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

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**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 Fis is modulated by the concentrations of DnaA protein and RNA polymerase; 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 binding of *oriC*, though binding without sequence specificity, and makes an equally efficient initiator structure as IHF in *vivo* (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 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
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. Ribonucleoside triphosphates, deoxyribonucleoside triphosphates, poly(dI–dC)·poly(dI–dC) and Sephadex G-50 Nick columns.

**Plasmid DNA**

Plasmid pBSoriC (3640 bp), also called pTB101 (4) contains a Plasmid DNA Sigma. were from Pharmacia; [poly(dI–dC)·poly(dI–dC) and 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 IIIβ 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 P70 promoter, and plasmid pCF351 (31) having expression of the fsp1 gene regulated by the T7 RNAP-inducible promoter P10. Strong overproduction of Fis protein is possible in this transformed strain. An overnight culture grown at 28°C for 20 min, and then precipitated with 500 µl cold 10% trichloroacetic 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 Sau96–ClaI restriction fragment (R-ori; Fig. 1) containing DnaA boxes R2, R3 and R4, and part of the mico open reading frame (32). The fragments were dephosphorylated with calf intestinal alkaline phosphatase and 5′-end-labeled with [γ-32P]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 NaCl (1 M) was included in the lysis reaction. The lystate 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 lystate (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 (1.21, 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 IIIβ (Pol III); 26 ng β subunit of Pol III; 8 ng HU and 32 ng DnA unless otherwise stated; dATP, dTTP, dCTP and dGTP each at 0.1 mM with [γ-32P]dATP 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% trichloroacetic acid. Total nucleotide incorporation was measured by liquid scintillation counting after filtration onto GF/C glass-fiber filters.
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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-32P]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 level found in vivo. Under these conditions DnaA protein is poor at melting the duplex DNA and must be aided by transcription by RNA polymerase (4,5). The effect of Fis was investigated in such an 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.

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
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 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 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
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 a loss of replication (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 replatively active initiation complex. Our data indicate that, in vitro, this may be achieved simply by providing more replatively 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.

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