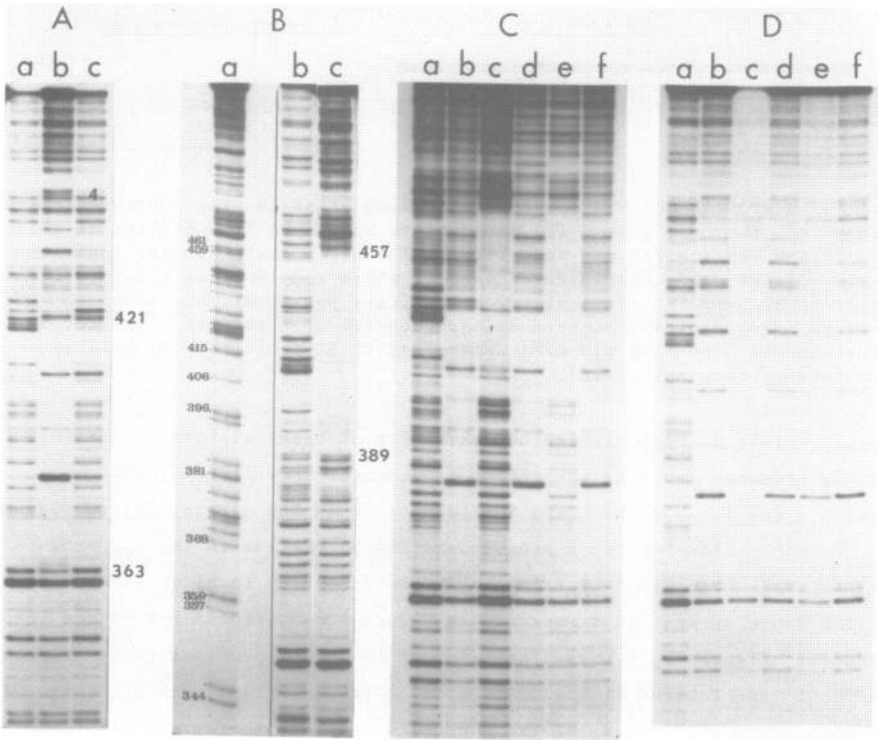


Fig. 4. Footprint analysis of π protein and RNA polymerase (RNA-P) bound to the operator-promoter region of the pir gene. All DNaseI experiments were carried out as described in Materials and Methods with the BglII-EcoRI restriction fragment from plasmid pRK690 (11) labeled at the 5'-end of BglII site. (A) Fragment digested in the presence of no protein (lane a); 20 ng of π protein (lane b); or 2 ng of π protein (lane c). (B) G DNA sequencing sample with some of the residues numbered (lane a); fragment digested with no π protein (lane b); or 6 ng of RNA-P (lane c). Footprints of π protein and RNA-P competing for promoter of the pir gene are shown in Fig. 4C and 4D. (C) Fragment digested without π protein (lane a); 20 ng of π protein (lane b); 6 ng of RNA-P (lane c); 12 ng of RNA-P (lane e); the π protein (20 ng) DNA fragment complex allowed to form at 37°C for 5 min and then 6 ng of RNA-P (lane d) or 12 ng of RNA-P (lane f) were added. (D) Fragment digested without π protein (lane a); 20 ng of π protein (lane b); 20 ng of RNA-P (lane c); RNA-P -DNA fragment complex was allowed to form at 37°C for 5 min and then 10 ng (lane d), 15 ng (lane e) or 20 ng (lane f) of π protein were added. Following incubation at 37°C for 15 min all samples were processed as described in Materials and Methods. DNase I cleavage products were resolved on 8% polyacrylamide, 8 M urea gels in TBE buffer.

Competition between RNA polymerase and π protein for binding sites in the pir regulatory region

In the experiment shown in Figure 4C, we examined binding of RNA polymerase alone (lane c) or to preformed DNA-π protein complexes. The pattern of DNaseI cleavage of the template when π protein was added before RNA polymerase (lanes Cd and Cf) is virtually identical with the footprint observed for π protein alone (lane Cb). It is of interest to note that the region not bound by π protein that constitutes part of the RNA polymerase footprint cannot be protected by RNA polymerase once π protein has bound to its own recognition sequence.

In previous reports of repressor protein binding to DNA, the binding site resided either entirely within the segment of DNA protected by RNA polymerase (17,18,19,20,21) or flanks the RNA polymerase site (22). The interaction of π and RNA polymerase at the pir promoter represents an unusual situation in that the two binding sites overlap but neither is contained entirely within the other. Since the RNA polymerase binding site does not include the eighth repeat of 22 bp (π binding site), the questions of whether an established RNA polymerase-pir_p complex can alter binding of the π protein and whether the RNA polymerase-pir_p complex can be affected by π protein were of importance. Comparison of the footprint for π protein alone (lane b; Fig. 4D) and for RNA polymerase alone (lane c) with the footprints of samples in which the RNA polymerase-pir promoter complex was allowed to form prior to the addition of 10 ng (lane d), 15 ng (lane e) or 20 ng (lane f) of π protein indicated that formation of the polymerase promoter complex has no effect on binding of the π protein to the eighth 22 bp repeat. Thus, it appears that π protein can disrupt the RNA polymerase-pir promoter complex in vitro.

DISCUSSION

It has been shown in previous studies that the plasmid pRK775 containing HindIII fragment 9 of plasmid R6K inserted upstream of the lacZ gene promoted expression of β-galactosidase and that the pir sequence substantially reduced the enzyme levels when provided in trans (9). Since HindIII fragment 9 contains two promoters in tandem (15) and two binding sites for the π protein, a new plasmid, pGD2, containing pir-lac fusion was constructed in this study. A major difference in the results obtained in plasmids pRK775 and pGD2 is the relative amounts of β-galactosidase synthesis specified by these constructs when the pir sequence is provided in trans. Greater than 95% repression of the enzyme synthesis from plasmid pGD2 was observed when the complete pir gene

was present *in trans*. On the other hand, repression of β-galactosidase expression from plasmid pRK775 was approximately 50%. Although the experiments involved a different source of the *pir* gene, the weaker repression observed when two tandem R6K promoters, namely γ-ori_p and pir_p, are present may indicate a contribution of both promoters to the regulation of the *pir* gene. Recent result, have shown that autorepression of the *pir* promoter by π protein can be abolished when a strong promoter is provided upstream (10; Filutowicz, unpublished data). This observation indicates that binding of π to the *pir* operator may not prevent synthesis of RNA initiated upstream of the π protein binding site. Unlike the *pir* promoter, expression from the promoter in the γ-origin region is not inhibited by the *pir* sequence provided *in trans* or *in vitro* by the presence of the π protein (15, data not shown). Since nucleotide sequence analysis (8) and *in vivo* experiments (15) indicate the absence of a transcriptional terminator between these two promoters, the possibility that the promoter in the γ-origin region contributes to the regulation of π protein synthesis by virtue of overriding repression exerted by the protein on the promoter proximal to the *pir* gene deserves further exploration (Fig. 1).

A second approach to the study of *pir* autoregulation involved using purified π protein and RNA polymerase to determine the ability of these proteins to bind their putative recognition sites. During the course of these studies it was reported that a π-β-galactosidase hybrid protein interacts with regulatory sequences of the *pir* gene (10). Comparison of DNaseI footprints of the wild type π protein (Fig. 2A) and the π-β-galactosidase hybrid protein at the regulatory region of *pir* shows many similarities in the protection pattern, although some differences were observed. Enhanced cleavage sites are seen at base positions 371, 397 and 421. Protection within a ten fold range of π protein is apparent although at the lowest protein concentration it is weaker and restricted to the segment between coordinates 397-421. In the case of the hybrid protein, protection at the lowest concentration of the region of the *pir* regulatory region is indistinguishable from that at the highest concentration (10). The π protein also causes strong enhancement at base position 421 which is not seen with the hybrid protein. The largest difference between the two proteins is protein concentration dependency of the appearance of enhancements and protection. In the case of the π protein enhanced cleavages are pronounced before full protection is observed, whereas the π-β-galactosidase hybrid protein protects first, and then at higher concentration it renders the phosphodiester bonds at positions 371 and 397 more susceptible to DNase cleavage. It is possible, therefore, that both proteins are not equivalent in

their binding properties. Alternatively, minor differences in experimental protocols may contribute to the differences observed in footprint patterns.

The binding site of RNA polymerase (coordinates 389-459) includes the sequences at the -10 and -35 regions of pir (Fig. 3). The segment of 32 bp between coordinates 389-421 is shared by RNA polymerase and π protein. In contrast to the binding patterns observed for the regulatory region of the pir gene, position of a repressor site for the ara (20), lac (18,21) and trp (19) is entirely within the segment protected by RNA polymerase; in the case of the crp gene the operator lies entirely outside the RNA polymerase site (22). Since the pir operator both overlaps and extends beyond the RNA polymerase binding site, the pir promoter-operator region appears to be somewhat unique. Competition studies indicate that binding of RNA polymerase requires an empty shared region (coordinates 389-422). When occupied by the π protein, RNA polymerase cannot displace π from its binding site or bind to that portion of its binding site not protected by π protein (coordinates 422-459). Therefore, prevention of RNA polymerase binding by π protein resembles typical repressor-polymerase competition. Surprisingly, the addition of the RNA polymerase before the π protein did not prevent the binding of the π protein to its binding site, including the shared region. The ability of the π protein to displace RNA polymerase is likely due to either direct protein-protein repulsion, destabilization of the RNA-polymerase-DNA complex brought about by π binding to the eighth repeat and propagated through DNA structure or competition for the shared region determined by relative binding affinities of the two proteins and the reversible nature of RNA polymerase binding.

The aforementioned abilities of the π protein resemble properties of transcription factor 1 (TF1), a basic protein of Bacillus subtilis bacteriophage SP01. This protein selectively blocks initiation of RNA synthesis; it interferes with the binding of RNA polymerase to DNA and it can destroy preformed promoter complexes of RNA polymerase with SP01 DNA (24). Further analyses using mutant π proteins should facilitate a better understanding of the regulation of pir gene expression and the interaction between RNA polymerase, π protein and their respective binding sites.

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