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Genetics

Positive and negative roles of an initiator protein at an origin of replication

(plasmid R6K/promoter deletions/replication control/autoregulation/immunoassays)

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ABSTRACT **The properties of mutants in the *pir* gene of plasmid R6K have suggested that the π protein plays a dual role; it is required for replication to occur and also plays a role in the negative control of the plasmid copy number. In our present study, we have found that the π level in cell extracts of *Escherichia coli* strains containing R6K derivatives is surprisingly high ($\approx 10^4$ dimers per cell) and that this level is not altered in cells carrying high copy number *pir* mutants. The wild-type and a high copy mutant (Cos405) *pir* gene were inserted downstream of promoters of different strengths to measure the copy number of an R6K γ replicon as a function of a 1000-fold range of intracellular π concentrations. The data demonstrate that reducing the intracellular level of π to 5% of its normal value can result in a substantial increase in copy number of a γ origin replicon and that a π level <1% of normal is still permissive for replication. Conversely, increasing the π level even a few-fold above normal results in a marked inhibition of replication of plasmids containing a single, two, or all three of the R6K origins (α, β, and γ). We have also shown that the replication inhibition mediated by excess π is greatly reduced by the *pir405* Cos mutation. These results demonstrate that the total level of π protein is not rate-limiting for a γ replicon. We have also determined the sensitivity of the *pir* gene promoter to a wide range of π concentrations. The activity of this promoter is stimulated by very low π levels and is almost entirely inhibited when the protein is overproduced 2-fold.**

The self-transmissible antibiotic resistance plasmid R6K of *Escherichia coli* is a member of a large group of bacterial plasmid replicons that share common features, including nucleotide sequence repeats at their replication origin and in several cases an autoregulated initiator protein that binds to these repeats (1, 2). Replication functions of plasmid R6K are clustered within a 4-kilobase-pair (kbp) segment (3–6). This region contains three replication origins designated α, β, and γ (7, 8) and the structural genes *pir* (9) and *bis* (10) that encode for the π and bis proteins, respectively. The 35-kDa π protein (9) is required for activity of all three origins (3, 11, 12), while the requirement for the 17-kDa bis protein appears to be restricted to the β origin of replication (10, 13). The minimal genetic information that is required for stable maintenance of this plasmid at its copy number of $\approx$15 per chromosome equivalent consists of the $\approx$400-bp γ origin (14) and the *pir* gene, whose product can function when supplied in trans (3, 11). The π protein purified in its native dimeric form (15), or as a hybrid with a β-galactosidase (16) or collagen (17) moiety, has been shown to bind to the seven 22-bp repeats in the R6K γ origin and to an eighth 22-bp repeat and a smaller inverted pair of repeats that are present in the operator–promoter region of the *pir* gene (15, 16, 18, 19). Binding of the π protein to these two regions appears to be essential for γ origin activity (20) and autogenous regulation of the *pir* gene, respectively (18, 21).

A negative role for the π protein in the control of R6K copy number was suggested originally by the isolation of a mutant of the R6K derivative plasmid pRK419, designated Cos405, that maintained itself at a greatly increased copy number as a result of a single amino acid substitution within the π protein. This mutational change was recessive to wild-type protein (22). The properties of this mutant protein along with the well-established positive function of π protein in R6K replication (3, 4) lead to the conclusion that the π protein plays a dual role; it is both required for R6K replication and yet is also capable of inhibiting it.

This study examines the effect of varying π concentration on plasmid copy number utilizing the wild-type *pir* gene and a copy-up mutant *pir* gene (Cos405) inserted downstream of promoters of different strength. The varying levels of π protein in *E. coli* carrying these constructs were determined by an immunological assay using antibody preparations raised against purified π protein. The data clearly indicate that there is not a linear relationship between π concentration and plasmid copy number and that excessive levels of π result in a substantial reduction in plasmid copy number. Our studies also suggest that the major role of autorepression of π expression in the R6K system is to prevent the π protein from accumulating to levels high enough to inhibit plasmid replication.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strains C600, MC1000 (21), C2100 (20), MB2 (15), P678-54 (2), YS1λ*pir**, ϕ11λ*pir**, ϕ41 (23), and ϕ51 (24) were used in the experiments indicated in the figure legends. Characteristics of plasmids R6K (25), pRK35 (24), pRK419 and pRK526 (3), pMF26 (15), and of other plasmids used in this study are indicated in the text or will be described elsewhere.

Preparation of Lysates for Protein and DNA Electrophoresis. Total cell lysates from 1- or 1.5-ml cultures were prepared for protein analysis according to Laemmli (26). Analysis of cellular DNA content in 0.6% agarose gels was carried out with total lysates prepared from 1 ml of cultures in logarithmic phase. Cells were harvested at room temperature, washed with buffer A (150 mM NaCl/100 mM Na_2EDTA/10 mM Tris·HCl, pH 10.2) and resuspended in 0.1 ml of the same buffer. The cell suspension was treated with 10 μl of lysozyme (5 mg/ml; Sigma), incubated for 10 min at 37°C followed by the addition of 2.5 μl of 10% $NaDodSO_4$ (Bio-Rad) and 10 μl of Pronase (20 mg/ml), and incubated at 37°C for 2 hr. The mixture was then incubated for 30 min at 65°C and after cooling to room temperature, 40 μl of solution B (10% urea/3% Ficoll/0.02% xylene cyanol/0.02% bromophenol blue) was added. Chromosomal DNA was sheered by

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Abbreviation: bp, base pair(s).

1 min of mixing in a Vortex followed by vigorous passing of lysate through a 200-μl Pipetman tip. Samples were frozen at −20°C, thawed, and 1- to 20-μl aliquots were electrophoresed under the conditions described in the figure legends.

DNA and Protein Blotting. Southern analysis was carried out as described (27) with the probes described in the figure legends. Autoradiographs were traced with an LKB Ultroscan XL laser densitometer. Plasmid copy number was determined by using an extract from strain YSI-λ*pir** containing plasmid pRK526 as the control. This strain contains a single copy of a γ-origin fragment inserted into the chromosome (3), and the copy number of the pRK526 plasmid was estimated to be 15 per chromosome equivalent. The transfer of proteins after $NaDodSO_4$/PAGE was carried out as described (28). After transfer, the nitrocellulose was washed three times with TBS buffer (150 mM NaCl/20 mM Tris·HCl, pH 7.5) and incubated at room temperature overnight with a purified π IgG preparation in TBS buffer containing 1% bovine serum albumin (fraction V; Sigma). The nitrocellulose was washed three times with TBS buffer and incubated for 1 hr at 37°C with a peroxidase-conjugated fraction of goat anti-rabbit IgGs at a concentration recommended by the supplier (Cooper Biomedical, Malvern, PA). The immunoblot was developed as described (10).

Purification and Standardization of π IgGs. Purification of monospecific antibodies was carried out by affinity chromatography on a π protein immunoabsorbent [100 μg of π per 1 ml of glutaraldehyde-activated Ultrogel Ac-22 (Pharma-industrie, Villeneuve-La-Garenne, France)], kindly provided by the laboratory of S. J. Singer. Crude serum (5 ml) collected from a rabbit that was immunized with highly purified π protein (15) was loaded onto 1 ml of the affinity column. The column was extensively washed with phosphate-buffered saline (PBS) (10 mM KPO_4, pH 6.0/150 mM NaCl), and the π IgGs were eluted with 0.2 M HCl into tubes containing an equal volume of 1 M Tris base. Pooled fractions were dialyzed against PBS containing 50% (vol/vol) glycerol at 4°C for 40 hr. These steps essentially eliminate all IgGs present in the crude serum that react with other *E. coli* proteins. Standardization of the antibodies was carried out with purified π protein mixed with an R6K-free cell lysate that was run on a $NaDodSO_4$/polyacrylamide gel. Analysis by the immunoblotting technique described above was carried out with an amount of π IgGs sufficient to detect 1 ng of π. Relative amounts of the protein were determined by microdensitometric tracing of film positives of the immunoblots. The actual amount of the π protein in cell lysates containing R6K or other plasmids containing the *pir* gene was estimated by a comparison with a standard curve.

***In Vitro* Recombinant Techniques.** Restriction enzymes and T4 ligase were obtained from New England Biolabs or Bethesda Research Laboratories and used according to the protocols recommended by the supplier. The 12-bp *Eco*RI linker and BAL-31 endonuclease were obtained from New England Biolabs and used according to Maniatis *et al.* (29).

RESULTS

Overproduction of the π Protein Inhibits R6K Replication. An immunological assay was used to measure levels of the π protein in *E. coli* cells containing derivatives of plasmid R6K. This assay indicated that an exponentially growing culture in LB or M9 medium of an *E. coli* strain carrying plasmid R6K or its smaller derivatives produces π protein at a level of ≈0.3% of the total protein. Since π protein is a dimer (15), this would correspond to 3500–10,000 molecules per cell. Genetic data have suggested that the π protein exerts a negative control on the R6K copy number in addition to its absolute requirement for plasmid replication (22). It was, therefore, of interest to determine whether the negative activity of π on DNA replication is exhibited within a particular range of π concentrations. To test this, plasmids that overproduce either the wild-type or a mutant π protein were constructed. The plasmid pairs pPT20, pPT21 and pPT32, pPT39 contain a *Fnu*dII–*pir* segment from the mutant *pir405*-Cos (22) and wild-type plasmids, respectively, cloned into the pBR322-derived vector pKJB825 (15). In one orientation (pPT20 and pPT39) the *pir* gene is under the control of a conditional promoter (λP_R), while in the opposite orientation (pPT21 and pPT32) it is under the control of a constitutive vector promoter. The level of π protein in the *E. coli* host P678-54 containing each of these plasmids was determined by Coomassie blue staining and immunoblotting analyses. As shown in Fig. 1*A*, plasmids with the constitutive promoter upstream of the *pir* gene overproduce the π protein at 30°C. A further increase in the cellular level of π can be seen in cell cultures carrying plasmids pPT20 and pPT39 when shifted to 37°C. Since the stained gel procedure did not allow for quantitative determination of the amount of π, the level of this protein in samples containing plasmids pPT32, pPT39, and pRK419 was determined by immunostaining (Fig. 1*B*). Clones containing pPT39 and pPT32 produce about 2-fold and 8-fold more π protein, respectively, than those containing the R6K derivative plasmid pRK419. The same ratios of expression were observed with plasmids containing the *pir405*-Cos mutation (data not shown).

To examine the effect of higher than normal levels of π protein on R6K replication, a transformation efficiency test was carried out at 30°C with plasmid-free cells and with *E. coli* recipients carrying either pPT20, pPT21, pPT32, or pPT39. As summarized in Table 1, the only recipient that did not allow the establishment by transformation of any of the four R6K replicons tested was the strain carrying as a

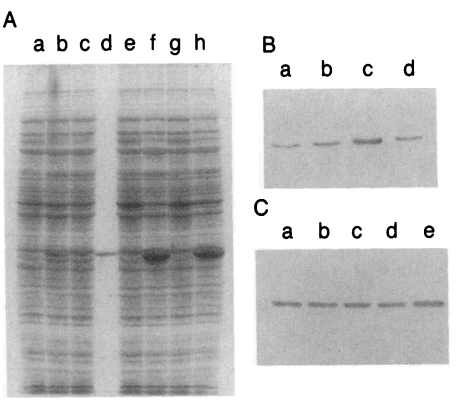

FIG. 1. Levels of π protein in *E. coli* cells containing wild-type or the *pir405*-Cos gene inserted in two orientations into the unique *Eco*RI site of the vector pKJB825 (15). (*A*) $NaDodSO_4$/PAGE analysis of total cell lysates prepared from strain P678-54 grown at 30°C without any plasmid (lane a) and with plasmids pPT21 at 30°C (lane b), pPT32 at 30°C (lane c), pPT20 at 30°C (lane e), pPT20 after a 20-min shift to 37°C (lane f), pPT39 at 30°C (lane g), and pPT39 after a 20-min shift to 37°C (lane h). Lane d contains purified π protein. (*B*) Immunoblotting of total cell lysates prepared from strain P678-54 harboring plasmids pRK419 (lane a), pPT39 at 30°C (lane b), pPT32 at 30°C (lane c), and 1/4th of the sample shown in lane c (lane d). All cultures analyzed in this experiment were grown in M9 medium supplemented with glycerol (0.5%), thymine (10 μg per ml), and Casamino acids (0.5%). (*C*) Level of the π protein in high copy number mutants of the *pir* gene. Cultures of strain MB2 harboring plasmids pRK419 (lane a), pR419 *pir405* Cos (lane b), pMF26 (lane c), pMF26 *pir1* Cop (lane d), and pMF26 *pir104* Cop (lane e) were grown at 37°C in LB medium. Cells were harvested at OD_{650} = 0.5–0.6, except for cultures containing plasmid pRK419 and pRK419 *pir405* Cos that before harvesting were shifted to 30°C for 1 hr to induce the increase in copy number (22). Cell lysates were analyzed by $NaDodSO_4$/PAGE and immunoblotting was carried out as described in *Materials and Methods*.

Table 1. Overproduction of π protein and its effect on establishment of different derivatives of plasmid R6K

Recipient *E. coli* MB2	Source of *pir*	Establishment of plasmids by transformation: pRK526 (γ ori)	pRK419 (β–γ ori)	pMF51 (β ori)	pRK35 (α–β–γ ori)
Plasmid-free	None	−	+	+	+
pPT39	WT	+	+	+	+
pPT32	WT	−	−	−	−
pPT20	*pir405* Cos	+	+	+	+
pPT21	*pir405* Cos	+	+	+	+

All transformation experiments were carried out at 30°C on LB plates supplemented with penicillin G (250 μg/ml) and kanamycin (50 μg/ml). A few penicillin-resistant kanamycin-resistant colonies from plates containing the MB2 pPT32 recipient transformed with the R6K-derived replicons were subjected to clonal analysis and were found to contain cointegrate plasmids. Such colonies were not observed when a *recA*$^-$ derivative of MB2 strain (MB4) was used in the transformation experiment. Plasmids pRK526, pRK35, and pRK419 are described in *Materials and Methods*. Plasmid pMF51 contains *Hin*dIII fragments 9, 15, and 2* consisting of a functional β origin (ori) only (10, 13) and a kanamycin-resistance marker.

resident plasmid pPT32. These transformation data show that an 8-fold increase of π levels over that produced by plasmid R6K prevents the establishment of an R6K replicon containing all three, two, or a single replication origin. This π protein level had no effect on the establishment of the compatible replicons derived from RSF1010, p15A, and RK2 (data not shown). Interestingly, unlike the wild-type protein the overproduction of the mutant pir405 Cos protein did not prevent the establishment of any of the plasmids used in this transformation experiment. These results are consistent with the previously published finding that excess wild-type π protein inhibits R6K replication as measured by pulse-labeling of plasmid DNA (2).

With regard to the question of whether or not levels of the π protein are rate limiting for R6K replication it was important to determine whether altered π levels are responsible for the high copy number phenotype of certain *pir* mutants. As shown in Fig. 1*C*, π levels in cells containing mutants pir405-Cos, pir1-Cop, or pir104-Cop are either unchanged or only slightly increased despite the fact that the copy number of these mutant plasmids is at least 10-fold greater than the wild-type level (15, 22). These data clearly indicate that it is not an alteration of the total π level that accounts for the phenotype of these *pir* copy-up mutants. In addition, the occurrence of approximately normal levels of π despite the increased copy number of these plasmids suggests that these mutant π proteins are still able to autoregulate their own synthesis.

Effect of a Broad Range of π Concentrations on the Copy Number of Minimal R6K Replicons. An earlier investigation that addressed the question concerning the relationship between π protein concentration and R6K origin activity involved a plasmid (pAS751) that contained the Trp promoter upstream of a *Hin*fI–*pir* fragment of R6K (23). Immunoassays carried out on cell extracts containing plasmid pAS751 have since shown that in both a *trpR*$^+$ and *trpR*$^-$ background there are two protein bands that cross-react with π IgGs. Considering the size of these two polypeptides, they most likely correspond to a *trpE–pir* fusion product and an NH_2-terminally truncated π polypeptide. In addition, the induction of π (*trpR*$^-$/*trpR*$^+$) was not greater than 15-fold and the π level in exponentially growing trpR$^-$ bacteria was similar to that found in wild-type R6K cells (data not shown). The strategy used to re-examine the question of the relationship of π concentration to R6K copy number was to remove or alter the *pir* promoter of an RK2 plasmid construct carrying the wild-type *pir* gene (pPR1) by using BAL-31 nuclease digestion. Isolates that provided substantially less or more π protein than the parent plasmid pPR1 were identified and used to determine the effect of different π concentrations on the copy number of R6K γ origins plasmids. Fig. 2 contains complementary Southern blots and immunoblots that show the response of the R6K γ origin plasmid pRK526 to several levels of π ranging from slightly below normal (Δ6R5) to severalfold above normal (Δ6R1, Δ6R3, Δ6R14, Δ6R14-3, and Δ6). Two conclusions are evident from these data. The first is that even modest levels of π overproduction are inhibitory, as judged by their ability to reduce pRK526 copy number. The fact that there is no level of π overproduction that produces a higher than normal copy number indicates that the total level of π is not rate-limiting for R6K replication. The second conclusion is that the presence of a γ origin plasmid has little or no effect on the total level of π in the cell. This conclusion is also true in the case of the π underproducers.

To examine the response of γ origin copy number to levels of π below the normal (R6K) level, various other deletion derivatives of pPR1 were examined. As shown in Fig. 3*B*, derivatives Δ10, Δ5, and Δ14 in strain C600 produce approximately 100, 20, and 7 times less π protein, respectively, than plasmid pMF42, which specifies a level of π comparable to R6K (data not shown). As shown in Fig. 3*A*, plasmids pMF5 and pMF36 are maintained at the highest copy number when the levels of π are 7–20 times less than normal (Δ5 and Δ14) and that even <1% the normal level is sufficient to maintain γ origin replicons at a relatively high copy number. It is also clear that for these plasmids the copy number decreases substantially when the protein concentration is approximately normal. Another unexpected result is the difference between plasmids pMF5 and pMF36 in their response to low π concentrations, with pMF36 being maintained at a sub-

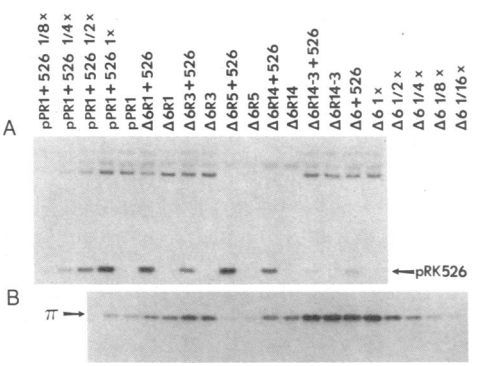

FIG. 2. Copy number of an R6K γ-origin plasmid in the presence of different levels of π protein specified by overproducing derivatives of pPR1. Plasmid pPR1 was constructed by the insertion of a *Bam*HI fragment containing the *pir* gene from plasmid pMF39 (15) into the RK2 plasmid derivative pTJS77. pPR1Δ6 is a BAL-31 deletion derivative of pPR1 that still contains the *pir* promoter but is deleted for a portion of the autoregulatory sequences. pPR1Δ6R1, pPR1Δ6R3, pPR1Δ6R5, pPR1Δ6R14, and pPR1Δ6R14-3 are rearrangements of pPR1Δ6 derived by cutting this plasmid with *Eco*RI and religating. C600 cells containing the various plasmids were grown in LB medium to an OD_{650} of 0.3, harvested, and divided into two samples for Southern and immunoblot analyses. Each pPR1 derivative is shown with and without the γ-origin plasmid pRK526. Southern analysis (*A*) was carried out using a probe that contained both the γ origin and part of the *pir* gene and, therefore, hybridized to both pRK526 and the pPR1 derivatives. As shown by the immunoblot (*B*), the various derivatives of pPR1 produce π levels that are approximately 1.5 (Δ6R1), 3 (Δ6R3), 0.4 (Δ6R5), 2 (Δ6R14), 7 (Δ6R14-3), and 6 (Δ6) times that produced by pPR1. The different levels of π produced are apparently due to different sequences upstream of the *pir* gene and/or different copy numbers of the RK2 vector.

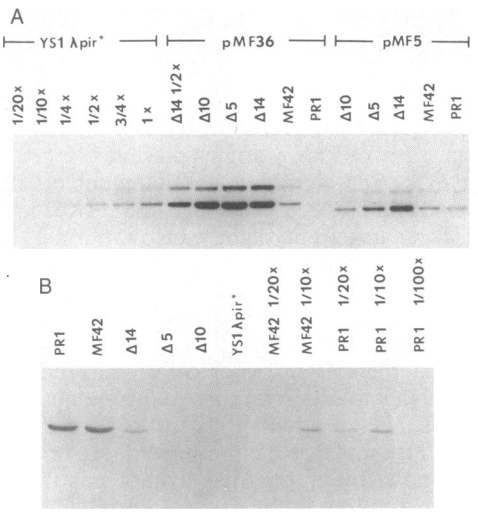

FIG. 3. Copy number of γ-origin replicons in the presence of different levels of the π protein specified by underproducing derivatives of pPR1. The construction of plasmid pPR1 is described in Fig. 2. Plasmid pMF42 produces a wild-type (R6K) level of π. pPR1Δ5, pPR1Δ10, and pPR1Δ14 are deletions into the *pir* promoter–operator region. Plasmid pMF5 consists of the same γ origin *Hae* II fragment that is present in plasmid pRK526 but contains an ≈2-kbp ampicillin fragment from R6K in place of the kanamycin-resistance fragment. Plasmid pMF36 contains a minimal R6K γ origin (≈400 bp) that lacks the adjacent *pir* promoter region and was obtained from the joint replicon pMF34 (15) by deletion of an *Hae* II fragment containing the pUC9 origin. C600 cells containing the various plasmids were grown as described in Fig. 2, harvested, and divided into two samples for Southern and immunoblot analyses. Southern analysis (*A*) was carried out using an *Hin*dIII γ origin fragment from plasmid pMF34 (15) as a probe. As indicated by the immunoblot (*B*), deletion derivatives of pPR1 produce π levels that are approximately 7- (Δ14), 20- (Δ5), and >100- (Δ10) times below that produced by pMF42.

stantially higher copy number than pMF5 over a wide range of π levels (7–100 times less than normal). The copy number of pMF5 achieves its highest level in the presence of the Δ14 construct (7 times less π than normal). The data in Fig. 3 allow the following conclusions. The first is that a very low percentage of the normal level of π produced by intact R6K is sufficient for the activity of a γ origin replicon. In fact, the highest copy number was obtained at a π level substantially lower than normal. Some exceptions have, however, been found to this latter point. In some strains, some γ replicons display their highest copy number at the wild-type level of π. The second conclusion is that a negative effect of π is exerted below or at the normal cellular concentration of π. Furthermore, sequences outside of the minimal origin apparently can (comparing plasmids pMF36 and pMF5) contribute to the manner in which a γ origin replicon responds to various π levels.

In addition, the π protein level was measured in the *E. coli* strain YS1λ*pir** that produces a truncated π protein. It is of interest that strains YS1λ*pir** (Fig. 3*B*), φ11, φ41, and φ51 (data not shown) that contain a single copy of the *pir* gene inserted into the *E. coli* chromosome, produce from 20 to 50 times less π protein than that produced by R6K.

Effect of the π Concentration on the Activity of the *pir* Gene Promoter. Both *in vivo* (23) and *in vitro* (18, 21, 23) data have indicated that the level of π protein in *E. coli* is regulated at the level of transcription initiation. The availability of various derivatives of plasmid pPR1 that specify different levels of π protein makes it possible to determine the minimum concentration of this protein that is required for a substantial inhibition of *pir* gene expression. Plasmid pRK775 contains a 935-bp fragment of R6K (*Hin*dIII fragment 9) inserted upstream of the β-galactosidase gene as a transcriptional fusion (23). This fragment contains the *pir* promoter whose expression has been shown to be repressed when the π protein is supplied in trans (23). The results of β-galactosidase measurements, carried out in strain MC1000 harboring plasmid pRK775 alone or harboring pRK775 together with the pPR1 derivatives Δ10, Δ5, Δ14, Δ6R5, and pPR1, are shown in Table 2. Surprisingly, levels of π protein substantially lower than normal stimulate synthesis of β-galactosidase. Conversely, at π protein levels near normal, the β-galactosidase activity is substantially reduced. These data indicate that relatively low levels of π enhance *pir* promoter activity and that the repression of the *pir* promoter occurs only near normal cellular levels of the π protein.

Table 2. Effect of different π levels on activity of the *pir* gene promoter

π source	Level of π, % wild type	β-Galactosidase activity, units	Activity, %
None	0	1032	100
Δ10	<1	1970	190
Δ5	≈2	1770	171
Δ14	≈5	1440	140
Δ6R5	≈50	890	86
pPR1	≈150	122	12

The activity of β-galactosidase was measured in exponentially growing cultures of strain MC1000 containing plasmid pRK775 alone or together with the plasmid pPR1 and its derivatives. Plasmid pRK775 produces a truncated π polypeptide that itself does not affect the activity of the *pir* promoter (data not shown). Relative levels of the π protein were estimated from immunoblotting of total cell lysates as described in *Materials and Methods*. Wild-type levels refer to the amount of π observed in strains carrying intact R6K. The extent of stimulation and repression of *pir* promoter activity in the presence of different π levels was similar in four independent experiments.

DISCUSSION

The absolute requirement of the π protein in R6K replication coupled with its two negative functions, one at the level of replication origin activity and the second at the level of initiation of transcription of its own gene, demonstrate the multifunctional role that this protein plays in R6K replication control. An earlier *in vivo* study of the regulatory properties of π (23) had concluded that the copy number of a γ origin plasmid remained unchanged despite a 95-fold variation in the π level. This study was complicated, however, by the fact that the π proteins involved in this analysis contained altered amino termini and have since been found to be altered in their regulatory properties. Another potential difficulty in this work was that π levels were measured by an indirect method that overestimated the range of π levels produced by the plasmid used. The goal of the present study was to construct a number of plasmids that would underproduce or overproduce a wild-type π protein in an exponentially growing culture and then to determine the effect of different protein levels on the copy number of R6K derivatives. Central to this goal was the use of an immunological detection method as an accurate method of determining the quantities of π present in the bacteria and the construction of plasmids that produce various levels of the π protein as the result of deletions into the *pir* promoter region or the presence of an additional promoter.

The initial use of the π immunoassay was to determine the absolute amount of π present in cells containing R6K, mini-R6K derivatives and copy-up mutant R6K derivatives. Two striking features emerged from these measurements. The first is that the total amount of π present in mid-logarithmic phase cells containing R6K or its smaller derivatives is very high; our estimates range from 3500 to 10,000

π molecules per cell. This is in sharp contrast to the common notion of an initiation protein being present in very low and rate limiting amounts. The second notable feature of these initial measurements was that π levels showed little or no change in cells containing copy-up pir mutants of R6K derivatives. This indicates that altered π levels are not the cause of the increased copy number in the case of these mutants.

Further evidence that the total amount of π normally in the cell is not rate-limiting and that there is no strict proportionality between π concentration and γ origin activity comes from the studies with the plasmids that underproduce or overproduce π. These studies involved two types of γ origin replicons: the minimal replicon (pMF36) and an extended γ origin region that contained adjacent R6K sequences (pRK526 and pMF5). Results with each of these origin plasmids clearly demonstrated that replication occurs at a π protein level that is <1% of the normal amount. Furthermore, decreasing π levels can actually result in an increase in γ replicon copy number (up to 70 copies per chromosome for pMF36 and 30 copies per chromosome for pMF5). The higher copy number at low π concentrations and the increased sensitivity to high π concentrations for pMF36 when compared to pMF5 conceivably are due to the presence of the additional R6K sequences present in pMF5. Our present studies with π underproducers were limited to γ origin replicons. It remains to be determined whether or not intact R6K or derivatives containing the α or β origins require as few π molecules as the γ replicons for replication.

Levels of π above normal result in a reduction in plasmid copy number for all R6K γ replicon constructs examined in this study. This is consistent with the results of the transformation experiments that demonstrated that excess levels of π prevented the establishment of R6K replicon constructs containing γ, β, or α origins of replication. Pulse-labeling experiments (2) demonstrated that excess levels of π actually result in an inhibition of γ origin replication and that a copy-up mutant π protein (Cos405) is substantially reduced in this inhibitory property. Finally, the finding that π protein can inhibit replication at or near its normal concentration suggests a reason why its synthesis is autoregulated. Utilizing the *pir* gene β-galactosidase transcriptional fusion, it was observed that a substantial reduction of *pir* promoter activity does not occur until the total π concentration is at or above normal levels. Thus, a role of π autoregulation is likely to be to prevent π concentration from climbing to a level that could inhibit R6K replication. The surprising finding that the *pir* promoter is stimulated at low π concentrations may be an important property of the *pir* gene to ensure sufficient π protein for the establishment of R6K replicons after conjugal transfer.

Our ultimate goal is to elucidate the mechanism by which R6K initiates and controls its replication. Two crucial components of this process are the π protein and the 22-bp direct repeats within the γ origin, both being required for replication yet both also capable of inhibiting replication. A model for R6K replication control that attempts to accommodate the multiple properties of these two components has been described elsewhere (30). This model, similar in certain aspects to that proposed by Trawick and Kline (31) for the regulation of F replication, proposes that two biochemically distinct forms of π account for the positive and negative roles of this protein. Further insight into the mechanism of regulation of R6K replication is critically dependent on a greater understanding of the biochemical properties of the π protein and the nature of its association with DNA in the R6K replication region.

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