Article

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Abstract

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Reference

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Replication of pSC101: effects of mutations in the *E. coli* DNA binding protein IHF

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Summary. We have shown that the plasmid pSC101 is unable to be maintained in strains of *E. coli* carrying deletions in the genes *himA* and *hip* which specify the pleitropic heterodimeric DNA binding protein, IHF. We show that this effect is not due to a modulation of the expression of the pSC101 RepA protein, required for replication of the plasmid. Inspection of the DNA sequence of the essential replication region of pSC101 reveals the presence of a site, located between the DnaA binding-site and that of RepA, which shows extensive homology with the consensus IHF binding site. The proximity of the sites suggests that these three proteins, IHF, DnaA, and RepA may interact in generating a specific DNA structure required for initiation of pSC101 replication.

Key words: Plasmid replication/IHF protein

Introduction

The heterodimeric *E. coli* protein, IHF, is the product of two genes, *himA* and *hip* (*himD*), located at 38 and 20.5 min on the standard linkage map (see Weisberg and Landy, 1983 for review). The protein was first detected as a factor in extracts of nonlysogenic cells necessary for in vitro integrative recombination of phage λ (Nash et al. 1977).

IHF is a DNA binding protein which protects λ*attP* DNA sequences from DNase and neocarzinostatin digestion in vitro (Craig and Nash, 1984). Similar protection of the *attP* region of other lambdoid phages such as φ80 and P22 has also been observed (Leong et al. 1985). These protection studies have revealed a consensus sequence (YAAnnnnTTGATA/T) implicated in IHF binding. The protein exhibits strong homology with the heterodimeric HU protein (Miller 1984), itself implicated in the formation of nucleosomelike structures (Rouvière-Yaniv et al. 1979) and is at least partially under the control of the *E. coli* S.O.S. system (Miller et al. 1981).

Although the genes *himA* and *hip* are not essential for cell viability, since both can be deleted, mutants in IHF affect a range of ancillary phenomena. Thus it has also been implicated in the translation of the cII message of λ (Hoyt et al. 1982), is involved in the expression of the early operon of phage Mu at the transcriptional level (Goosen and van de Putte 1984), influences the expression of the *ilv* and *xyl* genes of *E. coli* (Friden et al. 1984; Friedman et al. 1984), and may be involved in encapsidation of λ (Bear et al. 1984). Moreover, we have recently observed that IHF binds specifically in vitro to the extremities of IS*1* and to the main target region for IS*1* in the plasmid pBR322 (Gamas et al., in preparation), and that it is necessary for efficient conjugal transfer of the *E. coli* F-plasmid (Chandler et al., in preparation).

The plasmid pSC101 (Cohen and Chang 1977) is a low copy number non-conjugal plasmid sometimes used as a cloning vector. The mechanism governing its autonomous replication has attracted interest, in part, because it is one of the only known naturally occuring plasmids showing an absolute requirement for the host *dnaA* gene product (Hasunuma and Sekiguchi 1977; Frey et al. 1979). We report here that pSC101 also requires a second host protein, IHF, for maintenance.

Materials and methods

Bacterial strains. Bacterial strains used in this study are listed in Table 1. E582 was constructed by R. Weisberg and carries the *himA* deletion 82 marked with the transposon Tn*10* (Miller 1981) and a substitution of part of the *hip* gene for a gene specifying resistance to chloramphenicol. The *himA* and *hip* mutations were introduced into various recipient strains by P1 transduction (Caro and Berg 1971) by selection for the associated antibiotic resistance gene. The IHF character was determined by sensitivity or resistance to phage Mu. The *recA* deletion was introduced by P1 transduction from strain JV10289 (del *recA srl*::Tn*10*; Willis et al. 1981) into MC240 and a Tc^s derivative of MC253 which has presumably lost the *himA*-associated Tn*10* (kindly constructed by D. Lane).

Antibiotic concentrations were as follows: Spectinomycin (Sp)/Streptomycin (Sm), 10 and 20 μg/ml respectively; Chloramphenicol (Cm), 30 μg/ml; Tetracycline (Tc), 10 μg/ml; Ampicillin (Ap), 50 μg/ml; Rifampicin (rif), 100 μg/ml.

Plasmids. The constructions of the plasmids pLC712, pGB2 and pLC1983-36A have been described (Linder et al. 1983; Churchward et al. 1984; Linder et al. 1985) as have those of pHS1 (Hashimoto-Gotoh and Sekiguchi 1977) and of pOC15 (Gamas et al. 1985). pOX38Km::Tn*OC15* was con-

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Table 1. Bacterial strains

Strain	Genotype	Source
E582	$hsdR_{514}$ $supE_{44}$ $supF_{58}$ $lacY_i$ $galK_2$ $galT_{22}$ $metR_1$ $trpR_{55}$ himA[del82::Tn*10*] hip[del3:CAM]	R. Weisberg
MC210	thy thi pro recA rif^r/pOX38Km::*TnOC15*	this study
MC240	ara del (lac pro) nalA metB $argE_{am}$ rif^r thi supF	this study
MC251	ara del (lac pro) nalA metB $argE_{am}$ rif^r himA[del82::Tn*10*]	this study
MC252	ara del (lac pro) nalA metB $argE_{am}$ rif^r hip[del3:CAM]	this study
MC253	ara del (lac pro) nalA metB $argE_{am}$ rif^r himA[del82::Tn*10*] hip[del3:CAM]	this study
MC294	ara del (lac pro) nalA metB $argE_{am}$ rif^r del (recA) srl::Tn*10*	this study
MC296	ara del (lac pro) nalA metB $argE_{am}$ rif^r del (recA) srl::Tn*10* himA[del82] hip[del3:CAM]	this study

structed in vivo by transposition of Tn*OC15* from pOC15 to pOX38Km (Chandler and Galas 1983) by standard techniques. The plasmids pHO1 and pHO2 were constructed by ligation of a 2.9 kb *Hin*cII fragment from pHS1 which carries all essential pSC101 replication functions and a 2 kb *Sma*I fragment (omega; Prentki and Krisch 1984), carrying genes for resistance to Sp/Sm flanked by two strong transcription terminators, from plasmid pHP45. The purification of DNA fragments and conditions for ligation have been described (Gamas et al. 1985).

Transformation was according to Maniatis et al. (1982). Transformants were selected by plating on L. agar containing Sp/Sm (for pHO1, pHO2, pGB2, pOC15, and pLC1983–36A) or Ap and/or Tc (pLC712). Transformation with and growth of cells carrying the temperature sensitive replication mutants was at 30° C.

Transfer of pOX38Km::Tn*OC15* from MC210 to the test strains was accomplished by cross-streak mating on L. agar plates containing Sp/Sm and rif.

β-galactosidase was assayed as described previously (Chandler and Pritchard 1974) using cultures grown in V.B. medium supplemented with glucose, cas amino acids, and 20 μg/ml Sp. The levels are expressed as: OD_{420}/min/OD_{450}. Assays were performed on two independant pLC1983-36A transformant clones. Cultures were analysed for plasmid loss after each experiment by plating on V.B. agar containing glucose and cas-amino acids and supplemented with X-gal (Miller 1972).

Results and discussion

In the process of designing a sensitive transposition assay for an investigation of the effect of various host mutations on the transposition of the insertion sequence IS*1*, we constructed in vitro two derivatives of the temperature sensitive pSC101 replication mutant, pHS1 (Hashimoto-Gotoh and Sekiguchi 1977). These plasmids were constructed from two components. One, was a 2.9 kb *Hin*cII fragment of pSC101 carrying all the essential replication functions (Fig. 1; Churchward et al. 1983). The other was a DNA fragment, constructed by Prentki and Krisch (1984), the fragment omega, which carries a spectinomycin/streptomycin resistance gene (Sp^r/Sm^r) flanked by two strong transcription/translation termination signals. The resulting plasmids, pHO1 and pHO2 (carrying the omega fragment in opposite orientations), undergo temperature dependant segregation like the parental pHS1 plasmid (data not shown). While pHO1 and pHO2 efficiently transform strain MC240 and a *recA* derivative MC294 to Sp^r/Sm^r (Table 2), we were consistently unable to obtain transformants of strains MC251, MC252, and MC253 (or its *recA* derivative MC296). These strains carry a deletion of the *himA* and/or *hip* genes but are otherwise isogenic with MC240 and MC294 (Table 1).

Table 2. Comparison of wild-type and IHF mutant strains. Transformation frequencies are expressed as transformants per ml of culture. Transformation efficiencies were for plasmids pH01, pH02, pGB2 and pLC712 were approximately 4×10^5 per μgm of input DNA for strain MC240. Plasmid pOC15 showed a consistent reduction of about 2 orders of magnitude compared with the other plasmids. The reason for this difference is not understood at present.

Plasmid	Strain					
	MC240 IHF^+	MC251 *himA*	MC252 *hip*	MC253 *himA hip*	MC294 IHF^+	MC296 *himA hip*
pH01	5×10^4	<10	<10	<10	5×10^4	<10
pH02	5×10^4	<10	<10	<10	5×10^4	<10
pGB2	1.4×10^5	<10	<10	<10	8×10^4	<10
pLC712	2.5×10^5	3.9×10^4	9×10^4	4.5×10^4	5.5×10^4	4×10^4
pOC15	4.3×10^3	8.2×10^2	5.5×10^2	6.4×10^2	1.4×10^3	2×10^3
pOX38Km::Tn*OC15*[a]	+	+	+	+	+	+
pLC1983-36A[b]	165	n.t.	n.t.	183	n.t.	n.t.
	169	n.t.	n.t.	181	n.t.	n.t.

[a] Cross streak transfer of pOX38Km::Tn*OC15* is described in Materials and methods

[b] β-galactosidase levels as defined in Materials and methods. It should be noted that strains carrying pLC1983-36A grow with a doubling time approximately twice that of the parent or other plasmid carrying strains. A small but insignificant difference in doubling time between the wild-type and mutant strains was also observed. Selection for the plasmid was maintained throughout these experiments

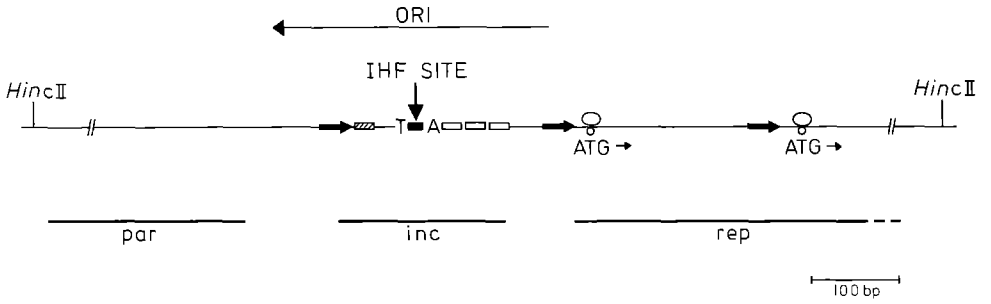

Fig. 1. Features of the nucleotide sequence of the essential replication region showing: the relationship between the potential IHF binding site, the DnaA binding site ▨; the blocks of repeated sequence, □, bound by RepA; and the asymmetric A + T-rich region. Promoters, both real and potential (Churchward et al. 1983; Linder et al. 1985) (*heavy horizontal arrows*), and the 5′ end of two open reading frames, the longer encoding the RepA protein, are also indicated

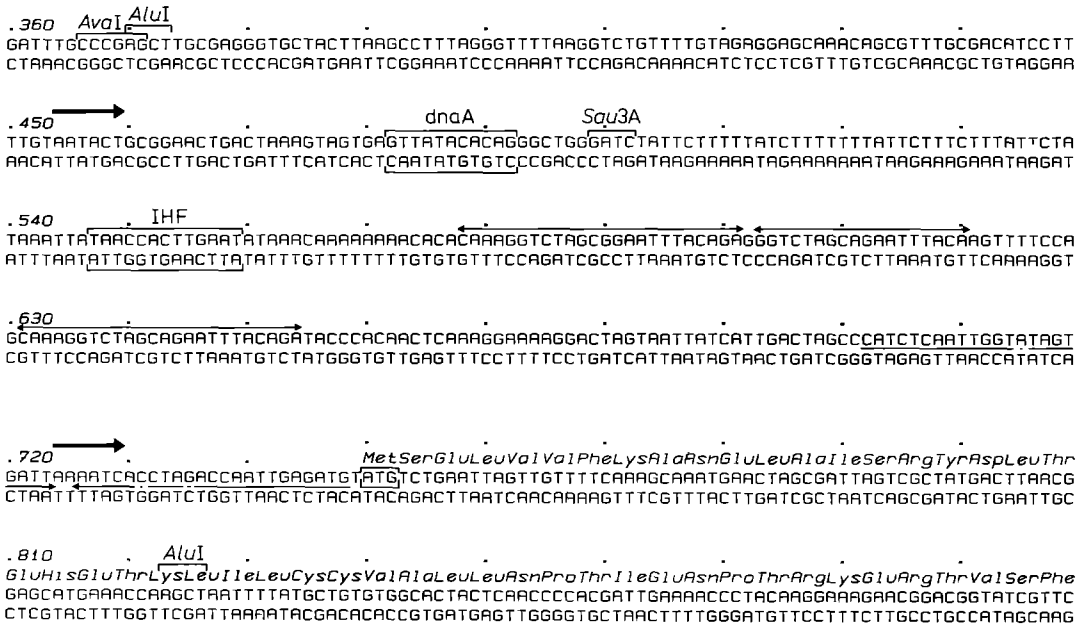

Fig. 2. Nucleotide sequence of part of the essential replication region. The figure shows: the relative positions of promoters (→); the proximity of the consensus DnaA and IHF sites and the repeated sequence blocks (↔); the N-terminal extremity of RepA, including the first 25 amino acids present in the pLC1983-36A fusion plasmid

The large difference in transformation frequencies does not arise from artefacts associated with the selection procedure. The strains exhibit comparable transformation efficiencies for a high copy number Sp^r/Sm^r pBR322 derivative plasmid, pOC15 (Gamas et al. 1985), and, as judged by cross-streak matings, both IHF mutant and wild-type strains accept and maintain an Sp^r/Sm^r derivative of the low copy number plasmid F, pOX38Km::Tn*OC15*, under similar selective conditions (Table 2).

To rule out the possibility that the inability to transform the IHF deletion strains is linked to the temperature sensitive replication lesion carried by pHO1 and pHO2, the strains were transformed with the plasmid pGB2 (Churchward et al. 1984). This plasmid is similar to pHO1 and pHO2 but carries wild-type pSC101 replication functions. The results (Table 2) are identical to those obtained with the temperature sensitive plasmids.

This suprising difference between wild-type and mutant strains could reflect either an inability of IHF mutants to maintain pSC101 or a lethal effect of pSC101 in these strains. To distinguish between the two possibilities, we investigated the transformation properties of the plasmid pLC712, a hybrid plasmid carrying both pBR322 and pSC101 replication functions (Linder et al. 1983). This plasmid transforms both mutant and wild-type strains with equal efficiency (Table 2). Thus the presence of pSC101 *per se* does not seem to be lethal to IHF^- cells.

The replication region of pSC101 (Fig. 1 and Fig. 2) includes three major components: *par*, a locus involved in plasmid partition; *inc*, a locus determining plasmid incompatibility; and *rep*, a region adjacent to the origin of replication and encoding an autoregulated protein RepA required for plasmid replication (see Churchward et al. 1983).

It is known that IHF can modulate gene expression both at the transcriptional (Goosen and van de Putte 1984) and possibly translational (Hoyt et al. 1982) levels. In order to determine whether IHF exerts its effect by modulating expression of the *repA* gene, we have measured *repA*-driven β-galactosidase levels determined by the plasmid pLC1983-36A (Linder et al. 1985) in wild-type and mutant strains. This plasmid is a derivative of pMC1403 (Casadaban et al. 1980) which carries a Sp^r gene and the *repA* promoter (from co-ordinate 662) together with the first 25 codons of the *repA* gene (Fig. 2) fused to a β-galactosidase gene. Both

wild-type and mutant strains exhibit comparable transformation frequencies with this plasmid (data not shown). The results of β-galactosidase assays are shown in Table 2. Clearly, IHF has no significant effect on the expression of *repA*.

Since, however, pLC1983-36A does not produce a functional RepA protein, and since it is known that RepA expression is autoregulated, it remains formally possible that IHF interacts with RepA in modulating *repA* expression.

It is interesting to note that a relatively good IHF consensus sequence occurs close to the origin of replication at coordinate 547 (T.A.A.N.N.N.N.T.T.G.A.-.T) of the *Hin*cII fragment (Fig. 2; Churchward et al. 1983). The absence of this site in the RepA/β-gal fusion plasmid pLC1983-36A reinforces the conclusion that IHF is not required for *repA* expression: the strength of the RepA promoter, as judged by β-galactosidase levels, is relatively high (Table 2; Linder et al. 1985) and sufficient RepA is produced by a functionally analagous plasmid carrying an intact *repA* gene, to complement pSC101 derivatives mutant in *repA* (Linder et al. 1985).

Our results do not rule out the possibility that the effects of IHF are indirect. IHF may modulate the expression of another protein, for example DNA gyrase, required in the replication and/or maintenance of pSC101 (Gellert et al. 1983; Danilevskaya and Gragerov 1980). The sequence environment of the potential site, however, suggests a direct effect. The environment is a region of high, extremely asymmetric, A+T density, approximately 20 bp upstream of a series of repeated blocks of sequence which appear to bind the RepA protein (Vocke and Bastia 1983), between 50 and 60 bp downstream from a consensus DnaA binding site and 90–100 bp downstream from a potential promoter (Churchward et al. 1983). An interesting feature of this region is that it is extremely asymmetric with respect to A and T tracts. To the left of the IHF site T is found predominantly in the "top" strand, while to the right, A tracts predominate (Fig. 2). It is possible that this type of sequence feature may significantly influence DNA structure (see for example: Zahn and Blattner 1985). The arrangement suggests two possible roles for IHF involvement in pSC101 maintenance or replication. IHF could act by modulating the synthesis of a transcript, yet to be demonstrated, from a promoter located at about 450 bp (Fig. 2). Alternatively, IHF could act in modifying the topology of the region, either directly or indirectly, permiting assembly of a replication complex and subsequent replication. It should be noted that the size of DNA regions protected by the DnaA protein (Fuller et al. 1984), IHF (Ross et al. 1979; Leong et al. 1985; Gamas et al., in preparation), and RepA (Vocke and Bastia 1983) are consistent with the possibility that these three proteins interact physically in the replication origin region. In this context, DnaA binding at the 4 repeated "DnaA boxes" in the *oriC* region of the *E. coli* chromosome generates condensed DNA/protein complexes (Fuller et al. 1984). In a similar way, a specific nucleosome-like DNA structure might be generated at the pSC101 origin by interaction between DnaA bound at the single site in pSC101, IHF, and RepA. Further analysis of these potential interactions will require the use of *in vitro* biochemical techniques such as DNA footprinting (Galas and Schmitz 1978).

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Note added in proof

We have recently shown that the 686 bp *Hin*PI fragment of pSC101 carrying the origin of replication is specifically retarded on acrylamide gels by purified IHF protein. This strongly suggests that the potential IHF binding site described above indeed binds IHF in vitro.