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Integration host factor interacts with the DNA replication enhancer of filamentous phage f1

(replication origin/initiator protein/DNA-binding protein)

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ABSTRACT We present data which show that the *Escherichia coli* integration host factor (IHF) is an activator of phage f1 DNA replication. Phage f1 poorly infects bacterial strains lacking IHF because IHF is required for efficient expression of F-pili, the receptor for f1 phage. However, when F⁻ strains are transfected with f1 DNA the phage replicates in IHF mutants (*himA*, *himD*, or *himA himD*) at a rate of only 3% of that in wild-type bacteria. A plasmid dependent on the f1 replicon fails to transform IHF mutants. By gel retardation analysis, we show that IHF specifically binds to the origin of replication. DNase I "footprinting" experiments demonstrate that IHF binds to multiple sites within the replication enhancer sequence, a cis-acting, A+T-rich sequence that potentiates f1 DNA replication. Moreover, the effect of IHF mutation on f1 growth is suppressed by initiator protein (f1 gene *II*) mutations that restore efficient replication from origins that lack a functional replication enhancer sequence. This genetic evidence supports the conclusion that the replication enhancer sequence is the site of action of IHF.

The replication origin of the filamentous phages (f1, M13, and fd) consists of two adjacent domains, A (core origin sequence) and B (replication enhancer sequence) (1, 2). The core origin sequence [about 45 base pairs (bp)] is absolutely required for replication (1) and contains inverted repeats which serve as the binding site for the phage-encoded initiator protein (gene *II* protein or gpII) (3, 4). gpII binds to the origin in two steps (4) and introduces a specific nick in the plus strand (5). The 3′-hydroxyl end of the nick serves as the primer for plus-strand replication, which proceeds by a rolling-circle mechanism. On the other hand, the replication enhancer sequence (about 100 bp) is not absolutely required, but rather serves to potentiate DNA replication (1, 2). It is A+T-rich and is located immediately downstream of the core origin sequence. Origins with deletions in the replication enhancer sequence replicate *in vivo* at about 1% of the wild-type level (1). Phage with lesions in the replication enhancer grow poorly and acquire compensatory mutations (6–8). Compensatory mutations that restore efficient replication by A⁺B⁻ origins (wild-type domain A, defective domain B) alter (7, 8) or overproduce gpII (6, 7). However, the mechanism of action of the replication enhancer sequence has remained elusive.

In this paper, we propose that the *Escherichia coli* integration host factor (IHF) plays a role in f1 DNA replication via interaction with the replication enhancer sequence. IHF is a heterodimer consisting of two nonidentical proteins, IHF-α and IHF-β (9), which are the products of the *E. coli* genes *himA* and *himD* (or *hip*), respectively (10–12). IHF functions in a number of processes (for a review see ref. 13), the best characterized of which is phage λ integrative recombination. IHF is a DNA-binding protein (9) that binds to three sites within the phage λ attachment site (*attP*) (14). This binding is required for formation of a higher-order structure called the *attP*-intasome (15, 16). We show that IHF is an activator of f1 replication and that IHF binds to multiple sites within the replication enhancer. Furthermore, we show that gene II mutations that restore efficient replication from origins with a defective replication enhancer also suppress the effect of IHF mutations on f1 replication.

MATERIALS AND METHODS

Bacteria, Phage, and Plasmids. K361 is a streptomycin-resistant derivative of *E. coli* W3110 (F⁻ λ^- *thyA36 deoC2*). E582 (*hsdR514 supE44 supF58 lacYi galK2 galT22 metR55 himA*[Δ82::Tn*10*] hip[Δ*3::cat*]) was from R. Weisberg (National Institutes of Health). K924 is HfrC *argH polA1*. K1173 (K361 *himA*[Δ82::Tn*10*] *hip*[Δ3::*cat*]) and K1175 (K924 *himA*[Δ82::Tn*10*] *hip*[Δ3::*cat*]) were constructed by P1 transduction using E582 as the donor. K91, K38 (17) cured of λ, was used for growth and titering phage f1. Phage f1 and its derivatives were from our laboratory collection. R209 (18) and M13mp1 (7, 19) were described. R348 containing an amber mutation at the mp1 mutation site (codon 40 of gene *II*) on the wild-type genetic background was constructed by mutagenesis using the oligonucleotide 5′-GATAAATTCTAGCCGGAAGA-3′. R350 was constructed by marker rescue of R348 using the M13mp1 *Taq* I C fragment containing the mp1 mutation (Met[40] → Ile). pLIIO+ is an f1 replicon plasmid containing gpII under control of the *lac* uv5 promoter, and the complete f1 intergenic region from the *Rsa* I site at position −295 to the *Dra* I site at position +161 relative to the nicking site (20). pFZY1 (21) and pBR322 (22) were described. The plasmids pD10-wt (pD16) and pD10-Δ40.56 were described (1). The mutant origin Δ40.56 has a deletion of 17 bp from position +40 to +56, counting from the gpII nicking site. A double-origin assay was performed to analyze origin function as described previously (23).

Phage Growth After Transfection. Phage supercoiled replicative form I DNA (RFI) was prepared as described (17) and further purified on CsCl/ethidium bromide density gradients. Competent cells of female strains (K361 and K1173) were prepared by the method of Hanahan and were transfected as described (24). After transfection, the infective centers at time zero (IC_0) were determined by diluting the culture and titering on an indicator lawn of K91. A 0.2-ml inoculum of transfected cells (10 ng of f1 RFI) was diluted into 50 ml of SOC medium (24) and grown with shaking at 37°C. At the indicated times, 1 ml of culture was withdrawn and centrifuged at 8000 × *g* for 30 seconds in an Eppendorf microcentrifuge. The supernatant was heated at 65°C for 5 min, and the phage were titered.

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Abbreviations: IHF, integration host factor; gpII, gene *II* protein; RFI, replicative form I (supercoiled) DNA; IC_0, infective center at time zero.

Binding of IHF to the Origin. Restriction enzymes and DNase I were from New England Biolabs and Boehringer Mannheim Biochemicals, respectively. Binding to a *Hpa* II–*Sau*96I digest of f1 RFI was analyzed by gel retardation (25, 26). Purified IHF (27) was kindly provided by H. Nash and C. Robertson (National Institutes of Health). Binding was carried out on ice in a 20-μl reaction volume containing 30 mM Tris·HCl (pH 7.5), 80 mM KCl, 5% (vol/vol) glycerol, 6 mM $MgCl_2$, 5 mM dithiothreitol, and Pentex bovine serum albumin at 220 μg/ml for 5 min. Binding of IHF to the origin was also analyzed by DNase I "footprinting" (28) using the f1 *Sau*96I–*Cla* I fragment containing the origin, which was end-labeled as described (4). Ten to 20 fmol of end-labeled origin restriction fragment was incubated in a 9-μl reaction volume containing 56 mM Tris·HCl (pH 7.5), 4.4% glycerol, 89 mM KCl, 5.6 mM $MgCl_2$, 5.6 mM dithiothreitol, and IHF (0–0.25 μM as indicated). After incubation at room temperature for 10 min, 30 ng of DNase I was added for 30 sec, after which 90 μl of stop mix (0.3 M NaOAc, pH 5.1/67 mM NH_4OAc/0.1% $NaDodSO_4$/25 mM EDTA) was added. The DNA was precipitated with ethanol and electrophoresed on standard 8% polyacrylamide sequencing gels (29). Autoradiography was carried out at −70°C on Fuji RX50 film with an intensification screen.

RESULTS

IHF Potentiates f1 Growth. Initial attempts to determine whether f1 phage growth is affected by IHF mutations were inconclusive because IHF mutations depress the expression of the F-pili. For reasons that remain unclear, *himA* or *himD* mutant strains produce F-pili at a reduced level; hence they poorly participate in conjugal transfer (30) and are inefficiently infected by the F-specific phages. Phage f1 infected IHF mutant strains with about 1/100th the efficiency for wild-type strains. [Experiments with radioactive f1 phage showed that the inefficient infection of IHF mutant strains could be explained by poor entry of the phage DNA into the cells (data not shown).] To determine whether IHF plays a role in the intracellular growth of f1, we introduced f1 replicative-form DNA into IHF mutant and wild-type strains by transfection and measured the kinetics of phage production during subsequent growth. Female strains (F^-) were used as recipients to ensure against reinfection. Fig. 1 shows the results obtained with an isogenic pair of wild-type (K361) and IHF mutant (K1173) strains. They indicate that upon transfection with the wild-type f1 DNA the IHF mutant strain produces phage at a reduced rate (approximately 3% of normal phage production at 2 hr). K1173 is a *himA himD* double mutant carrying a deletion and Tn*10* insertion in the *himA* gene (*himA*[Δ82::Tn*10*]) and a deletion and insertion in the *himD* gene (*himD*[Δ3::*cat*]), so it is unlikely that K1173 produces any active IHF protein. Growth curves identical to the curve for K1173 were obtained for single-mutant strains carrying the *himA*[Δ82::Tn*10*], the *himD*[Δ3::*cat*], or the *himD*[Δ1::*cat*] allele (data not shown). The reduced phage production of the IHF mutant strains was not due to differential cell growth after transfection, because the number of infective centers increased at a rate similar to the wild type. Transfection efficiencies (infective center formation) for the IHF mutant strains were generally 50–100% of the wild-type values.

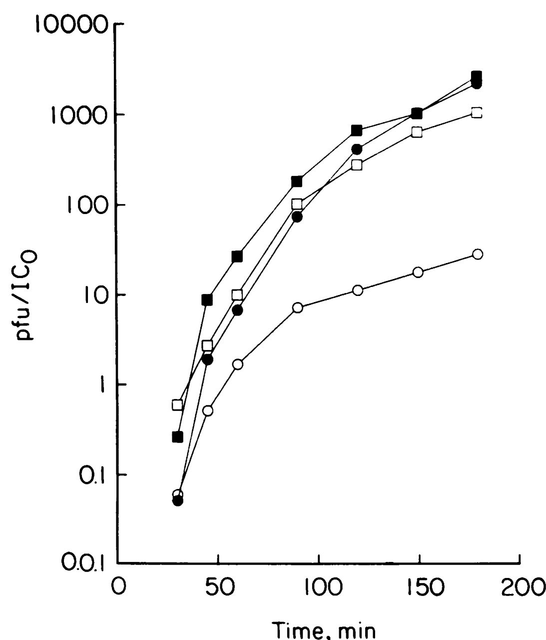

FIG. 1. Effect of IHF on phage growth. Phage growth was analyzed after transfection of female (F^-) strains. The growth curves show the plaque-forming units (pfu) per IC_0 at various times after transfection. The growth of f1 (circles) and R350 (squares) is shown in the wild-type (K361; filled symbols) and IHF mutant (K1173; open symbols) bacteria, respectively.

Table 1. Requirement of IHF for transformation with an f1 replicon plasmid

Host strain	Transformation efficiency pLIIO+	pFZY1	pBR322
K924 (*polA1*)	6.0×10^6	1.5×10^6	<250
K1175 (*polA1 himA himD*)	<500	4.0×10^5	<250

The transformation efficiencies (ampicillin-resistant colonies per μg of transforming DNA) of the plasmids pLIIO+ (20), pFZY1 (21), and pBR322 (22) are listed for K924 (*polA1*) and for K1175 (*polA1 himA himD*). Preparation and transformation of competent cells were performed as described by Hanahan (24). For transformation with pLIIO+, the medium contained 2 mM isopropyl β-D-thiogalactopyranoside (IPTG). For selection of transformants, ampicillin was used at 100 μg/ml for pLIIO+ and pBR322, and at 50 μg/ml for pFZY1. The values of <250 and <500 are given to indicate the limit of detection because in these cases no ampicillin-resistant colonies were obtained.

IHF Is Required for Transformation by f1-Replicon Plasmids. To establish whether IHF functions in f1 DNA replication, we analyzed the effect of IHF on transformation by an f1-origin-dependent plasmid, pLIIO+. pLIIO+ contains gene *II* under control of the *lac* uv5 promoter, and both the

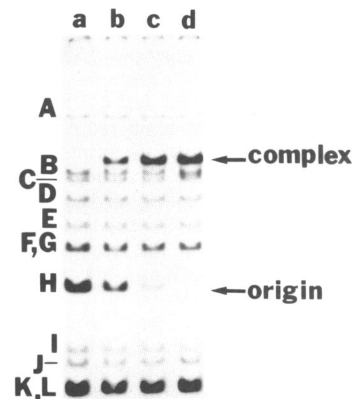

FIG. 2. Binding of IHF to the origin. The binding of IHF to a *Sau*96I–*Hpa* II digest of f1 RFI was analyzed by gel retardation (25, 26). Lanes a–d contained 0, 0.11, 0.22, and 0.55 ng of purified IHF, respectively, in addition to 23.3 ng of an end-labeled *Sau*96I–*Hpa* II digest of f1 RFI (5.5 fmol of each fragment). Binding was analyzed on a nondenaturing 5% polyacrylamide gel with 0.09 M Tris/0.09 M boric acid/2.8 mM EDTA (TBE) buffer.

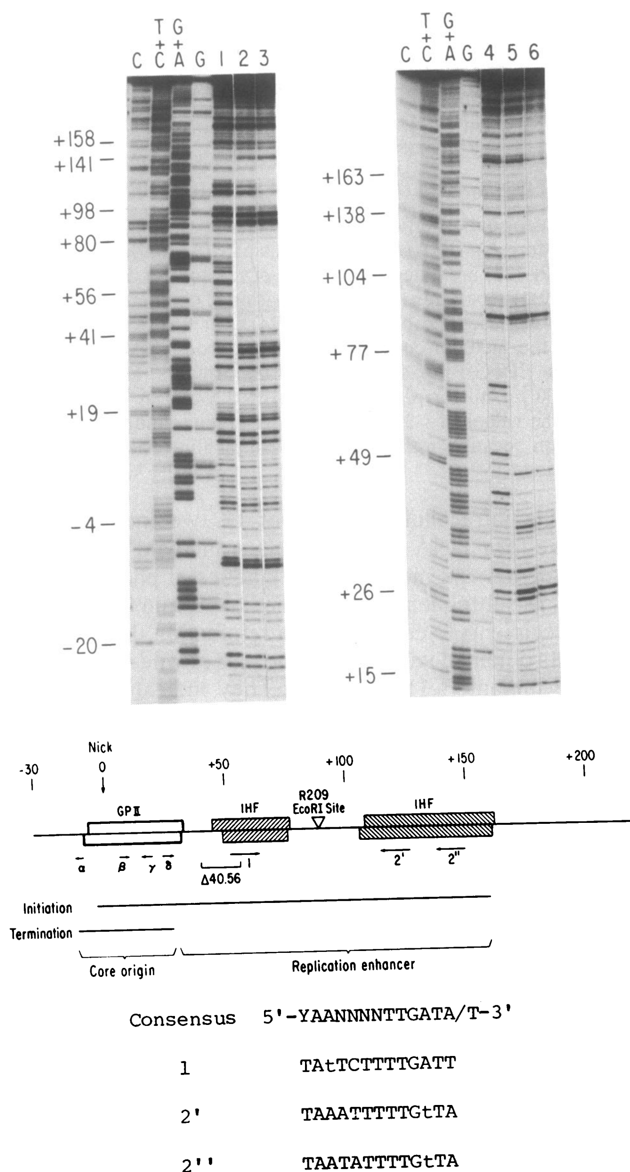

FIG. 3. Footprinting of IHF bound to the origin. The binding of IHF to the origin was analyzed by DNase I footprinting. (*Top*) Lanes 1–3, the reaction mixtures contained 1.6 nM f1 origin-containing restriction fragment (315-bp *Sau*96I–*Cla* I fragment) 5′-end-labeled on the plus strand and 0, 40, and 160 nM IHF, respectively. Maxam–Gilbert sequencing reaction products served as markers (lanes are marked to indicate the cleavage reaction). Lanes 4–6, the reaction mixtures contained 1.9 nM f1 origin-containing restriction fragment 3′-end-labeled on the minus strand and 0, 61, and 243 nM IHF, respectively. The nucleotides are numbered relative to the nicking site (position 0). (*Middle*) Summary of footprinting results. The origin region is shown as a horizontal line. A vertical arrow marks the site of nicking by gpII (0). DNA replication begins at the nick and proceeds rightward. The core origin sequence and the replication enhancer sequence (described in the text) are indicated. DNA sequences protected by IHF or gpII are shown by bars above (plus strand) and below (minus strand) the line. The left hatched bar shows the region strongly protected by IHF (site 1) and the right hatched bar shows the region weakly protected by IHF (site 2). The region protected by gpII is shown by the open bar. Repeated sequences within the core origin are indicated by arrows marked α, β, γ, and δ. Arrows marked 1, 2′, and 2″ are sequences within the replication enhancer that nearly match the consensus sequence for IHF binding (see below). The R209 *Eco*RI site is indicated. The deletion, Δ40.56, that disrupts site 1 is indicated by a bracket showing the extent of the deletion. Sequences required for initiation and termination of DNA replication are shown. (*Bottom*) Nucleotide sequence of IHF binding elements. The sequences present in the f1 replication enhancer (1, 2′, and 2″) are shown, as is the consensus sequence for IHF binding (13, 14, 31). Nucleotides that differ from the consensus sequence are shown in lowercase. Y denotes a pyrimidine nucleotide, N denotes any nucleotide, and A/T denotes that this nucleotide may be either an A or a T.

f1 and pBR322 origins (20). pLIIO+ can transform *polA*-deficient bacteria (K924; see Table 1), because it can replicate in an f1-origin-dependent fashion in the presence of isopropyl thiogalactoside. Transformation of *polA* strains by pLIIO+ is dependent on the presence of isopropyl thiogalactoside and on the Rep helicase function of the recipient. The result shown in Table 1 indicates that pLIIO+ cannot transform K1175 (*polA1 himA himD*), whereas pFZY1, a plasmid dependent on the F-episome origin, can. Therefore, replication of the f1 replicon plasmid requires IHF.

IHF Binds to the Replication Origin. To determine whether IHF has any binding sites in the f1 genome, we analyzed the binding of IHF to a *Sau*96I–*Hpa* II digest of f1 DNA by using gel retardation (25, 26). Fig. 2 shows that IHF binds specifically to the 271-bp fragment (fragment H) which contains the origin of replication. As the concentration of IHF is increased (lanes a–d), the intensity of the free origin fragment diminishes and the intensity of the complex increases. The remaining DNA fragments are unaffected by the presence of IHF except for the 818-bp fragment (C) and the 176-bp fragment (I), which slightly bind IHF at the highest concentration. Both of these fragments are from gene *I*, and the significance of their weak binding is unknown.

Replication Enhancer Sequence Has Multiple IHF Binding Sites. To further characterize the interaction of IHF with the origin, DNase I footprinting experiments (28) were carried out. Fig. 3 *Top* shows the results of this analysis. Lanes 1 and 4 are the DNase I controls (no IHF added) for the plus and minus strand, respectively. When IHF is added (lanes 2 and 5) a segment of DNA sequence from position +45 to position +77 (relative to the nicking site) is protected (site 1). As the concentration of IHF is increased (lanes 3 and 6) positions +106 to +162 are protected (site 2). Binding to site 2 is approximately 1/10th as strong as that to site 1. These results are summarized in Fig. 3 *Middle*. Site 1 contains one copy of a sequence that differs from the IHF consensus binding site at one position (the third base pair of the consensus sequence—see Fig. 3 *Bottom*). Site 2 contains two copies of a sequence that differs from the consensus sequence at a single position (the 11th base pair of the consensus sequence in each case—see Fig. 3 *Bottom*). The relative orientations of the two sequences in site 2 (2′ and 2″) are the same but opposite from the orientation of site 1.

To determine whether IHF binds to site 1 and site 2 in a cooperative or independent fashion, we footprinted the two sites individually, using the R209 *Eco*RI site (see Fig. 3 *Middle*) to separate the two sites. Binding to either site is not affected by the presence of the other, suggesting that IHF binds the two sites independently (data not shown). In contrast to the footprinting results, which indicate that IHF has multiple binding sites within the origin, the gel retardation results (Fig. 2 and unpublished data) show the formation of only one species of IHF-bound complex even at high IHF concentrations and under a variety of conditions. We are unable to detect binding of IHF to a fragment that contains only site 2 by gel retardation analysis. Possibly, binding to site 2 may be unstable under the conditions of gel analysis.

Initiator Protein Mutations Confer Independence of IHF. The mutation mp1 at codon 40 of gene *II* (Met → Ile) overcomes a defective replication enhancer sequence. This has been shown by using indirect methods such as phage growth not being interfered with in the presence of an active f1 origin on plasmids and the ability of these phage to produce transducing particles from cells containing f1 origin plasmids

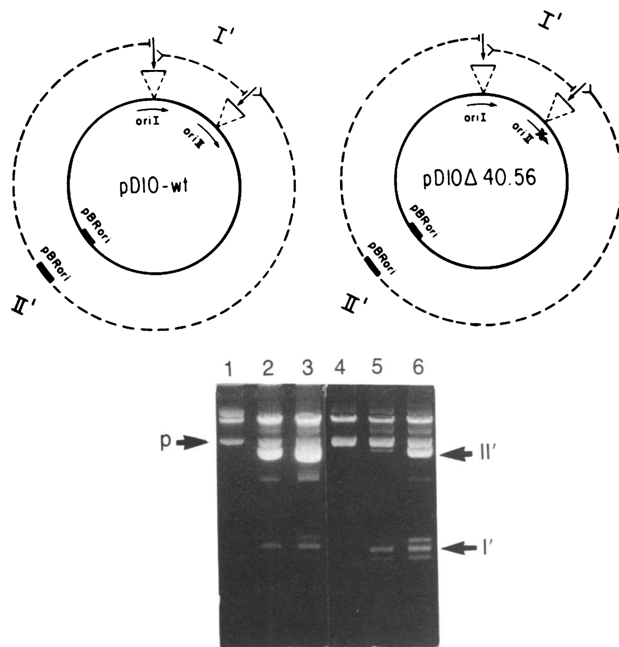

FIG. 4. Activation of a defective origin by an initiator mutation. (*Upper*) Double-origin assay of DNA replication. pD10-wt contains two wild-type f1 origins (indicated as oriI and oriII) in direct repeats. pD10-Δ40.56 contains a wild-type f1 origin (indicated as oriI) and a defective origin (indicated as oriII) with a deletion in the replication enhancer sequence (indicated with an X). Upon superinfection with helper phage, initiation at oriI and termination at oriII results in production of the small plasmid I'. Initiation at oriII and termination at oriI produces the larger plasmid II'. (*Lower*) Results of the double-origin assay. Cells containing pD10-wt (lanes 1–3) or pD10-Δ40.56 (lanes 4–6) were infected with f1 (lanes 2 and 5) or R350 (lanes 3 and 6) or were mock infected (lanes 1 and 4). At 35 min after infection, the cells were harvested and DNA was prepared and analyzed on a 1.0% agarose gel containing ethidium bromide at 0.5 μg/ml. The position of the superhelical form of the parental plasmid (p) and its resolution products I' and II' are indicated. Open circles and single-stranded circles of these plasmids are also seen.

(7). The experiment shown in Fig. 4 demonstrates this phenomenon more directly. Since the f1 origin contains the signals for both the initiation (32) and the termination (33) of DNA replication (see Fig. 3 *Middle*), a plasmid containing two wild-type origins in the same orientation (e.g., pD10-wt in Fig. 4 *Upper*) is resolved into two replicons when cells containing it are superinfected with f1 (23). Initiation at origin I and termination at origin II produces the small plasmid I', while initiation at origin II and termination at origin I produces the larger plasmid II' (Fig. 4 *Lower*, lane 2). When, however, origin II has a deletion in the replication enhancer sequence (e.g., pD10-Δ40.56 in Fig. 4 *Upper*), then production of plasmid II' is markedly reduced (Fig. 4 *Lower*, lane 5). The yield of plasmid I' is unaffected because, while the origin II is initiation-defective, it is termination-proficient (1). When R350, which carries the mp1 mutation, was used instead of the wild-type f1 phage, both plasmid I' and plasmid II' were produced from pD10-Δ40.56 (Fig. 4 *Lower*, lane 6). This indicates that the gpII with the mp1 mutation can initiate replication from the enhancer-defective Δ40.56 origin, as well as from the wild-type origin.

It was therefore crucial to test if the mp1 mutation also confers IHF independence. Growth of phage carrying the mp1 mutation was measured after transfection of IHF-deficient bacteria. The result, shown in Fig. 1, clearly indicates that R350 phage grows normally upon transfection of both the wild-type (K361) and IHF mutant (K1173) bacteria, in contrast to the wild-type phage, which requires IHF for optimal growth. This observation strongly suggests that IHF stimulates f1 DNA replication by interacting with the replication enhancer sequence. The result also indicates that the low growth rate of the wild-type phage in the IHF mutants is not due to an artifact of the transfection procedure, because R350 grows with normal kinetics in the IHF mutants.

Several gene *II* mutations other than mp1 have also been shown to overcome defects in the replication enhancer sequence. They are at codon 73 of gene *II* (ref. 8; unpublished data). Using the double origin assay described above (Fig. 4), we have demonstrated that phages carrying an alanine or a cysteine residue at codon 73 of gene *II* (glycine in the wild type) initiate replication from the Δ40.56 origin, as does R350. Furthermore, upon transfection, these codon 73 mutants grow normally on both wild-type (K361) and IHF mutant (K1173) bacteria (data not shown).

DISCUSSION

We have shown that IHF stimulates the intracellular replication of phage f1 (Fig. 1). IHF is not absolutely required for f1 growth, because *himA* or *himD* mutants can produce phage, albeit at a reduced rate (3% of the wild type). We propose that IHF functions at the level of DNA replication because an f1 replicon plasmid (pLIIO+) fails to transform IHF-deficient bacteria (Table 1). In addition, a mutant phage, R350, containing a mutation (mp1) in gene *II* ($Met^{40} \rightarrow Ile$) grows normally on *himA* or *himD* strains. Since the mp1 mutation suppresses the effect of both the absence of IHF (Fig. 1) and the absence of the replication enhancer sequence (Fig. 4), IHF most likely functions through the replication enhancer. This notion is supported by footprinting experiments, which show that IHF specifically binds to multiple sites within the replication enhancer sequence (Fig. 3). Since the only known function of the replication enhancer sequence is the initiation of DNA replication, the results indicate that IHF enhances f1 DNA replication.

IHF plays a role in a number of cellular processes, including site-specific recombination, transcription, translation, transposition, phage morphogenesis, and DNA replication. It appears that IHF acts in all these processes as an accessory or enhancing factor rather than an absolutely required factor (see ref. 13 for a review). As described above, this also holds true for f1 replication. In its best-characterized function, IHF plays a role in λ integrative recombination (9) by binding to three sites within *attP* (14, 34), leading to the formation of a higher-order structure termed the *attP*-intasome (15, 16). Interestingly, just as the lack of IHF in f1 replication is bypassed by certain gene *II* mutations (e.g., mp1), the lack of IHF in λ integrative recombination is mitigated by some λ integrase mutations (10, 35). We imagine that IHF functions in f1 DNA replication in an analogous manner. We have shown that both IHF and gpII bend the replication origin (our unpublished results). Thus, higher-order structures involving bent/wound DNA–protein complexes are likely to play a role in initiation of f1 DNA replication.

At which step in the initiation of DNA replication IHF functions is unknown, though it is likely to be at a step beyond nicking by the initiator protein gpII. IHF and gpII bind independently to the origin. In addition, IHF has no effect on the efficiency of nicking by gpII *in vitro* (unpublished data). Since the lack of IHF is suppressed by the mp1 mutation in gene *II*, IHF probably functions in a step in which gpII participates. However, gpII is a multifunctional protein and is known to play several different key roles in f1 DNA replication. In particular, gpII is required for initiation of replication on a template-primer molecule that has already been nicked by gpII itself (36). Thus, IHF may play a role in the initiation of unwinding, in the coupling of unwinding with synthesis of DNA, or in the entry of DNA polymerase III holoenzyme to the initiation complex.

IHF has been shown to function in the replication of pSC101 (37). It has recently been shown that IHF binds to a bent region at the pSC101 origin and bends it further (38). Although the precise step at which IHF functions in pSC101 replication remains unclear, Stenzel *et al.* (38) have proposed that IHF might facilitate the interaction of other replication proteins (DnaA, DnaB, and DnaC) with the origin or with each other. The observation that IHF promotes DNA replication from two different origins (pSC101 and f1) that employ different replication proteins supports the idea that IHF acts by altering DNA conformation rather than directly interacting with other replication proteins. Better understanding of the function of IHF in the two replication systems will require *in vitro* analyses to determine at which step in initiation of replication IHF acts.

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