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Integration Host Factor of *Escherichia coli* Reverses the Inhibition of R6K Plasmid Replication by π Initiator Protein

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Integration host factor (IHF) protein is the only host-encoded protein known to bind and to affect replication of the γ origin of *Escherichia coli* plasmid R6K. We examined the ability of R6K origins to replicate in cells lacking either of the two subunits of IHF. As shown previously, the γ origin cannot replicate in IHF-deficient cells. However, this inability to replicate was relieved under the following conditions: underproduction of the wild-type π replication protein of R6K or production of normal levels of mutant π proteins which exhibit relaxed replication control. The copy number of plasmids containing the primary R6K origins (α and β) is substantially reduced in IHF-deficient bacteria. Furthermore, replication of these plasmids is completely inhibited if the IHF-deficient strains contain a helper plasmid producing additional wild-type π protein. IHF protein has previously been shown to bind to two sites within the γ origin. These sites flank a central repeat segment which binds π protein. We propose a model in which IHF binding to its sites reduces the replication inhibitor activity of π protein at all three R6K origins.

Since the replicon model was first proposed by Jacob and coworkers (33), studies of a large group of replicons have revealed the direct interaction of an initiator protein with a replication origin (for current reviews, see references 5 and 17). The *Escherichia coli* plasmid R6K is a member of this group. As shown in Fig. 1, a 5.5-kbp replication region of R6K contains three origins termed α, β, and γ (8, 9, 32, 43, 60) and the *pir-bis* operon that encodes the π and Bis replication proteins (56). The majority of DNA replication in vivo initiates at the primary α and β origins, with approximately 10% initiating at the γ origin (8). However, the minimum genetic information necessary for stable maintenance of R6K at its characteristic copy number (15 per chromosome equivalent) consists of two elements: the 400-bp γ origin and the π protein encoded by the *pir* gene (30, 31, 39). The π protein can be supplied in *trans* (39). While it is possible to physically separate the DNA defining the γ origin from the α or β origin, the primary origins require the entire γ origin, or part of it, in *cis* to permit replication (56, 60, 61).

Genetic and biochemical analyses have shown that the π protein binds seven 22-bp direct repeats at the γ origin (18, 24, 48). Previous work has established that recessive mutations in a coding segment of the *pir* gene could produce a copy-up phenotype of the R6K origin plasmids. This finding suggested that π protein might have a role in the negative control of plasmid R6K replication in addition to its positive role in initiation (18, 64). Experiments in which excessive levels of π protein inhibit R6K replication clearly demonstrate that π also has a replication inhibitor activity (15, 16). Even normal π levels of 3,500 to 10,000 dimers per cell appear to partially suppress replication, since a decrease in π concentration results in a concomitant increase in copy number for most γ origin replicons (16).

Examination of several copy-up *pir* mutations has shown that they do not consistently alter either the total amount of π in a cell (16) or the relative binding constant for the direct repeats (13a, 18). The *pir* copy-up mutations are clustered in a 40-amino-acid region (17) outside the DNA-binding domain of π (25). The biochemical basis of inhibition of γ origin activity by π protein is not known. However, replication inhibition could be visualized as the repulsion of host proteins from the origin DNA. Inhibition could occur through π-π interactions, as proposed in the "handcuffing" model (47), which might sterically hinder the access of host proteins to the origin. Alternatively, π binding could alter the origin DNA structure in a way that indirectly inhibits host protein binding.

The only host-encoded protein which has been shown both to bind and to be required for replication of the R6K γ origin (14) is the *E. coli* DNA-bending protein integration host factor (IHF) (7, 59, 68). IHF protein is a heterodimer consisting of an α and a β subunit encoded by the *himA* and *hip* (formerly *himD*) genes, respectively (19, 49, 51). This histonelike protein is utilized in a broad spectrum of processes (20), which include phage λ recombination (7, 53), expression of several genes (21, 40), DNA packaging (2), replication (3, 6, 23, 28, 50, 67), and plasmid partitioning (22). In many of these cases, IHF is thought to assist in the assembly of specialized nucleoprotein structures which provide the required accuracy for locating the active site in high-fidelity DNA transactions (11).

DNA protection experiments using neocarzinostatin (14) have shown that IHF binds to two sites within the γ origin, termed site 1 and site 2 (Fig. 1). These sites flank the seven 22-bp direct repeats which bind π protein. Cells lacking functional IHF protein cannot support replication of plasmids containing only the γ origin of R6K, while plasmids containing the primary α or β origin are able to replicate (14). Thus, the goal of this study centered on two questions. (i) What is the role of IHF protein in the replication of R6K derivatives containing only the γ origin? (ii) Does IHF influence the replication of plasmids containing the intact replication region of plasmid R6K?

In this paper we present evidence supporting the role of IHF in the regulation of replication for all three R6K origins. We show that replicons containing the γ origin can propagate

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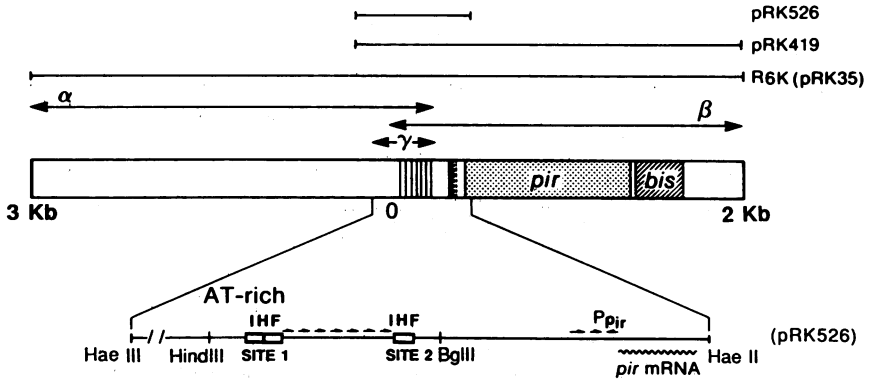

FIG. 1. R6K replication region. The locations of the seven direct repeats which bind π protein and an eighth direct repeat and two smaller inverted repeats in the operator region of the *pir* gene are indicated in the lower part of the figure by direct and inverted half-arrowheads, respectively. The π and Bis protein structural genes are designated *pir* and *bis*, respectively. IHF-binding sites 1 and 2 are indicated by open boxes. Site 1 contains two IHF consensus binding sequences, while site 2 contains one (14). Also shown is the AT-rich (80%) 90-bp segment to the left of the seven repeats. The plasmids indicated at the top of the figure contain the following segments of the R6K replication region ligated to a restriction fragment providing kanamycin resistance: pRK526, γ origin; pRK419, γ and β origins; and pRK35 α, γ, and β origins. Plasmid pRK526 (γ origin), shown in detail at the bottom of the figure, requires π protein supplied in *trans* for replication. Plasmids pRK526 (65) and pRK419 (38) share the same left boundary of the R6K sequence. The restriction sites shown reside at coordinates −700 (*Hae*III), 0 (*Hin*dIII), +277 (*Bgl*II), and +572 (*Hae*II) of the R6K sequence (65).

in IHF-deficient cells when the intracellular level of wild-type π protein is decreased or in the presence of normal levels of π protein variants deficient in negative control of the R6K copy number. Furthermore, we demonstrate that a small increase in the intracellular π concentration causes inhibition of all three R6K origins in an IHF-deficient background. We propose that the activity of all three origins of plasmid R6K is determined in vivo by competitive interactions between IHF and π for the central regulatory segment in the plasmid replication region.

MATERIALS AND METHODS

Bacterial strains and media. Strains K37 (*himA*$^+$) and K2691 (*himA* Δ*Sma*) were described elsewhere (27) and kindly provided by D. Friedman. Strains GM 1859 (*hip*$^+$) and GM 1859 (*hip-3*) (which contains a chloramphenicol resistance cartridge in the coding region of the *hip* gene) were provided by W. Reznikoff's laboratory. For all experiments, bacteria were grown in LB medium.

Plasmid construction and DNA preparation. Previous reports have described the construction and/or origin of plasmids pMF34 (18), pRK35 (37), pRK419 (39), and pRK526 (39), which contain various R6K origin segments. Plasmids pRK35, pRK419, and pRK526 contain the kanamycin resistance gene ligated to the origin fragments shown in Fig. 1. The construction of plasmid pPR1 and plasmids Δ22, Δ14, Δ5, and Δ10, which were derived by a series of *Bal* 31 digestions into the *pir* promoter of pPR1 and produce different amounts of π protein, was also described elsewhere (16). The Δ22 and Δ14 copy-up *pir* derivatives were constructed by M. J. McEachern (46a) by replacing a 307-bp *Eco*RI*-*Bgl*II fragment (R6K coordinates 497 to 804 [66]) from Δ22 or Δ14 with the equivalent fragment isolated from the original plasmid source of each *pir* mutant. All π protein plasmids used in this study contain an RK2 replicon and thus are compatible with R6K and ColE1 (pBR322) replicons. The construct pHX4-34, which has not been described in the literature and which contains the *himA* and *hip* structural genes downstream of the Tac promoter, was kindly provided by J. Gardner.

Isolation of *pir* copy-up mutants. The isolation of *pir-405 cos* (64), *pir-1*, and *pir-104* (18) mutants has been described elsewhere. The *pir-13* mutant was isolated and sequenced by M. J. McEachern (46a). The nitrosoguanidine-induced *pir-200* mutant was isolated and sequenced by P. Mukhopadhyay. Isolation of mutant *pir-116* will be described elsewhere (27a). The single-amino-acid substitutions in the *pir* mutants used in this study are to be reported elsewhere (27a).

Preparation of lysates. Total cell lysates were prepared from logarithmically growing cells and subjected to DNA and protein blotting as described previously (16).

DNA hybridization and immunoblotting. Southern hybridization analysis was carried out as described previously (63) with a *Hin*dIII γ origin fragment isolated from plasmid pMF34 and labeled by nick translation (45) as a probe. Plasmid copy numbers were determined by comparing autoradiographs containing serial dilutions of the appropriate samples. Plasmid pRK526, with a copy number of 15 per chromosome in cells harboring helper plasmid Δ22, served as the control (16). Western immunoblotting analysis was carried out as described previously (16).

RESULTS

IHF is dispensable for replication at reduced intracellular levels of π. It has been previously shown that π protein binds to the seven 22-bp repeats in the γ origin, producing a characteristic pattern of DNase I cleavage (18, 24). In addition, π binding results in enhanced sensitivity to DNase I at coordinates +15 and +51 within the AT-rich block that is located to the left of the repeats (80% AT within 90 bp [Fig. 1]) (18). It is not known whether π protein can bind to each of these distinct domains of the γ origin or whether the enhanced sensitivity to DNase I at coordinates +15 and +51 reflects the indirect effect of π binding to the seven 22-bp repeats. It has also been shown (14), by using neocarzinostatin as the DNA-cleaving agent, that both π protein and IHF protein can bind to the same γ origin fragment only if IHF protein is added to the reaction mixture before π protein. When π protein is added first, IHF protein is excluded from the protection pattern. It remains to be determined whether

TABLE 1. Transformation efficiency of plasmids pRK526 and pBR322 at different π protein levels in wild-type and *hip-3* mutant bacteria

Transforming DNA	Resident plasmid	No. of transformants of strain[a]: GM 1859 (wt)[b]	GM 1859 (*hip-3*)
pRK526	Δ22	700	0
	Δ14	1.6×10^4	1.9×10^4
	Δ5	2.9×10^4	2.0×10^4
	Δ10	3.7×10^4	1.5×10^4
pBR322	Δ22	3.0×10^5	4.0×10^5
	Δ14	4.0×10^5	3.5×10^5
	Δ5	3.0×10^5	4.0×10^5
	Δ10	4.0×10^5	4.0×10^5

[a] Competent cells (10^9) harboring one of the indicated π protein plasmids were transformed with pBR322 and pRK526 closed circular plasmid DNA. Cells (4×10^7 per strain) were plated on the appropriate drug-resistant plates. Data show numbers of transformants obtained per microgram of DNA.
[b] wt, Wild type.

TABLE 2. Relative transformation efficiency

Resident plasmid	Relative transformation efficiency (%) with strain[a]: GM 1859 (wt)[b]	GM 1859 (*hip-3*)
Δ22	0.2	0
Δ14	3.9	5.5
Δ5	9.6	5.0
Δ10	9.4	3.7

[a] Each value is calculated as the number of pRK526 transformants divided by the number of pBR322 transformants obtained for each of the resident π helper plasmids and expressed as a percentage.
[b] wt, Wild type.

the order-of-addition phenomenon is caused by simple occlusion. Alternatively, π protein binding to the seven 22-bp repeats may indirectly alter the conformation of the AT-rich block, thus preventing interaction of IHF with site 1 (Fig. 1).

It was appealing to us to assume that the competitive interactions between IHF and π for origin DNA can also occur in vivo and that the positive effect of π protein on R6K replication can change to replication inhibition as the intracellular concentration of π increases (15, 16). Thus, IHF as part of a nucleoprotein structure formed at the origin may (i) act as a direct positive factor necessary for replication or (ii) interfere with the inhibition of replication by π.

It was possible to test our model by examining γ origin activity in wild-type and IHF-deficient cells producing different π levels. The intracellular π concentration is at least 100-fold higher than that needed for γ origin replication (16). If IHF is absolutely essential for initiation, then reducing π protein levels should have no effect and IHF-deficient cells will still be unable to support replication of a γ origin plasmid. On the other hand, if the role of IHF is to allow replication at an otherwise inhibitory concentration of π protein, then the γ origin might function in the absence of IHF if π levels were reduced.

This experiment used *Bal* 31 deletion derivatives of the *pir* promoter of plasmid pPR1. The resulting plasmids, Δ22, Δ14, Δ5, and Δ10, produce approximately 100, 5, 2, and <1% of the wild-type π level, respectively (16). The Δ plasmids were each established in a *hip-3* mutant strain and its isogenic parent. These cells were then transformed with a γ origin replicon, pRK526. As indicated in Tables 1 and 2, there were no transformants in IHF-deficient bacteria producing normal levels of π protein. However, in the same genetic background, kanamycin-resistant transformants were obtained when π was supplied at a reduced level. It is also important to note that the lowest transformation frequency of the γ origin plasmid pRK526 was observed with the wild-type recipient which harbored plasmid Δ22 (producing normal π levels). This was not the case with the control plasmid, pBR322. These data are consistent with the observation that Δ22 produces π at a level limiting the γ origin plasmid copy number even in IHF-proficient cells (16). Clonal analysis was carried out with randomly chosen transformants, and an autonomously replicating pRK526 (γ origin) plasmid was detected in all clones examined (Fig. 2 and data not shown). Therefore, a decrease in the intracellular concentration of π permits replication of the γ origin in the absence of a functional IHF heterodimer. These data demonstrate that *E. coli* cells deficient in the production of IHF protein are unable to support replication of a γ origin plasmid

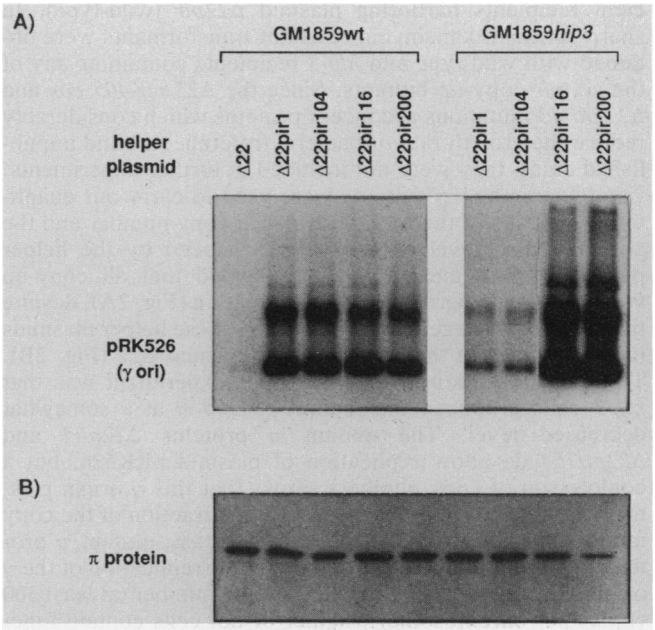

FIG. 2. Copy number of a γ origin plasmid in wild-type and *hip-3* mutant bacteria harboring helper plasmids producing wild-type π and its copy-up variants. One-milliliter aliquots of logarithmically growing cells (5×10^8 cells) were harvested by centrifugation and lysed according to the protocols described in Materials and Methods. Half of each sample served to analyze either the plasmid DNA level or the π level. Equivalents of 5×10^7 bacteria for each sample were loaded onto agarose gels, and equivalents of 1×10^7 bacteria were loaded onto sodium dodecyl sulfate-polyacrylamide gels and subjected to either DNA hybridization (A) or immunoassay (B). pRK526 plasmid copy numbers were estimated from Southern analysis, assuming that the Δ22 helper permits replication of a γ origin replicon at a copy number of 15 per chromosome equivalent (17). The higher-molecular-weight bands observed are multimeric forms of the pRK526 plasmid. The relative copy number of plasmid pRK526 was determined by comparison of signal intensities produced by serial dilutions of each sample (data not shown) and matching these dilutions with reference samples. These procedures were determined experimentally to be as adequate as the determination of plasmid copy number and π levels by laser scanning of film negatives (16).

unless the level of π protein is reduced below normal. The need for IHF in the replication of the γ origin is dependent on the concentration of π protein, which suggests a regulatory role for IHF during replication.

Cells producing variants of π protein relaxed in copy number control allow replication of a γ origin replicon in the absence of functional IHF protein. If the role of IHF in γ origin activity is to hinder the concentration-dependent negative function of π, then normal levels of π proteins with altered regulatory function might allow replication in the absence of IHF. To test this idea, we used the existing copy-up *pir* mutants. In wild-type cells, these copy-up π proteins are relaxed in negative control and result in greatly increased copy numbers of a γ origin plasmid (17).

Copy-up helper plasmids were created by replacing the 307-bp *Eco*RI*-*Bgl*II fragment from the wild-type *pir* gene of plasmid Δ22 (17) with the equivalent fragment from copy-up variants containing single-amino-acid substitutions (*pir-1*, *pir-13*, *pir-104*, *pir-116*, *pir-200*, and *pir-405 cos*) (47). These helper plasmids and Δ22 were established in a *hip-3* mutant strain and its wild-type counterpart. These recipients were then transformed with a pRK526 (γ origin) plasmid. As expected, we failed to obtain transformants with IHF-deficient recipients harboring plasmid Δ22*pir* (wild-type). In sharp contrast, kanamycin-resistant transformants were obtained with wild-type and *hip-3* recipients containing any of the six *pir* copy-up mutants. Since the Δ22 *pir-405 cos* and Δ22 *pir-13* mutations produce π proteins which considerably reduce the growth rate of bacteria (reference 64 and unpublished data), they were not included in further experiments. The four remaining mutants were used to carry out quantitative analysis of the pRK526 γ origin copy number and the corresponding level of π protein produced by the helper plasmid. These measurements indicated that all copy-up variants of π do permit plasmid replication (Fig. 2A), despite the fact that the level of π produced by these helper plasmids is very similar to that produced by plasmid Δ22 (Fig. 2B). The only exception in this particular experiment was that *hip-3* cells produced the mutant *pir-200* π at a somewhat decreased level. The mutant π proteins Δ22*pir1* and Δ22*pir104* do allow replication of plasmid pRK526, but a comparison of copy numbers shows that this γ origin plasmid replicates in IHF-deficient cells at a fraction of the copy number seen in wild-type cells. In contrast, mutant π proteins Δ22*pir116* and Δ22*pir200* both allow replication of the γ origin-containing plasmid at a high copy number (at least 300 copies per chromosome) whether or not cells contain functional IHF protein.

We also examined the response of the γ origin copy number to subnormal levels of mutant π proteins. The Δ14 derivatives of the wild type, *pir-1*, *pir-104*, *pir-116*, and *pir-200* all produce wild-type or mutant π protein at approximately 1/20 of the normal level (16). As shown in Fig. 3A, the pRK526 γ origin copy number remains greatly increased (approximately 300 copies per chromosome) in both wild-type and *hip-3* bacteria producing Δ14*pir116* and Δ14*pir200* mutant proteins. However, a comparison of the Δ22 and Δ14 levels of the *pir-1* mutant (and the *pir-104* mutant; data not shown) shows that the plasmid copy number is clearly influenced by the amount of mutant π protein made in IHF-deficient bacteria. By comparing Δ22*pir1* in GM 1859 (*hip-3*) (Fig. 2A) with Δ14*pir1* in GM 1859 (*hip-3*) (Fig. 3A), it can be seen that the pRK526 γ origin copy number is substantially higher in *hip-3* cells underproducing the *pir-1* (and *pir-104*; data not shown) mutant protein. This variance in copy number is not observed in the wild-type counterpart

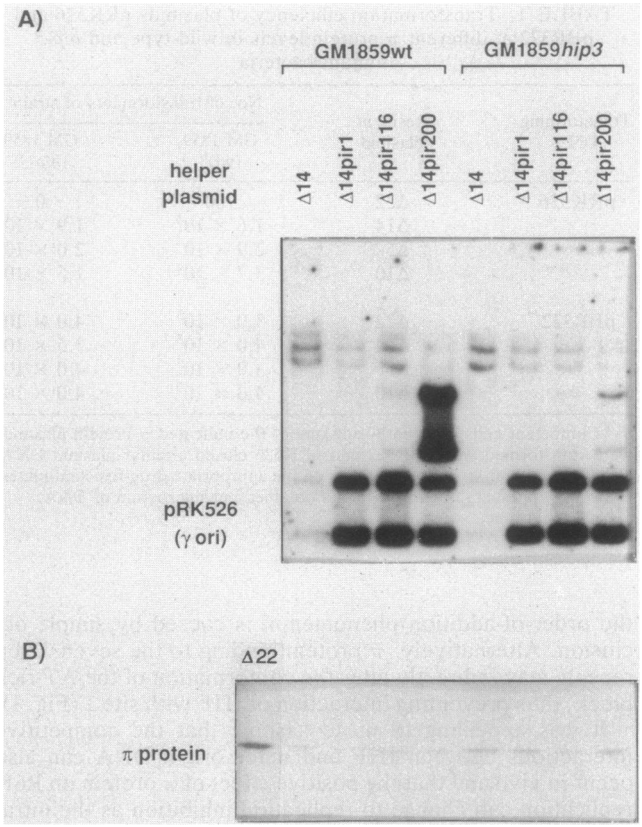

FIG. 3. Copy number of a γ origin plasmid (A) in cells underproducing wild-type and copy-up π protein variants (B). The experiment was carried out with strains carrying Δ14 helper plasmids (16) bearing wild-type or mutated variants of the *pir* gene. The wild-type level of π protein (Δ22), from a sample prepared simultaneously, is provided for comparison. Assay conditions were as described in the legend to Fig. 2.

(compare Δ22*pir1* in wild-type GM 1859 [Fig. 2A] with Δ14*pir1* in wild-type GM 1859 [Fig. 3A]). These data clearly show that the *pir-1* and *pir-104* mutants are still able to inhibit the γ origin in a concentration-dependent manner in cells lacking functional IHF protein.

The major conclusions from the data presented in this section and the previous section are that (i) IHF protein is not absolutely essential for initiation from the R6K γ origin and (ii) replication can occur in situations in which π protein has a reduced ability to control the copy number. Therefore, all data presented thus far are consistent with the model in which IHF protein assists in the regulation of γ origin replication by reducing the replication-inhibiting activity of π protein.

IHF is essential for replication of a plasmid containing all three replication origins under the conditions of increased intracellular levels of π. As previously reported (14), the entire R6K plasmid or replicons containing two origins (γ and β) can be established in cells lacking functional IHF protein, but γ origin plasmids cannot. The difference between the ability of the primary origins (α and β) and that of the secondary origin (γ) to replicate without IHF could reflect the greater vulnerability of the γ origin to elevated π levels in vivo. Experiments performed previously with wild-type bacteria had shown that plasmid pRK419(γ + β) is able to replicate at a π protein level that prevents pRK526 (γ

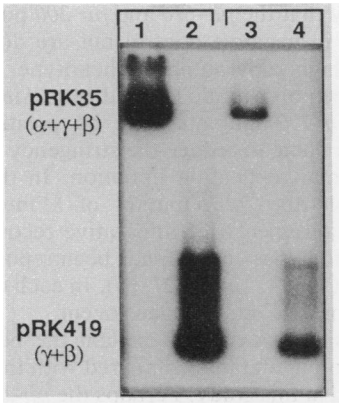

FIG. 4. The copy numbers of extended-origin plasmids pRK35 and pRK419 are shown for the wild-type strain (lanes 1 and 2, respectively) and *hip-3* mutant (lanes 3 and 4, respectively). The copy number was assayed by Southern hybridization as described in the legend to Fig. 2.

origin) replication (13a). Perhaps the primary replication origins do not require IHF at normal π levels but need IHF to replicate at elevated π levels. This hypothesis was tested in the experiments described below.

One group of isogenic strains (*hip-3* and *himA* Δ*Sma* mutants and their wild-type counterparts) was transformed with helper plasmid pPR1, which produces approximately 1.5 times the normal R6K π level (16). This group and a second group of plasmid-free isogenic strains were used as recipients in transformation with the R6K derivatives pRK419(γ + β) (39) and pRK35(α + γ + β) (37). Both of these replicons are able to produce normal levels of wild-type π protein (Fig. 1). Neither plasmid pRK419(γ + β) nor pRK35(α + γ + β) could be established in IHF-deficient cells containing plasmid pPR1. In contrast, when either the wild-type cells or IHF-deficient mutants lacking plasmid pPR1 were transformed with plasmid pRK419(γ + β) or pRK35(α + γ + β), transformants were obtained with each recipient. Subsequent analysis of the plasmid copy numbers revealed that, despite the ability to replicate, there is a substantial reduction in pRK419(γ + β) and pRK35(α + γ + β) plasmid DNA content in both the *hip-3* (Fig. 4; compare lane 1 with lane 3 and lane 2 with lane 4) and *himA* (data not shown) mutants. The additional π supplied by pPR1 causes a lack of γ + β or α + γ + β replicon transformants in IHF-deficient cells.

Since the lack of IHF resulted in a lack of transformants, we tried to prevent the replication inhibition by supplying IHF protein. Plasmid pHX4-34 contains both the *himA* and *hip* structural genes under the control of an inducible Tac promoter. Plasmids pHX4-34 and pPR1 were established as resident plasmids in the *himA* and *hip-3* strains and their isogenic parents. These bacteria were then transformed with replicon pRK419(γ + β) or pRK35(α + γ + β). Again, no transformants were obtained in IHF-deficient bacteria that contained only pPR1. However, both *himA* and *hip-3* cells containing plasmids pPR1 and pHX4-34 (induced with IPTG [isopropyl-β-D-thiogalactopyranoside] to produce IHF protein) were now able to support replication of pRK419(γ + β) and pRK35(α + γ + β). The number of transformants was equal to that of the wild-type parent strains also containing pPR1 and pHX4-34. Thus, supplying intact IHF enabled the IHF-deficient mutants to replicate at elevated π protein levels.

From these data we infer that the reduction in plasmid copy number in cells lacking IHF is caused by an increased susceptibility of primary R6K origins to the replication-inhibiting activity of π. The additional π protein supplied by plasmid pPR1 prevented replication of the primary origins in cells lacking functional IHF. These experiments demonstrate that IHF is important for replication control in all three origins. The activity of each of the three R6K origins is reduced to various degrees in the absence of functional IHF, with the γ origin exhibiting the most stringent dependency on IHF and the greatest sensitivity to the level of π protein in the cells.

DISCUSSION

In this article, we present evidence that IHF protein neither is required for replication initiation of plasmid R6K nor affects replication by altering the level of the π replication initiator protein; instead, IHF protein serves as an essential host component in the copy number control mechanism of all three R6K origins.

Previous work on R6K had shown that the majority (90%) of in vivo initiations occur at the α or β origin (8). The γ origin, which contains the known IHF-binding sites, is an essential DNA segment for initiation at the α or β origin (56, 60, 61). It is unknown whether relative origin usage is altered in IHF-deficient cells. However, the π protein level necessary for inhibition of plasmid replication is reduced in IHF-deficient bacteria, regardless of which origins are present on a plasmid. The isolated γ origin is able to replicate at the copy number characteristic of the entire R6K plasmid when a normal level of wild-type π protein is supplied in *trans*, but this origin replicates in the absence of the IHF heterodimer only when the π protein level is substantially reduced. This reduced amount of π protein will produce an elevated plasmid copy number if the γ origin is replicating in cells containing IHF (Fig. 2 and 3; 16). The plasmid constructions containing either the α, β, and γ origins (pRK35) or the β and γ origins (pRK419) produce normal levels of wild-type π protein. Unlike the γ origin plasmid, these replicons can function in IHF-deficient bacteria, but their copy number is reduced. In wild-type cells these primary origins can function with π levels up to eight times higher than the normal level (16). However, the 1.5-fold increase in π protein in IHF-deficient cells prevents replication of the plasmids containing the α and β origins. The inability of these α and β origin plasmids to replicate in IHF-deficient cells when extra π protein is present can be compared qualitatively to the inability of a γ origin plasmid to replicate in IHF-deficient cells at normal π protein levels. The relationship between π protein and IHF protein in R6K replication can be viewed as follows: while π protein is required for replication of all three origins, a level inhibiting γ origin replication is soon reached unless IHF protein is present. The primary origins are able to continue replication at higher levels of π protein, but eventually their ability to function is also reduced in the absence of IHF.

Any analysis of R6K origin function (see reference 17 for the most recent review) has to take into account the differences in π protein levels required for initiation from the three R6K origins. From the measurements of γ origin replicon copy number at various intracellular concentrations of π, it had been concluded that this origin is able to replicate at a wide range of π protein levels below the normal level (16). A long-range cooperativity in the binding of π to its primary targets in the γ origin and a weaker secondary target in the β

origin requires a higher level of protein than that needed for binding only the γ origin (54). The *pir* structural gene, which is required in *cis* for β origin function (47), also contains other secondary π-binding sites (13a). The α origin contains sequences homologous to the 22-bp repeats in the γ origin (60); these may also prove to be secondary π-binding sites. The extended replicons pRK419 and pRK35 are less susceptible than the minimal γ origin to replication inhibition by π (16); this could reflect the requirement for higher π levels to bind these secondary sites. At higher π levels, a π-mediated loop could form between the γ origin and the secondary sites in the β origin (54), allowing replication machinery which has assembled at the γ origin to be transferred to the β origin. A similar scenario is possible between the γ and α origins. Perhaps the extended origins are less affected by the absence of IHF because they require a higher initial π concentration for replication initiation.

IHF protein assists in the control of γ origin replication by acting directly at the origin rather than indirectly by altering the level of π protein produced by the helper plasmids. Both the Δ22*pir* constructions (containing a wild-type operator-promoter region) and the Δ14 helpers (Fig. 2B and 3B, respectively) produce the same amount of π protein in cells containing or lacking IHF. This is consistent with previous results (the only exception in this particular experiment, but not others, was that *hip-3* cells produced the mutant Δ22*pir200* π at somewhat decreased level [Fig. 2B]). In contrast is site-specific recombination in phage λ, in which IHF directly participates in the recombination reaction and also regulates the amount of integrase protein available (by directly affecting the production of *c*II protein [44, 57]).

The effects of IHF on replication of the primary R6K origins may present a more complex situation than that of the γ origin. pRK419 and pRK35, the plasmids which were used to study the primary origins, contain the *pir* gene in *cis*. It is possible that IHF influences the level of π protein produced by these constructs. Directly upstream of IHF site 2 lies a promoter approximately 1/10 as strong as the *pir* promoter (56a). IHF binding to site 2 may thereby decrease transcription of the *pir* gene. This possibility and potential effects on R6K replication will be investigated in the future.

An additional argument supporting the role of IHF in a negative control circuit of plasmid copy number is derived from genetic experiments with π mutants. Helper plasmids producing any of six copy-up variants of π all allow IHF-independent replication, despite the fact that none were specifically selected to do so. The *pir-116* and *pir-200* mutants produce a high-copy-number phenotype in a γ origin plasmid, irrespective of π protein levels in wild-type or IHF-deficient hosts. The *pir-1* and *pir-104* mutants also produce a copy-up phenotype in wild-type cells irrespective of π protein levels. However, the *pir-1* and *pir-104* mutants can fully express the high-copy-number phenotype in IHF-deficient cells only at 1/20 of the normal π protein level; thus, in IHF-deficient cells, these two π mutants retain the concentration-dependent ability of wild-type π to inhibit replication.

In a complementation test, these pairs of π mutants also differ in their ability to increase the copy number of pRK419, a γ + β plasmid which contains the wild-type *pir* gene in *cis*. The *pir-1* and *pir-104* mutations are recessive, while the *pir-116* and *pir-200* mutations are dominant (27a). The meaning of this complementation test is somewhat ambiguous, considering the dimeric structure of π protein (18). All of the copy-up mutations map within a 40-amino-acid region of the *pir* gene responsible for negative control of the copy number (17). It is likely that the *pir-116* and *pir-200* polypeptides are also defective in negative control but are dominant in expressing the high-copy-number phenotype when present with a wild-type polypeptide in a π dimer. Alternatively, the *pir-116* and *pir-200* mutants may be positive regulatory mutants that are able to reduce the stringency of the requirements for normal replication initiation. In that case, they would resemble the *int-h* mutant of λ integrase, which relaxes the requirements for integrative recombination (42, 52). The genetic bypass of IHF has been reported in several other systems (1, 3, 4, 12, 13, 27, 69). In each case there was selection for the IHF-independent event.

A final possibility exists to explain the behavior of the dominant π protein mutants compared with that of the other π mutants used in this study. Perhaps the *pir-116* and *pir-200* dominant π proteins are able to utilize an alternate mode of replication that bypasses the need for IHF. Likewise, increasing the concentration of mutant replication protein may not inhibit this alternate pathway. Replication options are known to exist for several replicons. Plasmid ColE1 is the best-characterized example of alternate modes of DNA initiation (10, 35, 46). Replication of the *E. coli* chromosome during "stable DNA replication" (34) is independent of *oriC* and DnaA protein (36). Also of interest is recent work in our laboratory which shows that *oriC* minichromosomes in cells lacking IHF protein require DNA polymerase I for replication (17a). It is unknown what the requirements would be for an alternate replication mode by the dominant mutant π proteins or which mechanisms would be utilized.

While it is possible that IHF protein serves as a structural element directing interaction with another DNA-binding protein, there is as yet no evidence supporting such a mechanism at the R6K origin. The close proximity of IHF-binding sites to the π-binding sites in the R6K origin would certainly permit direct π-IHF interactions, as discussed earlier (14).

In contrast, several independent lines of evidence have shown that DNA bending is a common phenomenon underlying a diverse array of processes affected by IHF (29, 41, 58, 62, 67). As shown recently for one of the three IHF-binding sites in *attP* of phage λ, the requirement for IHF can be functionally substituted by a sequence-induced bend (26). Thus, this property may be the sole function of the protein in other processes as well. Since IHF binding also causes R6K origin bending (14a), it seems appealing to think that the origin inhibition by π protein could be reversed by altering the architecture of a region adjacent to the π-binding repeats. Therefore, it is unclear whether the bending or unwinding of origin DNA facilitated by the binding of π protein (55) would necessarily produce a reactive replication substrate. It is known that π protein is able to bind simultaneously to the γ and β origins, looping out the intervening DNA (54). However, it is not known whether this π protein "action at a distance" (54) serves solely to transmit a positive signal from the γ origin to the β origin. One could imagine that within a narrow range of increase in π concentration, at which origin inhibition can occur, there could be a major structural change resulting in a substrate which would not be able to participate in replisome assembly. This inert product could resemble a nucleosomelike structure and result from π-mediated origin bending (55) or could be produced by the formation of "handcuffed" molecules (47). Each of the primary origins requires γ origin domains where IHF protein binds, in addition to the repeat segment. Thus, it is conceivable that the γ origin in a nonactive conformation

has a reduced ability to engage in contact with the α and β origins unless IHF protein is provided.

We have shown that IHF protein is an important component in plasmid R6K replication, acting to reduce the replication-inhibiting activity of the multifunctional π initiator protein. It remains to be determined whether the role of IHF in the activity of all three origins of plasmid R6K is mediated by a fundamentally similar mechanism at the molecular level.

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