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The integration host factor of *Escherichia coli* binds to multiple sites at plasmid R6K γ origin and is essential for replication

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ABSTRACT

Examination of the effect of the himA and himD mutants of E. coli on the maintenance of plasmid R6K has revealed that the γ origin-containing replicons cannot be established in any of the mutants deficient in the production of E. coli Integration Host Factor (IHF). Contrary, the R6K derivatives containing other origins of the plasmid (α and/or β) replicate in a host lacking functional IHF protein. We show that IHF protein binds specifically to a segment of the replication region which is essential for the activity of all three R6K origins. Mapping the IHF binding sequence with neocarzinostatin showed that the protein protects three segments of the origin: two strong binding sites reside within an AT-rich block, while the third, considerably weaker site is separated from the other two by a cluster of the seven 22 bp direct repeats. These seven repeats have been shown previously to bind the R6K-encoded initiator protein π. We also demonstrate that the establishment of π-origin complexes prior to IHF addition prevents the binding of the IHF protein to the γ origin. The binding sequences of IHF and π proteins do not overlap, therefore, we propose that the binding of π protein alters the structure of the DNA and thereby prevents the subsequent binding of IHF protein.

INTRODUCTION

An increasing amount of evidence has accumulated over the past several years, indicating similar overall structures of the replication region for a number of plasmids (e.g. 1). They consist of origin of replication containing nucleotide sequence repeats and a closely spaced open reading frame for a replicon-specific initiator protein. Binding of replicon-specific initiator proteins to origin repeats has been shown for several plasmids (2-7) oriC of Escherichia coli (8) and eucaryotic origins of SV40 (9) and Epstein-Barr virus (10). Another common but less conserved feature of repeat-containing replicons is the presence of multiple origins of replication, indicating a composite structure of their replication regions.

The self-transmissible antibiotic-resistance plasmid R6K of E. coli contains in its 4Kb replication region three replication origins designated α, β and γ (11,12) and the structural genes pir (13) and bis (14) that encode for

the π and Bis proteins, respectively. The minimal genetic information, as demonstrated thus far, that is required for stable maintenance of R6K at its copy number of about 15 per chromosome equivalent includes a 400 bp γ origin (15) and the pir gene, whose product can function when supplied in trans (16). The π protein plays a negative (17,18,19) role in functioning of all three R6K origins in addition to its absolute requirement for their activity, whereas the Bis protein appears to be essential only for the β origin of replication (14,20). The repressor-like role of the π protein in autoregulated expression of its own gene has been shown both in vivo and in vitro (21-23). To accomplish replication initiation and the repression of its own synthesis requires binding of π protein to the seven origin repeats, to an eighth repeat and to a smaller, inverted pair of repeats, respectively (3,22).

Despite a lack of homology at the nucleotide sequence level, it seems interesting that a great majority of minimal replicons contain stretches of DNA rich in AT residues (24). In the case of the R6K γ origin a 90 bp AT-rich block has been identified to the left of the repeat cluster (25). It is intriguing that the consensus binding site for IHF protein of E. coli usually resides within AT-rich segments.

The IHF protein belongs to a group of DNA binding proteins, termed DNA binding proteins II (DBP II), whose structural similarities and function in procaryotic organisms have been the subject of recent review by Greene et al. (26). Since the discovery of IHF as a sequence-specific DNA binding factor (27) playing an accessory role in λ recombination (28), it has also been implicated in a number of other processes in E. coli, such as the regulation of gene expression at the transcriptional (29,30) and post-transcriptional (31,32) levels, the packaging of lambdoid phage DNA (33) and DNA replication of plasmid pSC101 (34,35). An IHF binding site is a common feature of these diverse processes.

In this study, we demonstrate that replicons containing only the γ origin of R6K cannot replicate in E. coli cells containing mutations in either the himA or hip (himD), the structural genes for the two subunits of IHF heterodimer (36). Immunoassay of the π level in the IHF-deficient strain ruled out the possibility that the lack of γ origin activity is due to an altered level of π protein. Instead, direct evidence for IHF binding sites, adjacent to the π binding sites in the γ origin, is provided. This suggests that possibly physical contact between both π and IHF at the R6K γ origin is required for initiation of R6K replication.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The E. coli strains K5185 (himA82), K5242 (himA81), K5248 (hip157), DS1255 (himA82), DS1256 (himA63) and the parent strain K37 were obtained from Dr. D.W. Smith (University of California, La Jolla). Characteristics of plasmids R6K (37), pRK35 (38), pRK419 and pRK526 (16), pMF51 and pMF36 (19), pMM3 (3) and of other plasmids used in this study are indicated in the text or will be described elsewhere.

Measurements of π protein levels.

Preparation of the total cell lysates and the quantitative immunoassay of the π were carried as described (19).

Neocarzinostatin and DNase I footprinting assay.

DNA sequencing was performed as described (4). Reaction with NCS and DNase I protection experiments were carried out in a 100 μl reaction mixture containing 20 mM KPO4 (pH 7.4), 100 mM NaCl, 1 mM Na EDTA, 5 mM dithiothreitol (prepared fresh daily) 50 μg per ml of bovine serum albumin (Pentex) and 1-5 ng of uniquely 5' end labeled with polynucleotide kinase (BRL) and (^{32}P) ATP restriction fragment containing the γ origin of plasmid pMF34 (4). The reaction was incubated without or with IHF or/and π protein at 25C, and 2.8 mM NaAc (pH 5.0) containing 0.5 U NCS (Bristol Laboratories, kindly provided by Dr. P.E. Geiduschek) or 0.1 μg of DNase I was added and incubation continued for 1 minute. To stop either DNase or NCS cleavage, a 100 μl of 1.0 M NH_4Ac, 0.1 M Na EDTA and 20 μg per ml sonicated calf thymus DNA was added. The samples were then extracted with phenol/chloroform/isoamyl alcohol (1:1:0.04) and the aqueous phase collected and DNA precipitated with equal volume of isopropanol. The resulting pellet was washed with 70% ethanol, dried and resuspended in formamide sequencing mix and subjected to urea-polyacrylamide gel electrophoresis.

Gel retardation assay.

Plasmid constructs, indicated in Figure 2 legend, were cleaved with a combination of the following enzymes, BglII, EcoRI, HindIII and/or SnaBI (New England Biolabs), resulting fragments purified by phenol extraction followed by isopropanol precipitation. Gel retardation assay was performed in 0.8% agarose as described (4).

Purification of IHF protein.

IHF protein was purified from the strain HN880 containing plasmids overproducing both subunits of the protein heterodimer constructed by Dr. H.

Miller. The cell paste was kindly provided by Dr. H. Nash. The original procedure of purification of the protein described by Nash and Robertson (39) was modified, taking advantage of the very strong binding of IHF to heparin. A French-press lysate obtained from 20 gm of cell in 100 ml of buffer containing 24 mM Tris-HCl (pH 7.4), 1 mM Na EDTA, 3 mM B-mercaptoethanol, 100 mM NaCl, 10 mM MgCl2, was cleared at 4°C by high speed centrifugation at 42 rpm for 4 hours and subjected to ammonium sulfate fractionation. The precipitate obtained (0.334-0.564 g/ml) was resuspended in buffer A [25 mM Tris-HCl (pH 7.4), 1 mM Na EDTA, 3 mM B-mercaptoethanol and 50 mM NaCl] and the suspension was dialyzed overnight against 2 liters of the same buffer. The lysate was then applied to a heparin Sepharose CL6B column (Pharmacia) equilibrated with buffer A. A 300 ml NaCl gradient (0.05-1.6 M) in buffer A was applied with speed 25 ml/hr yielded electrophoretically pure IHF protein, as judged by silver staining procedure. Furthermore, no differences between the IHF protein purified according to these two independent procedures were noted either in the Int-mediated recombination of phage lambda (H. Nash, personal communication) or in NCS-IHF protection pattern at R6K γ origin (data not shown).

RESULTS

One of three R6K origins requires IHF protein.

As shown in Figure 1, three replication origins reside within a 4Kb replication region of plasmid R6K (11,12). Two of these origins, namely β and γ, can be studied individually, whereas the α origin contains the entire γ origin sequence and an adjacent HindIII 4 fragment (15). The availability of different R6K derivatives containing one, two, or all three replication origins allowed us to examine the requirement of the IHF protein for their activity. Initially, we transformed IHF-deficient strains [(himA or himD (hip), see Materials and Methods for the strain genotypes] with intact plasmid R6K or its derivative pRK35, which also contains all three replication origins. Despite using a number of different mutants, including deletions of the himA gene (ΔhimA82 and ΔhimA63), we could not detect any effect of the mutations on the transformation frequency when compared to that of the isogenic parent strain. Similarly, we failed to detect a requirement of IHF protein for the activity of replicons containing only the β origin (pMF51) or the combination of the β and γ origins (pRK419). Interestingly, however, the ΔhimA82 strain, harboring either of two helper plasmids, pMF42 or pPR1 that produce π protein (14,19), could not support replication of pMF36 or pRK526

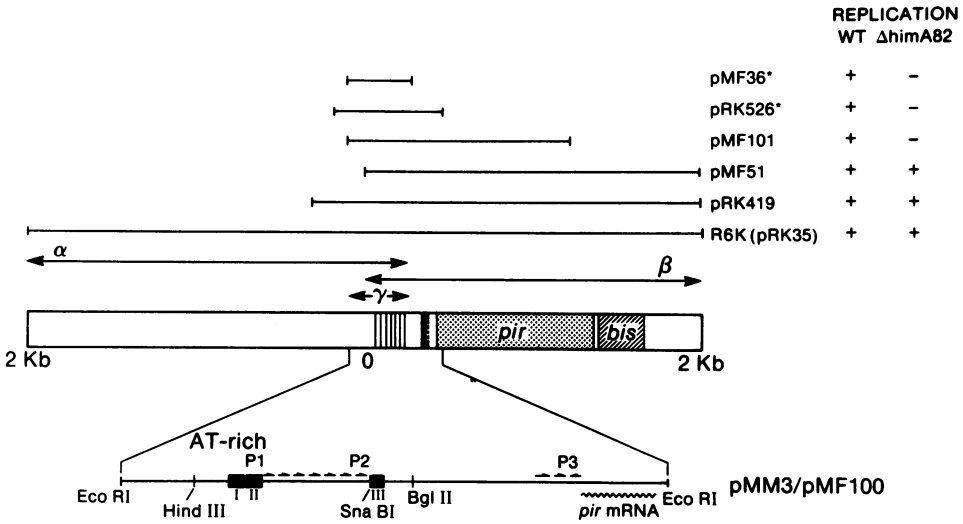

Figure 1: Localization of the R6K origins of replication and the effect of IHF-deficient strain DS 1255 (himA82) on their activity α, β and γ indicate the location of three origins of replication. The relative locations of the seven direct repeats and the eighth and two inverted smaller repeats in the operator region of the pir gene are indicated by direct and inverted arrows, respectively. The π structural gene and a second open reading framed for Bis protein are designated pir and bis, respectively. Two functional promoter regions P1 and P2 (P. Mukhopadhyay et al., in preparation) are indicated in the minimal γ origin replicon. The IHF binding sites (I, II and III) are indicated by solid boxes. The 90 bp segment 80% AT-rich to the left of the cluster of the seven repeats is indicated by the AT-rich mark. The restriction sites indicated EcoRI, BglII, HindIII and SnaBI are provided to help interpret the data shown in Figure 2. The plasmids shown at the top of the figure contain different segments of R6K replication origin ligated to a restriction fragment providing an antibiotic-selective marker. (+) and (-) indicate the ability or lack of plasmid maintenance, as determined by DNA transformation analyses. * indicates plasmids whose replication activity was examined in a host containing the helper plasmid pMF42 that produces the π protein.

(Figure 1). Plasmids pMF36 and pRK526 contain only the R6K γ origin. Additional support for IHF being essential for the γ origin comes from a transformation test with plasmid pMF101. This plasmid contains the π gene in cis with the γ origin, but lacks a functional β origin. The protein produced by pMF101 lacks 26 C-terminal amino acids, but is capable of supporting replication both in vivo (Fig. 1) and in vitro (21) and of binding to the repeats in vitro (M.F., unpublished data).

Although the π protein is not a rate limiting factor in γ origin activity, it does inhibit replication at normal and higher than normal

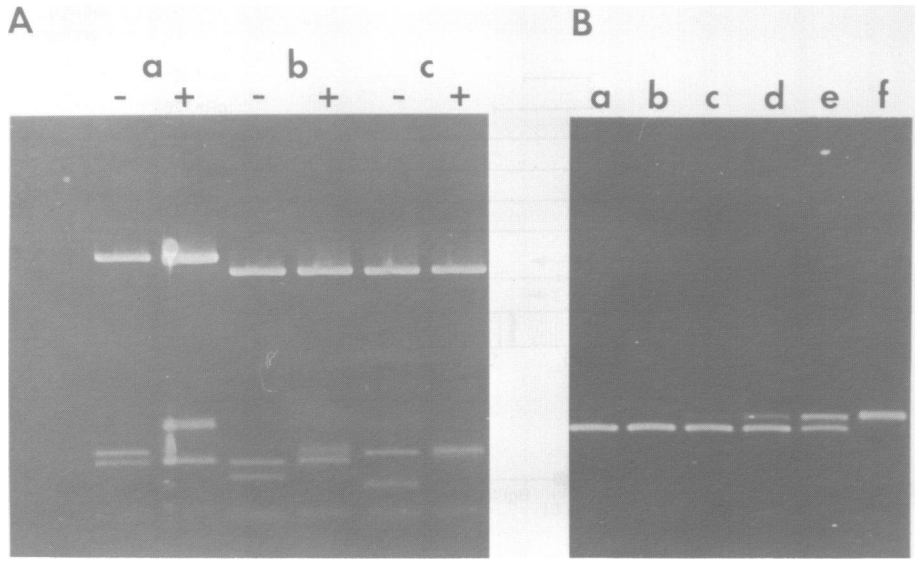

Figure 2: (A) Association of the IHF protein with restriction fragments of plasmid R6K present in plasmids pMM3 or pMF100, measured by the agarose gel retention assay. Approximately 1.5 μg of the plasmid DNA digested with; BglII and EcoRI (a), HindIII, EcoRI and BglII (b) or HindIII, EcoRI and SnaBI (c) were electrophoresed without (-) or after (+) prior incubation with 0.05 μg of purified IHF protein. (B) 0.05 μg of the HindIII γ origin containing fragment isolated from plasmid pMF34 (4) and incubated with 0.01, 0.02, 0.04, 0.06, and 0.08 μg of IHF protein (lanes b-f) each in 15 μl reaction volume and compared to the electrophoretic mobility of the naked DNA (lane a). The experimental details of the assay are described in Materials and Methods.

intracellular levels (18,19). To rule out the possibility that the inability to establish a γ origin replicon in the IHF-deficient strain is due to π protein overproduction, we carried out an immunoassay analyses of the total cell lysates prepared from wild-type and Δ*him*A82 mutant cells bearing either of the two π-producing helper plasmids. Neither pPR1 nor pMF42 produced different levels of the π protein in either bacterial hosts (unpublished data). Thus, we concluded that it is not an altered level of π that accounts for the lack of γ origin functionality in the IHF-deficient strains; rather it is direct involvement of the protein in the γ origin activity.

Binding of the IHF protein to a restriction fragment containing the R6K γ origin sequence.

Availability of highly purified IHF protein (see, Materials and Methods) allowed us to determine if IHF protein interacts with restriction fragments containing the entire γ origin or part of it. Two vector plasmids containing

the same R6K sequence cloned into pBR322 [pMM3(3)] or pUC8 (pMF100, this study) were cleaved with a combination of enzymes (described in the legend to Figure 2) and subjected to agarose electrophoresis either with or without prior incubation with IHF protein. Results of this gel retention analysis indicate that IHF protein interacts with the part of γ origin sequence residing between HindIII and SnaBI restriction sites (Fig. 1). IHF protein did not bind to the 115 bp EcoRI-HindIII fragment to the left of the HindIII 4/9 junction nor the operator-promoter region of the pir gene present in either fragment EcoRI-BglII or EcoRI-SnaBl (Fig. 1). Further experiments were carried out with the γ origin obtained from a HindIII digestion of plasmid pMF34. In comparison to the HindIII-SnaBI fragment just described as binding IHF protein, the HindIII fragment contains an additional 37 bp to the right of the repeats. This short region is essential for the γ origin activity (3,25) and has been found to contain a weak IHF binding site (see next section). Interestingly, however, the presence of this binding site did not alter electrophoretic mobility of the EcoRI-SnaBI fragment incubated with IHF (Figure 2, lane C+). Examining the electrophoretic mobility of the HindIII origin fragment as a function of IHF concentration, we found (Figure 2B) that unlike the π protein, which forms up to seven discrete bands upon addition of increasing amounts of the protein (4), the complexation of the IHF with the same fragment resulted in a one step shift. These data suggested that either the IHF binds a single site or that it binds in a cooperative manner.

Localization of IHF protein binding sites.

Because two putative IHF binding sites were localized to the AT-rich region to map the segment(s) of the origin that make contact with IHF, we chose Neocarzinostatin (NCS), known to specifically cleave AT residues (40) as a probing agent in a protection experiment. As shown in Figure 3A, at a low IHF to DNA ratio, NCS induced cleavage is substantially reduced in the AT-rich region to the left of the repeats (coordinates 61-88). These regions are termed IHF binding sites I and II (Fig. 1 and 4). A two-fold increase in the IHF protein concentration does not change the pattern of IHF footprint, however, a five-fold increase in the protein concentration resulted in the further protection of the region immediately to the right of the repeats (coordinates 251-263). The IHF protection of this sequence (in the complementary strand), termed IHF binding site III with more resolved NCS cleavage products, is displayed in Figure 3B.

Taking into account IHF-NCS protection data, and the average size of IHF binding site (41), we conclude that the γ origin segment contains three IHF

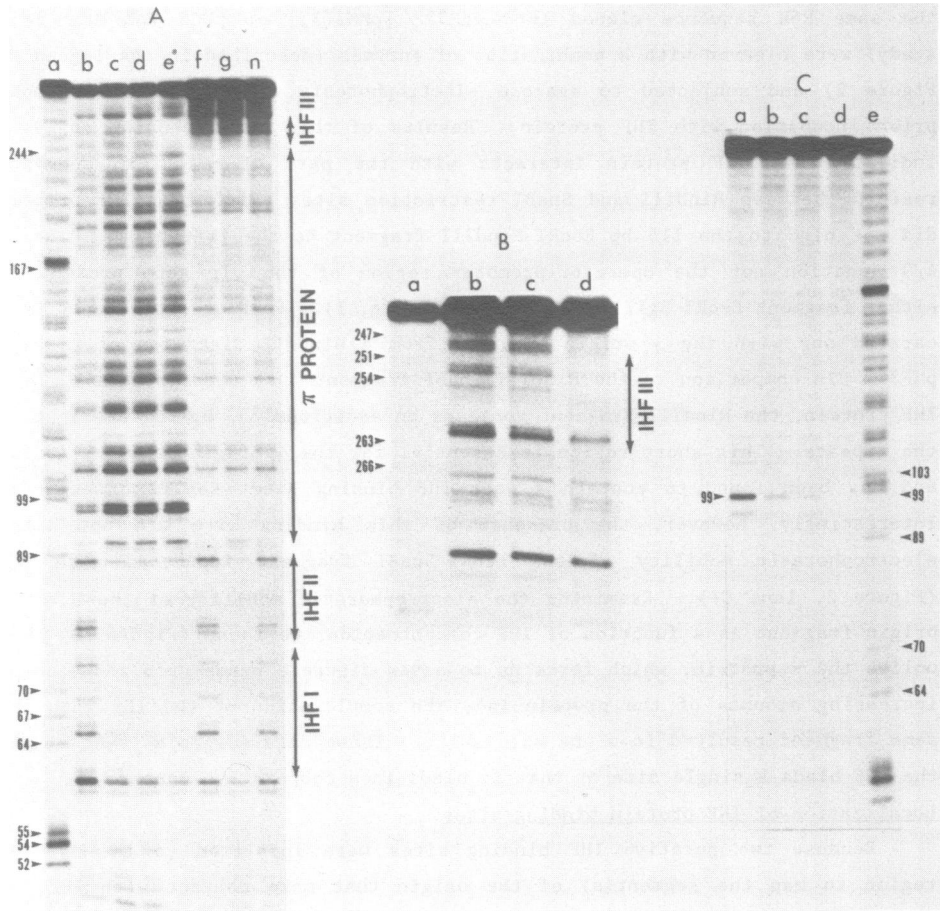

Figure 3: Neocarzinostatin and DNase I protection experiments of the IHF and/or the π protein bound to the γ origin of plasmid R6K. HindIII γ origin fragment from plasmid pMF34 (4), labeled at the 5′ end as described in Materials and Methods, was cleaved with PstI and used in NCS protection assay. (A) Lane a, G-specific DNA sequencing sample; lane b, no protein; lanes c-e, 5, 12 and 25 ng of IHF added, respectively; lane f, 50 ng of π; lane g, 12.5 ng of IHF followed by (after 2 minutes) 50 ng of π; lane h, 50 ng of π followed by (after 2 minutes) 12.5 ng of IHF. (B) IHF protection of binding site III obtained with HindIII origin fragment, labeled at the 5′ end, and cleaved with SnaBI. The small fragment used in the protection experiments [strand complementary to that shown in (A) and (C)]. Lane a, G-specific DNA sequencing sample; lane b, no protein; lane c, 20 ng of IHF; lane d, 40 ng of IHF. (C) Fragment shown in (A) incubated with decreasing concentrations of IHF protein and subjected to DNase I cleavage. Lane d, no protein; lanes a-c, contained 25, 12.5 and 5 ng of IHF protein; lane e, G-specific DNA sequencing sample. The numbers on the left of each group of gels corresponds to the coordinates given by Stalker et al. (22). IHF and π binding sites are designated by arrows.

	1	2	3	4	5	6	7	8	9	10	11	12	13
	C												A
Consensus		A	A	N	N	N	N	T	T	G	A	T	
	T												T
site I γ ori	T	A	A	G	T	T	G	C	T	G	A	T	T
site II γ ori	T	A	T	T	A	A	T	T	T	T	A	T	T
site III γ ori	C	A	A	C	A	G	G	T	T	G	A	A	C

Figure 4: The consensus sequence proposed by Leong et al. (46) is aligned with three IHF binding sites within R6K γ origin, determined in this study.

binding sites: two at the AT-rich segment next to the first repeat, and one next to the seventh repeat.

DNase I protection analysis of the π protein bound to the origin has been previously reported (4). It revealed a DNA sequence recognized by the π protein that included the seven 22 bp direct repeats and suggested weaker interaction with the AT-rich region, as judged by the enhanced frequency of DNase I cleavage at R6K coordinates 15 and 51 (4). It was of interest, therefore, to determine whether or not both proteins, π and IHF, simultaneously bind to this region. As shown in Figure 3A (lane f) the most indicative sign of π interaction with the repeats is the disappearance of two prominent cleavage products in all of the repeats except the 3rd and 4th. The alignment of a G sequencing sample (lane a) with the NCS protection samples indicate that those two bands correspond to the AT residues at the 5th and 6th position of each repeat. This dinucleotide is preceded by the sequence AAAC in the 1st, 2nd, 5th, 6th and 7th repeats and by the sequence AGCC in the 3rd and 4th repeats. Although the reason for this effect is unclear at the present time, it is possible that local changes in DNA conformation caused by the replacement of three consecutive A's in the sequence AAAC by GC bases in the sequence AGCC accounts, in the latter case, for the lack of cleavage of the neighboring AT bases. In contrast to the π protection patterns obtained at the γ origin with DNase I (4), the methylation protection (unpublished data), and the NCS protection analyses reported here show no sign of an

interaction of the π protein with the AT-rich region. Nevertheless, the establishment of π-origin complexes prior to IHF addition resulted in failure of the latter protein to interact with the γ origin (Figure 3A, lane h). Conversely, when the order of addition was reversed, with IHF added first followed by the addition of π, the characteristics protection pattern of both proteins were obtained (Figure 3A, lane g). The DNase I protection experiment was carried out despite the poor DNase I-induced cleavage with the AT-rich region of the γ origin and the relatively high ionic strength optimal for the IHF binding assay (see Materials and Methods), which diminishes DNase I activity. It has been suggested that the seven prominent enhancements, representing the most characteristic feature of the DNase protection pattern, reflects the increased susceptibility of specific residues to DNase I cleavage, due to the induced bending of the origin sequence by the binding of π protein (2). Although, the DNase I protection experiment did not provide substantially new information regarding the boundaries of the IHF binding sites, it did show that the unique residue within the first repeat (the G at coordinate 99) became highly susceptible to DNase I cleavage with increasing amounts of IHF protein (Figure 3C). This enhancement may reflect an alteration in the structure of the DNA helix at one of the boundaries of the IHF binding sites (see Discussion).

NCS and DNase I protection data presented in this section clearly indicate that two proteins that are required for γ origin functionality, the R6K-encoded π protein and the host-encoded IHF protein, each bind to the γ origin sequences. The total size of the protected DNA corresponds to approximately 50% of the R6K γ origin sequence that is required for replication. It is of interest that the 154 bp segment containing the seven repeats is flanked by IHF binding sites. It is also clear that, when added in a proper order, both proteins bind different segments of γ origin region. Considering the close proximity of their target sites, it is possible that these two proteins interact with each other when they are bound to the γ origin DNA. Finally, the results of competition experiments suggest that the inability of IHF protein to bind to the preestablished π-origin complexes is due to an altered structure of the DNA helix in the AT-rich segment facilitated by the π interaction with the repeat sequences (see Discussion).

DISCUSSION

Examination of the effect of the himA and himD mutants of E. coli on plasmid R6K maintenance has revealed that only the γ origin-containing

plasmids cannot be established in any of the E. coli mutants deficient in the production of IHF protein. By measuring the intracellular level of π protein in himA82 mutant carrying any of the helper vectors bearing the pir gene, we discounted altered production of the π initiation protein as the causal agent of lack of replication in the IHF mutants. Instead, we provided direct evidence for a sequence-specific interaction of purified IHF protein with a central regulatory segment of the R6K replication region, which contains the seven bp direct repeats and is known to be essential for the activity of all three origins of the plasmid. We would like to emphasize at this point that the ability to obtain transformants of the ΔhimA82 strain with plasmid R6K or its derivatives replicating via α and/or β origin does indicate that the IHF protein is not absolutely essential for these plasmids. It is clear, however that a quantitative analysis of plasmid copy number will be required before final conclusion could be made as to whether or not the IHF protein contributes to the replication of these plasmids.

Two out of the three IHF binding sites discovered in the R6K replication region reside in exceptionally AT-rich block to the left of the γ origin repeats cluster, while the third, considerably weaker site is separated from the other two by a cluster of the seven 22 bp direct repeats. None of these three sites shows perfect match with the consensus IHF sequence (Fig. 4). These observations and the alignment of several previously described IHF binding sites suggest that consensus IHF recognition sequence may be less stringent then previously proposed (Leong et al., 1985) and that IHF binding is influenced by the composition of neighboring bases. It would not be surprising if altered geometry of the DNA helix in AT-rich region (42,43) will appear to be critical during stage(s) of the IHF protein-DNA recognition that proceeds hydrogen bonding of the protein to DNA. It is tempting to speculate that in plasmid systems, other than R6K, that contain unusually AT-rich blocks of DNA adjacent to the iterons, IHF protein or perhaps other structurally homologous type II DNA binding proteins, such as HU, could play fundamentally similar functions.

The AT rich segment, common for the repeat-containing origins, also may serve as a structural element of the DNA helix that could adopt, upon binding of an initiator protein, an altered secondary structure more readily than any other nearby DNA sequences. Two facts seem to support this notion. First, the unwinding of the AT-rich segment of the phage λ origin brought about by the binding of replication O protein (44). Secondly, the results of the competition experiments which involved the π and IHF proteins indicate that

they bind the same DNA molecule only when IHF is added prior to π. The lack of the IHF binding with proteins, added in the opposite order, suggests that it could be due to an altered structure of the DNA helix in the AT-rich segment facilitated by the π interaction with the repeat sequence.

Two possible mechanisms by which IHF may participate in a number of diverse processes have been suggested by Craig and Nash (27). In their proposal, IHF protein may serve as a factor promoting a transition in the structure of DNA and/or may serve as a structural element directing interaction with another DNA binding protein. A greatly enhanced bending of the DNA upon the IHF binding to the replication origin of plasmid pSC101 (35), ends of IS1 (45) and attP of phage λ (46) provides strong support for the first of the two proposed functions of IHF in protein-DNA transactions. It is also conceivable that the greatly enhanced susceptibility of the G residue (coordinate 99) at the right-most boundary of the IHF binding site II in the R6K γ origin is due to DNA kinking or bending. Gel electrophoresis and electron microscope analyses of IHF-origin complexes will be carried out to test this possibility. There is no evidence supporting the second proposed mechanism other than the intriguing proximity of the IHF binding sites to the binding sites of π in the R6K γ origin, integrase Int in the phage λ attachment site "attP" and RNA polymerase in the gene expression systems influenced by the IHF.

Our previous studies on the γ origin mutants shed some light on the functional relevance of a possible π-IHF direct interaction. It has been shown that alteration of GC base pairs at both the 7th and 9th positions of the first repeat of γ 117 mutant and the sixth repeat of mutant γ 120 results in loss of π binding to the mutated repeat <u>in vitro</u> and concomitant loss of γ origin activity (3). Interestingly, isolation of revertants of the above origin mutations demonstrated that precise deletions of the mutated repeat restored origin function. Analyses of these revertants, as well as those of other origin deletion mutants (15) demonstrated that the γ origin does not require all seven repeats in order to function. Thus, we proposed (3) that the primary defect of mutant γ 117 and γ 120 is due to improper spacing between the origin π binding sites and essential neighboring sequences of the origin providing targets for putative, host-encoded factor(s). The arrangement of π-IHF binding sites at the γ origin suggests that π and IHF proteins may contact each other when bound to DNA and that alterations in their contact could cause the replication deficiency of the origin mutants discussed. Interestingly, the IHF binding site III consists of a DNA segment

that overlaps the mutation site in γ 111, which is deficient in replication (3). Preliminary gel retention experiments suggest a non-altered IHF binding to a restriction fragment containing the mutation. However, because the γ 111 mutation resides in the nonconserved segment of the protein recognition site, detection of its effect on IHF-origin interaction, if any, may require more sensitive DNA binding assay.

Finally, we would like to discuss the possibility of a putative IHF involvement in transcriptional events taking place in the region of the R6K γ origin. A rightward transcription from the minimal γ origin has been demonstrated in vivo (47). There are two consensus RNA polymerase binding sites predicted from the nucleotide sequence (25, P. Mukhopadhyay, unpublished). Interestingly, they either overlap (promoter P1) or lie next to (promoter P2) the IHF binding sites (Figure 1). In addition, direct contact of RNA polymerase with P1 was demonstrated by DNase I protection experiments (M.F., D.M. Stalker and D.R. Helinski, in preparation). Because a transcriptional step appears to be required for R6K in vivo (48) and in vitro replication (49), the second possible function of the IHF protein could be to serve as a modulator of transcription or as a structural element interacting with P2-directed transcript or a DNA-RNA hybrid. In fact, the interaction of IHF with cII mRNA of phage λ represents a single example of post-transcriptional involvement of the protein in gene expression (31). In vitro transcription and DNA binding experiments are underway that should shed more light on a role of the IHF protein in the replication of plasmid R6K.

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