The *Escherichia coli* Fis protein prevents initiation of DNA replication from *oriC in vitro*

Sture Wold, Elliott Crooke[1] and Kirsten Skarstad*

Department of Biophysics, Institute for Cancer Research, Montebello, 0310 Oslo, Norway and [1]Department of Biochemistry and Molecular Biology, Georgetown University Medical Center, Washington, DC 20007, USA

ABSTRACT

Fis protein participates in the normal control of chromosomal replication in *Escherichia coli*. However, the mechanism by which it executes its effect is largely unknown. We demonstrate an inhibitory influence of purified Fis protein on replication from *oriC in vitro*. Fis inhibits DNA synthesis equally well in replication systems either dependent upon or independent of RNA polymerase, even when the latter is stimulated by the presence of HU or IHF. The extent of inhibition by Fis is modulated by the concentrations of DnaA protein and RNA polymerase; the more limiting the amounts of these, the more severe the inhibition by Fis. Thus, the level of inhibition seems to depend on the ease with which the open complex can be formed. Fis-mediated inhibition of DNA replication does not depend on a functional primary Fis binding site between DnaA boxes R2 and R3 in *oriC*, as mutations that cause reduced binding of Fis to this site do not affect the degree of inhibition. The data presented suggest that Fis prevents formation of an initiation-proficient structure at *oriC* by forming an alternative, initiation-preventive complex. This indicates a negative role for Fis in the regulation of replication initiation.

INTRODUCTION

Initiation of chromosomal replication is a crucial regulatory event in the cell cycle and must be precisely timed in response to varying external conditions. In *Escherichia coli*, replication is initiated at the unique origin, termed *oriC*. Within *oriC*, DnaA protein binds to its binding sites (DnaA boxes), and then promotes separation of the DNA strands in the AT-rich region (Fig. 1; 1,2). This duplex melting step is termed open complex formation. DNA structure and architectural proteins are important at this stage; the opening depends on negative supercoiling in the origin region, and the presence of the histone-like proteins HU or IHF (3–6). IHF binds specifically to one site in *oriC* (Fig. 1; 7), and presumably facilitates open complex formation by building a proper nucleoprotein structure. HU protein also facilitates bending of *oriC*, though binding without sequence specificity, and makes an equally efficient initiator structure as IHF *in vitro* (5).

The Fis protein shares certain properties with HU and IHF in being a small, abundant DNA-bending protein involved in formation of higher order nucleoprotein complexes. It was first identified as a factor for inversion stimulation in site-specific DNA recombination (8–10). Later, Fis has been shown to also participate in transcriptional activation of tRNA and rRNA genes and other genes encoding proteins involved in translation (11–15). It binds DNA at specific sites, thereby introducing a bend. The binding consensus sequence is, however, permissive; the DNA structure at the binding site is probably as important as the sequence itself [reviewed in (10)].

Fis binds to *oriC* between DnaA boxes R2 and R3 with high affinity (16–18). DNase I footprinting experiments indicate also an additional high affinity Fis binding site to the right of DnaA box R4, as well as sites with less affinity in the left and central region of *oriC* (Fig 1; 17). Footprinting data indicate that binding of Fis between R2 and R3, and binding of DnaA to R2 and R3 are mutually exclusive (16).

Several lines of evidence suggest a role for Fis in DNA replication *in vivo*: (i) *oriC*-dependent plasmids cannot transform *fis* mutant strains efficiently (16–18); (ii) *fis* null mutants form filamentous cells, show aberrant nucleoid segregation, and have inhibited DNA synthesis at high temperatures (17); (iii) cells carrying a deletion of DnaA box R4 need Fis protein to be viable (19); and (iv) the synchrony of initiation appears to be dramatically reduced in *fis* mutants (20; U. von Freiesleben and K. V. Rasmussen, personal communication). Furthermore, *in vivo* footprinting studies indicate that Fis remains bound to *oriC* through most of the cell cycle, but is released at the time of initiation of replication (21).

In contrast, no positive effect of Fis protein on replication *in vitro* has been observed. Hiasa and Marians (22) found that Fis is unable to stimulate replication from *oriC in vitro*. Instead, high concentrations of Fis inhibited replication in the absence of HU and IHF protein. However, this inhibition was relieved when either of these two proteins was present (22).

We describe here further studies on the effect of Fis protein on replication *in vitro* using two reconstituted enzyme systems for replication of supercoiled *oriC* plasmids: one that requires transcriptional activation by RNA polymerase (RNAP) and one that does not. Inhibition by Fis was found to occur both in the presence and absence of IHF or HU, with the extent of inhibition

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* To whom correspondence should be addressed

governed by the level of DnaA protein and the amount of transcriptional activation.

MATERIALS AND METHODS

Reagents

Ribonucleoside triphosphates, deoxyribonucleoside triphosphates, poly(dI–dC)·poly(dI–dC) and Sephadex G-50 Nick columns were from Pharmacia; [γ-^{32}P]ATP (>5000 Ci/mmol) and [α-^{32}P]dTTP (800 Ci/mmol) from Amersham; cellulose phosphate P11 from Whatman; polyvinyl alcohol (MW 30–70.000) from Sigma.

Plasmid DNA

Plasmid pBSoriC (3640 bp), also called pTB101 (4) contains a 678 bp *Hin*cII–*Pst*I fragment spanning *oriC* (–189 to +489 bp) cloned into the pBluescript vector. pBSoriC-fis1 is a pBSoriC derivative containing six base substitutions in Fis site I in *oriC* [Fis → *Bam*HI; (23)]. Plasmid pOC170 (3853 bp) contains the *oriC* region –176 to +1497 (18). pOC170-fis2 (pOC170*oriC*131), contains six base substitutions (different from the fis1 mutation) in Fis site I (18).

The plasmids were purified by two successive equilibrium centrifugations in CsCl–ethidium bromide density gradients as described (24), followed by desalting over Sephadex G-50 Nick columns.

Enzymes

Replication proteins gyrase B subunit, SSB, primase, β-subunit of DNA polymerase III holoenzyme and HU [purified as described in (25,26)] were a gift from A. Kornberg. DnaA protein, DNA polymerase III* and gyrase A subunit were purified as described by Sekimizu *et al*. (27), Maki *et al*. (28) and Kruklitis and Nakai (29), respectively. DnaB–DnaC in equimolar complex (N. P. J. Stamford, *et al*., manuscript in preparation) was provided by N.Dixon. IHF protein was a gift from H. E. Nash. *Escherichia coli* RNA polymerase was bought from Pharmacia.

Purification of Fis

Fis protein was overproduced in the *E.coli* strain JM83 harboring a T7 expression system: plasmid pGP1-2 (30) expressing T7 RNAP under control of the temperature sensitive λP$_L$ promoter, and plasmid pCF351 (31) having expression of the *fis* gene regulated by the T7 RNAP-inducible promoter φ10. Strong overproduction of Fis protein is possible in this transformed strain. An overnight culture grown at 28°C in Terrific Broth [17 mM KH$_2$PO$_4$, 72 mM K$_2$HPO$_4$, 2.4% (w/v) Bacto-yeast extract and 0.4% (v/v) glycerol] containing ampicillin (40 μg/ml) and kanamycin (20 μg/ml) was diluted 1:1000 into fresh medium (6 l), and grown to an optical density (OD$_{600}$) of 1.4–1.5. Expression of Fis protein was then induced by shifting the culture to 42°C. After 40 min, the cells were harvested by centrifugation (4200 *g*; 0°C), resuspended in 50 mM Tris–HCl/10% sucrose and frozen in liquid nitrogen. Thawed cells were resuspended in buffer A (25 mM HEPES–KOH pH 7.6, 0.1 mM EDTA, 2 mM DTT, 15% glycerol), and sonicated before addition of lysozyme, as this increased the yield of Fis protein. The lysis was performed according to the procedure by Dixon and Kornberg (3), except

NaCl (1 M) was included in the lysis reaction. The lysate was clarified by centrifugation (25 400 *g*; 0°C), and the supernatant was diluted with buffer A to a conductivity equal to that of buffer A containing 200 mM NaCl. The diluted lysate (445 ml) was applied to a cellulose phosphate column (bed volume 150 ml) equilibrated in buffer A containing 200 mM NaCl. Bound proteins were eluted with a linear gradient of NaCl (800 ml, 200 mM–1 M, in buffer A). Fractions were tested for *oriC* binding activity in an electrophoretic mobility shift assay (see below); active fractions eluted at ~750 mM NaCl. These were pooled (Fr II), diluted in buffer A to a conductivity equal to that of buffer A containing 200 mM NaCl, and applied to a heparin–agarose column (bed volume 120 ml) equilibrated in buffer A containing 200 mM NaCl. Bound proteins were eluted with a linear gradient of NaCl in buffer A (1.2 l, 200 mM–1 M). Fis protein (Fr III; 77 ml; 1.22 mg/ml) eluted as a single peak (550 mM NaCl) of high purity (>99% as judged by silver stained SDS–PAGE). Contaminating DNase activity was not detectable in the preparation of purified Fis protein.

The purified Fis protein was sensitive to dilution; a >10-fold dilution into the same buffer (buffer A containing 550 mM NaCl) reduced the specific activity for binding to *oriC*, possibly due to precipitation of the protein. Inclusion of polyvinyl alcohol at 5% (w/v) in the dilution buffer reduced the loss of binding activity, and was therefore included in all dilutions.

In vitro replication reaction

The RNAP-independent reaction (25 μl) contained 30 mM Tricine–KOH (pH 8.2); 12 mM magnesium acetate; 2 mM ATP; 0.04% polyvinyl alcohol; 200 ng supercoiled DNA template (pBSoriC unless otherwise stated; equal to 600 pmol nucleotides, or 84 fmol molecules); 125 ng DnaB–DnaC in equimolar complex; 180 ng gyrase A subunit; 180 ng gyrase B subunit; 450 ng SSB; 23 ng primase; 112 ng DNA polymerase III* (Pol III); 26 ng β subunit of Pol III; 8 ng HU and 32 ng DnaA unless otherwise stated; dATP, dTTP, dCTP and dGTP each at 0.1 mM with [α-^{32}P]dTTP at 30–200 c.p.m./pmol of deoxynucleotides.

The RNAP-dependent reaction (25 μl) additionally contained UTP, GTP and CTP each at 0.5 mM, 100 ng HU, and RNAP at the indicated amounts. DnaA protein was added at the indicated amounts. In this replication system, RNaseH is sometimes added to inhibit DnaA-independent initiations originating outside *oriC*. However, the amount of DnaA-independent replication in our assay was <10% of the total DNA synthesis. RNaseH was therefore omitted.

Mixtures were assembled at 0°C and incubated at 29°C for 20 min, and then precipitated with 500 μl cold 10% trichloro-acetic acid. Total nucleotide incorporation was measured by liquid scintillation counting after filtration onto GF/C glass-fiber filters.

Electrophoretic mobility shift assay

During purification of Fis we used a 270 bp *Sau*96–*Cla*I restriction fragment (R-ori; Fig. 1) containing DnaA boxes R2, R3 and R4, and part of the *mioC* open reading frame (32). The fragments were dephosphorylated with calf intestinal alkaline phosphatase and 5′-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. For testing the affinity of Fis for the mutated pBSoriC-fis1 *oriC*, we used 350 bp PCR-amplified DNA fragments containing the whole *oriC* region (coordinates –20 to
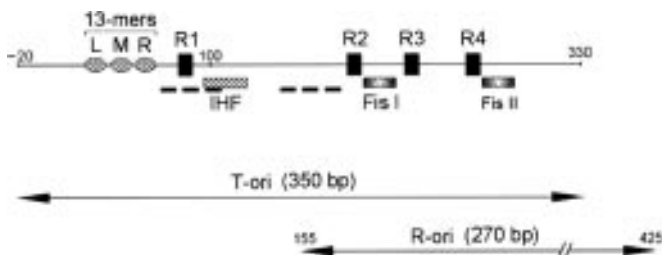
Figure 1. The *oriC* region. The three AT-rich 13mers (Left, Middle and Right) and the binding sites for DnaA protein (DnaA boxes R1–R4) are shown. The binding sites for proteins Fis and IHF are indicated with boxes. Broken lines: low affinity binding sites for Fis (17). The DNA fragments used in electrophoretic mobility shift assays are indicated. The coordinates are as in references (40) and (41).

+330; T-ori, Fig. 1). The fragments were purified with Spin-bind columns (MedProbe) before 5′-end-labeling with [γ-^{32}P]ATP. The mobility shift reaction mix (20 μl) contained 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 75 mM KCl, 2 mM DTT, 10% glycerol, 2 μg poly(dI–dC)·poly(dI–dC), 1.5 fmol labeled DNA fragments and the indicated amounts of corresponding, unlabeled DNA. Protein was added to the mixture at the indicated amounts. Reactions were incubated at 30°C for 20 min and subjected to electrophoresis through 5% polyacrylamide gels. The gels were dried and subjected to autoradiography. The relative intensities of the bands were quantified by scanning densitometry (Molecular Dynamics Computing Densitometer, Model 300A).

RESULTS

Fis protein can inhibit replication from wild-type *oriC* *in vitro*, even in the presence of HU or IHF

Replication of *oriC* plasmids *in vitro* can be reconstituted with purified replication proteins, among which DnaA is the key initiator protein. As long as the plasmid is sufficiently negatively supercoiled, DnaA is able to separate the two strands of the double helix. However, this DNA opening does not occur readily with DnaA as the sole actor. IHF and HU proteins both facilitate bending of *oriC*, and their presence strongly stimulates the opening reaction (3,5,6). We first examined the effect of purified Fis protein in a replication reaction independent of RNA polymerase [also known as the solo primase system (33,34)] where separation of the strands was facilitated by the presence of either HU (8 ng; Fig. 2A) or IHF (8 ng; Fig. 2B). Fis protein significantly inhibited replication, and the effect was equally strong whether HU or IHF was used. Replication reactions containing both HU and IHF (8 ng of each) were inhibited to the same extent by Fis as the reactions containing either HU or IHF (data not shown). Omitting HU or IHF reduced the activity by >95%. Fis protein had the same inhibitory effect in such 'unstimulated' reactions (data not shown). Low concentrations of Fis were not able to stimulate the existing feeble activity in systems lacking HU or IHF (see Discussion).

In vivo, free supercoils are restrained by the binding of HU and other structure-modifying proteins [reviewed in (35)], and initiation of replication is dependent on transcription by RNA polymerase (4,36,37). This situation can be mimicked *in vitro* by including a level of HU that reduces the free superhelicity to the
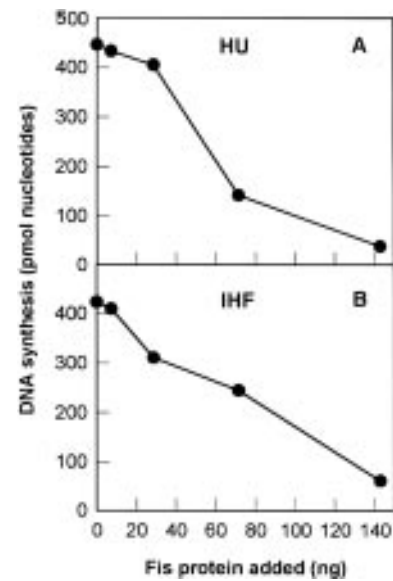

Figure 2. Effect of Fis protein on *in vitro* replication in the RNAP-independent replication system stimulated with HU (**A**) or IHF (**B**). The indicated amounts of Fis were added to the complete replication mixture on ice before incubation at 29°C. 2.1 ng of Fis correspond to one Fis dimer per oriC-plasmid.
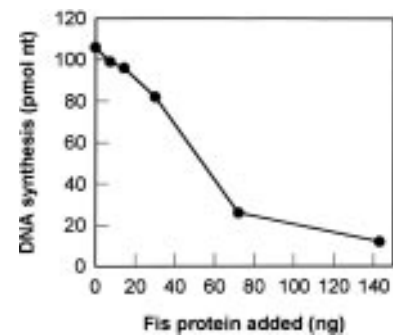

Figure 3. Effect of Fis protein on *in vitro* replication in the RNAP-dependent replication system. Fis was added as in Figure 2. The amounts of DnaA, RNAP and HU were 32 ng, 1.0 U, and 100 ng, respectively.

level found *in vivo*. Under these conditions DnaA protein is poor at melting the duplex DNA and must be aided by transcription by RNAP (4,5). The effect of Fis was investigated in such an RNAP-dependent, and thus physiologically more relevant, replication system (Fig. 3). Fis inhibited also this replication reaction; the degree of inhibition being similar to that observed for the RNAP-independent reaction (Fig. 2).

The concentrations of DnaA protein and RNA polymerase influence the degree of Fis-mediated inhibition

The negative effect of Fis suggests that it does not contribute to the formation of an initiation-proficient nucleoprotein structure. Rather, it prevents its formation, possibly by forming an alternative structure at *oriC*. If so, such an inhibitory structure might predominate when positively acting initiation factors are at low levels. To investigate this, we tested the effect of Fis at
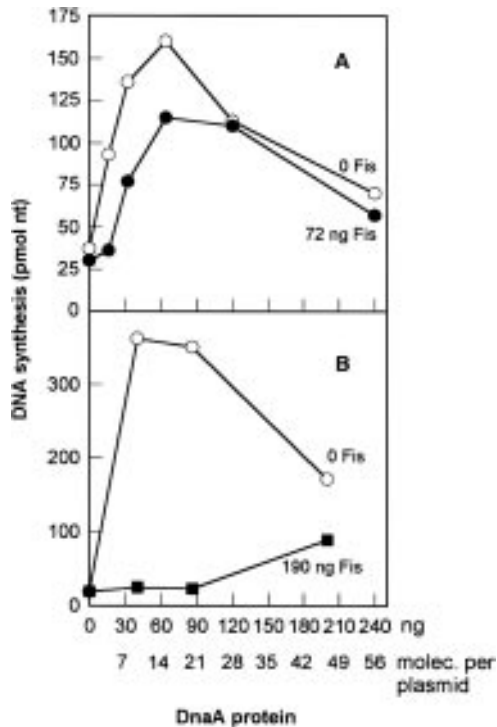
Figure 4. The degree of Fis-mediated inhibition depends on the concentration of DnaA protein. The indicated amounts of Fis protein (filled symbols) and DnaA were added to (**A**) the RNAP-dependent reaction (1.0 U RNAP) and (**B**) the RNAP-independent reaction. The abscissa shows the amount of DnaA in units of nanograms in the upper row, and in molecules per plasmid in the lower row.

limiting amounts of (i) DnaA protein and (ii) RNA polymerase. First, we varied the amount of DnaA protein in the RNAP-dependent reaction in the absence or presence of a moderate level of Fis (72 ng; 35 dimers per oriC-plasmid) (Fig. 4A). A reaction containing 16 ng DnaA (~four molecules of DnaA per plasmid) was inhibited >90% by 72 ng Fis (considering a basal level of ~30 pmol nucleotides of dNTPs incorporated; Fig. 4A). With an increase of DnaA to its optimal concentration (64 ng), the inhibition by Fis decreased to ~30%. Excessive levels of DnaA partially inhibited replication even without Fis, and alleviated the Fis-mediated inhibition. This lessening of inhibition by Fis at high DnaA concentrations was clearly demonstrated in a similar experiment in the RNAP-independent reaction (containing 8 ng HU; Fig. 4B). Here, even the complete inhibition at optimal DnaA concentrations by a high level of Fis (190 ng) could be partly overcome with excessive amounts (200 ng) of DnaA (Fig. 4B).

Next, the concentration of RNA polymerase was varied (in the RNAP-dependent reaction) while DnaA was kept constant at either an optimal level (64 ng), or at a limiting level (16 ng). With the optimal amount of DnaA the degree of inhibition correlated inversely to the RNA polymerase concentration (Fig. 5, upper curves). Excess RNA polymerase inhibited the reaction lacking Fis somewhat, such that at six times the optimal concentration (3.0 U) Fis no longer contributed with an additional inhibition. At limiting amounts of DnaA, Fis was inhibitory at all levels of RNA polymerase tested (Fig. 5, lower curves). These data suggest that
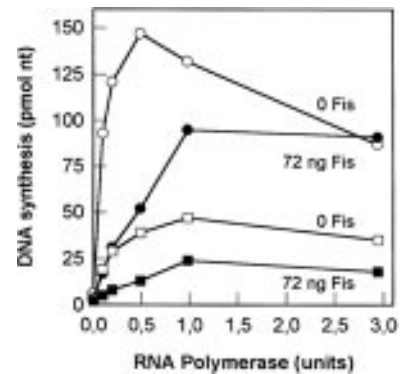

Figure 5. The degree of Fis-mediated inhibition depends on the concentration of RNA polymerase. DnaA protein (16 ng, squares, or 64 ng, circles) and Fis protein (72 ng, filled symbols) was added to the RNAP-dependent replication system containing the indicated amount of RNAP.

the inhibition by Fis is greatest when the system is balanced on the edge of being able to separate the DNA strands.

The inhibition by Fis is independent of a functional Fis site I

Plasmid pBSoriC-fis1 is a derivative of an *oriC* plasmid and contains base substitutions in the primary Fis binding site between DnaA boxes R2 and R3 (Fis site I; Fig. 1) (23). The ability of this *oriC* sequence to bind Fis protein was examined using an electrophoretic mobility shift assay with Fis protein and DNA fragments containing mutated or wild-type *oriC* (Fig. 6A). Quantification of the autoradiogram by scanning densitometry demonstrated a considerably reduced binding efficiency of Fis to the mutated *oriC* (Fig. 6B). The complex containing mutated *oriC* migrated more slowly than the complex with wild-type *oriC*, indicating possible structural differences. We do not know whether this is due to altered protein–DNA contact at site I, or to Fis binding at another site.

Surprisingly, with pBSoriC-fis1 serving as the template for replication *in vitro*, the effect of Fis was the same as that observed with the unmodified template (Fig. 7). Thus, Fis protein was capable of preventing replication even when its binding to *oriC* was not optimal. Also, replication of another template with base substitutions in Fis site I (pOC170-fis2), showing no protection by Fis at this site in methidiumpropyl–EDTA footprinting (18), was inhibited by Fis to the same degree as the wild-type control (data not shown).

DISCUSSION

We have shown that Fis protein inhibits replication from *oriC in vitro*. The level of inhibition depended on conditions that affect open complex formation, indicating that Fis inhibits the strand separation reaction. An interesting feature of the inhibition by Fis was the independence of a functional primary Fis binding site I between DnaA box R2 and R3. Fis was able to form inhibitory complexes with *oriC* in spite of alterations to this site and thus reduced binding to the origin. In electrophoretic mobility shift assays with *oriC* fragments containing the fis1 mutation at Fis site I, an amount of Fis equal to six dimers per DNA fragment gave a strong primary shift and a faint secondary shift. The higher order
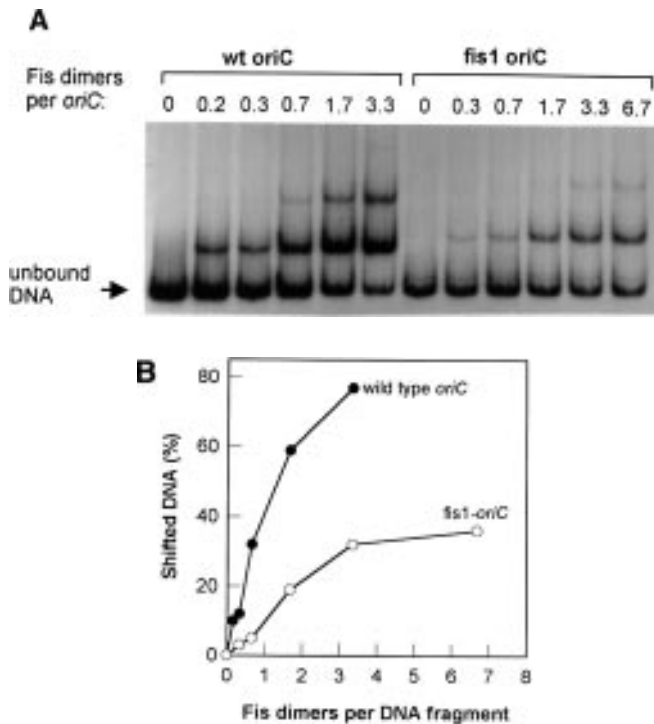
Figure 6. Electrophoretic mobility shift assay comparing Fis binding with wild-type *oriC* and to *oriC* containing the fis1 mutation. Fis protein was added at the indicated amounts to 376.5 fmol wild-type *oriC* DNA, and to 189.5 fmol fis1-*oriC* DNA, both of which 1.5 fmol DNA was ^{32}P-end-labelled. The DNA fragments (T-ori, see Fig. 1) were made by PCR, and cover the whole *oriC*. (**A**) Autoradiogram of the gel after electrophoresis; (**B**) quantification of the amount of shifted DNA (sum of all shifted bands) by scanning densitometry of the autoradiogram in (A).
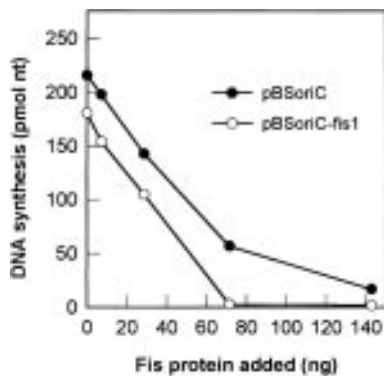

Figure 7. Fis prevents replication even of templates lacking a functional Fis binding site I. Fis protein at the shown amounts was added to the RNAP-dependent reaction containing wild-type pBSoriC template (closed symbols) or mutated pBSoriC-fis1 template (open symbols). The amounts of DnaA and RNAP were 64 ng and 1.0 U, respectively.

shifts are probably a result of Fis binding to several sites on the fragment. Increasing the concentration of Fis to 35 dimers per *oriC*, a ratio that significantly inhibited the replication reactions, resulted in a pattern with multiple shifted bands (not shown). Thus, at the ratios of Fis to *oriC* used in the replication reactions (7–90 dimers per *oriC* plasmid), Fis probably binds at multiple

sites, both within the normal and mutated origins, as well as outside *oriC*. Also, Fis protein is shown to be capable of inducing conformational changes even in DNA apparently lacking Fis binding sites (38). It is thus possible that several Fis dimers contribute to the formation of an initiation-deficient protein–*oriC* structure, and that the primary Fis binding site is dispensable for building this complex. This may explain why Fis inhibited replication of templates having mutated Fis I sites in a manner indistinguishable from that of wild-type plasmids.

Hiasa and Marians (22) reported that high Fis concentrations inhibit *in vitro* replication in the absence of HU or IHF, but that stimulatory amounts of HU or IHF can overcome this inhibitory effect. This contrasts with our findings that Fis is an efficient inhibitor of initiation in HU- and IHF-stimulated replication assays. However, we found that the inhibition by Fis was dependent on the levels of RNA polymerase and DnaA protein, being more pronounced the more limiting these factors were. The inhibition was alleviated when the levels of DnaA or RNA polymerase exceeded the concentrations optimal for replication. The level of DnaA used by Hiasa and Marians corresponds to >100 molecules per *oriC*-plasmid, whereas the optimal concentration in our assay is much lower, ~15 DnaA molecules per *oriC*-plasmid. This may explain the discrepancy. However, as our replication systems differ somewhat in total composition, it is difficult to draw conclusions based solely upon a comparison of this single parameter.

In the above mentioned work, it was found that, in contrast to HU and IHF, low amounts of Fis do not stimulate replication *in vitro* (22). We also investigated this issue. No stimulation was detectable; the only effect of Fis was to reduce the amount of replication. It has been suggested (18) that some DnaA protein preparations are contaminated by Fis protein. The lack of stimulation by adding Fis to *in vitro* replication reactions could thus be due to residual Fis being present in the replication mixture, already exerting a positive effect. However, immunoblot analysis with a detection limit of 0.1 ng Fis failed to detect Fis in a sample containing 30 times a normal complement of replication proteins (not shown). Hence, the purified replication proteins were, for all practical purposes, free from Fis contamination.

In vivo, Fis is needed for efficient transformation of *oriC* plasmids, as lack of Fis or Fis binding site I on the plasmid lead to feeble transformation (16–18). Also, introduction of a DnaA box R4 deletion into a *fis* mutant renders the mutant cells inviable (19). These seemingly positive effects of Fis on replication *in vivo*, contrasting its demonstrated negative effect on replication *in vitro*, raise interesting questions about this protein's role in control of replication initiation. Evidently, Fis has a negative effect; whether it also has a directly positive effect remains unclear.

Cassler *et al.* (21) have shown that a Fis–*oriC* complex exists throughout the cell cycle, but is replaced by an IHF–*oriC* complex as cells initiate replication. We suggest that Fis protein contributes to the formation of a structure at *oriC* that is incapable of promoting strand opening. In order for initiation of replication to occur, the preinitiation structure containing Fis protein must give way to a replicatively active initiation complex. Our data indicate that, *in vitro*, this may be achieved simply by providing more replicatively active DnaA protein or more transcriptional activity near *oriC*.

An important aspect of Fis being part of an inactive complex at *oriC*, is that with varying growth conditions, the cell experiences

large fluctuations in Fis concentration (39). Inasmuch as a large increase in the level of Fis protein would permit the inactive complex to persist longer, and thus cause a delay in initiation, Fis protein may also be involved in adjusting the initiation frequency in response to changes in growth rate.

ACKNOWLEDGEMENTS

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