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Biochemistry

Escherichia coli host factor for site-specific DNA inversion: Cloning and characterization of the *fis* gene

(DNA-binding protein/enhancer/recombination)

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ABSTRACT **The *Escherichia coli* (*Es. coli*) protein Fis (factor for inversion stimulation) stimulates site-specific DNA inversion of the G segment in phage Mu by binding to a recombinational enhancer. By using synthetic oligonucleotides deduced from the amino-terminal amino acid sequence, we have cloned the gene (termed *fis*) encoding this specific DNA-binding protein. The DNA sequence shows that the Fis protein is basic and contains 98 amino acids. A helix–turn–helix sequence motif characteristic of many DNA-binding proteins is located at the carboxyl-terminal end of the protein. By marker exchange, we have constructed an insertion mutation of *fis*. Fis is nonessential for *Es. coli* growth; however, inversion of the G segment of a Mu prophage was not detected in the *fis* mutant. The *fis* gene is located between 71 and 72 min on the *Es. coli* genetic map.**

Site-specific recombination has been shown to be involved in a variety of biological processes including viral integration, antigenic variation, and gene regulation (1). In phage Mu, site-specific inversion of the G segment determines the host range of the phage (2, 3). Recombination occurs between two 34-base-pair (bp) inverted repeats and is catalyzed by the phage Mu-encoded recombinase Gin. Gin belongs to a class of largely homologous DNA invertases, including Gin, Hin, Pin, and Cin, that complement each other (4). A distinctive property of this class of recombinases was revealed by the discovery of recombinational enhancer sequences capable of stimulating inversion independent of their position or orientation relative to the recombination sites (5–7). The stimulatory effect of the enhancer is mediated by a 12-kDa DNA-binding protein Fis (factor for inversion stimulation), identified as an *Escherichia coli* host factor required for *in vitro* DNA inversion (7–9). Fis recognizes three sites within the Mu enhancer and induces structural alterations: the DNA is bent and a certain degree of bending is crucial for enhancer function (C.K., F. Rudt and R.K., unpublished results).

Host factors other than Fis have been found to be involved in a number of site-specific recombination systems. One of these factors is integration host factor, a sequence-specific DNA-binding protein originally shown to be essential for λ site-specific recombination (10). Another factor is the histone-like protein HU, that is required for transposition of phage Mu (11). The two proteins are heterodimeric, share considerable homology on the amino acid level, and are members of the type II class of DNA-binding proteins (10).

In addition to their role in site-specific recombination, these host factors participate in other cellular processes. HU is a highly abundant protein thought to be involved in condensation of chromosomal DNA through nonspecific interactions (10). In contrast Fis and integration host factor bind to specific DNA sites, are present in low amounts, and thus have properties characteristic of proteins regulating gene expression (9, 12, 13). Indeed integration host factor is involved in transcriptional control of several genes (10). Up to now Fis has been shown to participate in one other site-specific recombination event, the excision of phage λ, which is stimulated by Fis at low Xis concentrations. Furthermore, the amount of Fis in the cell is growth-regulated (14), an aspect that makes it tempting to speculate about a more global regulatory function of Fis in *Es. coli*. Studies in this direction were restrained by a lack of genetic information about the gene encoding Fis. In this paper we report the isolation and characterization of this gene, which we designate *fis*.‖

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages. *Enterobacter cloacae* (*En. cloacae*) and the following *Es. coli* K12 strains were used: JM83 (*Δpro-lac rpsL thi φ80d lacZΔM15*); JC7623 (*recB21 recC22 sbcB15*) (15); CSH50 (*ara Δpro–lac thi*); K12ΔHIΔtrp (9); W3110 (F^- λ^-) (16). Plasmids pGp1-2 and pT7-5 were kindly supplied by Stan Tabor (Harvard University, Cambridge, MA) (17). pT7-5 differs from pT7-1 (17) by the orientation of the β-lactamase gene. pAK3 is a tester plasmid for DNA inversion and has been described (18). Cloning and sequencing vectors were from commercial sources.

Proteins and *in Vitro* Recombination Assay. Fis was prepared from strain K12ΔHIΔtrp as described (9); fraction V was used for the determination of the amino-terminal sequence. The isolation of Gin, analysis of recombination *in vitro*, and preparation of crude protein extracts were done as described (9, 19).

General Methods. Methods for the growth of bacterial strains, genetic manipulations, cloning of DNA, and colony hybridization followed established protocols (20, 21). Hybridization with 5′-end-labeled oligonucleotides was performed for 10 hr in 6× SSC (21), 10× Denhardt's solution (21), 0.1% $NaDodSO_4$, containing 5′-end-labeled deoxyoligonucleotides (0.5–2 pmol/ml; ≈2 × 10^6 cpm/pmol). DNA sequencing was carried out with the dideoxy method (22).

DNA Synthesis. The following oligonucleotides were synthesized on an automated DNA synthesizer (Applied Biosystems model A380): MC1 (5′ ITGTTCGAICAICGCGTCAA 3′, MC2 (5′ ITGTTCGAICAICGCGTIAA 3′), MC3 (5′ ITG-

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Abbreviation: ORF, open reading frame.

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‖The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03816).

TTCGAICAICGIGTCAA 3'), MC4 (5' ITGTTCGAICAICGIGTIAA 3'), MC5 (5' ITGTTTGAICAICGCGTCAA 3'), MC6 (5' ITGTTTGAICAICGCGTIAA 3'), MC7 (5' ITGTTTGAICAICGIGTCAA 3'), MC8 (5' ITGTTTGAICAICGIGTIAA 3'), MC9 (5' ITGTTTGAICAIAGIGTIAA 3'), MC10 (5' ITGTTTGAICAIAGIGTCAA 3'), MC11 (5' ITGTTCGAICAIAGIGTCAA 3'), MC12 (5' ITGTTCGAICAIAGIGTIAA 3'), and MC15 (5' ACIGTICIIACIGTIAGIACITCICIITTIACICITTGTTCIAACAT 3').

Protein Sequencing. Fis fraction V (9), containing ≈7 μg of protein, was subjected to final purification on a 20%/NaDodSO$_4$/polyacrylamide gel. Proteins were recovered by electroblotting on Polybrene-coated glass-fiber membranes and sequenced as described (23).

RESULTS

Amino-Terminal Amino Acid Sequence of Fis and Cloning of the *fis* Gene. Since only biochemical data on the effect of Fis on G inversion were available, it could not be anticipated whether a *fis* mutant would have a selectable phenotype. We, therefore, decided to clone the gene with oligonucleotide probes. By the microsequencing scheme described (23), the first 17 positions could unambiguously be determined to be NH$_2$-Met-Phe-Glu-Gln-Arg-Val-Asn-Ser-Asp-Val-Leu-Thr-Val-Ser-Thr-Val-Asn.

The amino acid sequence was used to devise a set of 12 deoxyinosine-containing oligonucleotides representing the first 20 nucleotides of the coding strand (oligonucleotides MC1–MC12). Another oligonucleotide (MC15) was designed to represent 47 nucleotides of the noncoding strand. In MC15 deoxyinosine was incorporated for A, T, and G at ambiguous positions; the remaining sequence possibilities were chosen randomly. These oligonucleotides were hybridized to Southern blots of chromosomal DNA from *Es. coli* strain K12-ΔHIΔtrp cleaved with various restriction enzymes (Fig. 1). Oligonucleotides MC1–MC12 were used individually (data not shown) and as a mixture (Fig. 1*B*). As can be seen in Fig. 1*B*, several specific sequences (more than five in lane 6) were detected, albeit with various intensities. With oligonucleotide MC15 only one band was detected in each restriction digest, and the smallest hybridizing fragment was generated by *Eco*RV (Fig. 1*C*). Since this signal coincides with one of the weaker bands detected by oligonucleotides MC1–MC12, it was analyzed for the presence of the *fis* gene. *Eco*RV fragments (1500–2000 bp long) were cloned into the *Hin*cII site of pUC19. By colony hybridization to radiolabeled MC15, three positive plasmid clones were detected. For one of them, we demonstrated that it hybridized to the same fragment as MC15 in Southern blot hybridization (21) (data not shown). To test if these plasmids contained the *fis* gene, we determined a partial DNA sequence with a mixture of MC1–MC12 as sequencing primer. All three clones gave identical sequencing patterns. The deduced amino acid sequence for the region next to the primer was Val-Ser-Thr-Val-Asn, which is in agreement with the protein sequence. Only one clone, pCF221, was used for further characterization.

DNA Sequence and Expression of the Cloned Gene. We have determined the DNA sequence of clone pCF221 (see Fig. 4*A*) around the unique *Hin*cII site. The sequence is shown in Fig. 2. An open reading frame (ORF) with a coding capacity for 98 amino acids was found. The amino-terminal part of the predicted protein sequence shows a perfect match to the sequence determined for the Fis protein. The predicted molecular mass of the protein is 11.2 kDa, which is in agreement with the apparent molecular mass of 12 kDa found in NaDodSO$_4$/polyacrylamide gel electrophoresis (9). The amino acid composition deduced from the DNA sequence is close to the composition determined experimentally for the Fis protein (data not shown). The protein is basic, having a calculated pI value of 9.7. The sequences preceding the ORF contain a weak homology to the Shine–Dalgarno sequence (Fig. 2). Within 150 nucleotides upstream of the ORF, no significant homology to the consensus *Es. coli* promoter elements was detected.

To verify the identity of the cloned gene, to test its functional integrity, and to have a convenient source of Fis protein for further studies, the phage T7 RNA polymerase expression system (17) was employed, in which expression of the cloned gene is driven by a T7 promoter. The *Eco*RI–*Pst* I fragment of pCF221 was cloned into the respective sites of pT7-5 DNA to yield plasmid pCF351. As shown in Fig. 3 induction of T7 RNA polymerase leads to the expression of

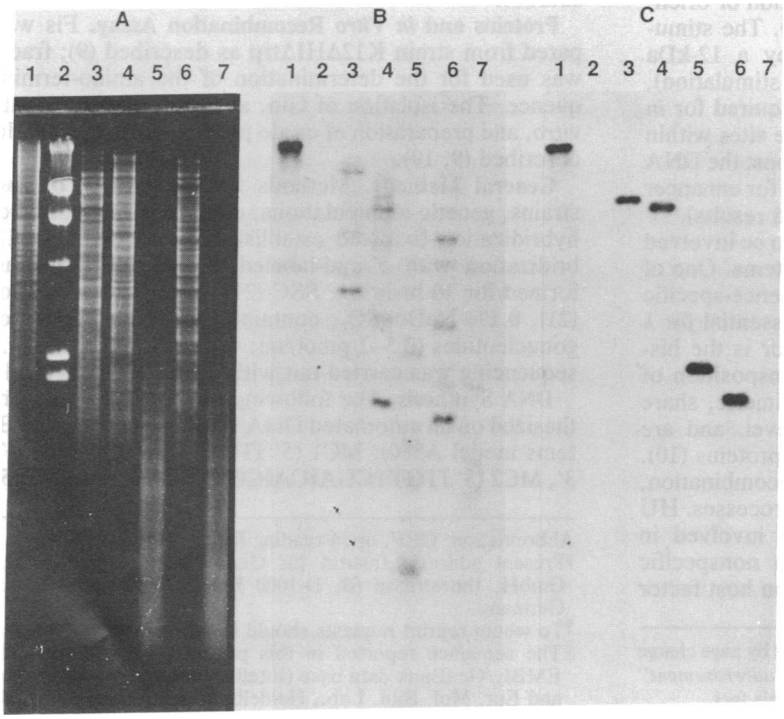

FIG. 1. Southern blot hybridization of *Es. coli* DNA from strain K12ΔHIΔtrp to synthetic oligonucleotides. (*A*) DNA (5 μg per lane) was digested with various restriction enzymes and analyzed on a 1% agarose gel. Lanes: 1, *Eco*RI; 2, λ *Hin*dIII marker; 3, *Pvu* II; 4, *Hpa* I; 5, *Hae* III; 6, *Eco*RV; 7, *Taq* I. After electrophoresis the DNA was transferred to a Hybond filter and hybridized to various probes. (*B*) Hybridization of blot shown in *A* to an equimolar mixture of oligonucleotides MC1–MC12. The filter was hybridized to the probe at 42°C. A high-stringency wash was performed at 48°C for 2 min in 6× SSPE/0.1% NaDodSO$_4$. (1× SSPE = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA.) (*C*) Hybridization of blot shown in *A* to oligonucleotide MC15. Hybridization and high-stringency washes were done at 52° C.

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      -160                -140                -120
 KpnI  .          .          .          .          .          .
GGTACCGAATTGCACGTAAACACGTTTCCTGGTATCTCCAGGAACACGCTCCAAATGACC

      -100                -80                 -60
        .          .          .          .  PvuII .          .
AGTTTCGGCGCACATTCAACGCCATTGAGGATGCCAGCGAACAGCTGGAGGCGTTGGAGG

       -40                 -20                  1
        .          .          .          .          .          .
CATACTTCGAAAATTTTGCGTAAACAGAAATAAAGAGCTGACAGAACTATGTTCGAACAA
                                  •••  ••
                                                  MetPheGluGln

        20                  40                  60
        .          .          .     HincII       .          .
CGCGTAAATTCTGACGTACTGACCGTTTCTACCGTTAACTCTCAGGATCAGGTAACCCAA
ArgValAsnSerAspValLeuThrValSerThrValAsnSerGlnAspGlnValThrGln

        80                  100                 120
        .          .          .          .          .          .
AAACCCCTGCGTGACTCGGTTAAACAGGCACTGAAGAACTATTTTGCTCAACTGAATGGT
LysProLeuArgAspSerValLysGlnAlaLeuLysAsnTyrPheAlaGlnLeuAsnGly

       140                 160                 180
        .          .          .          .          .          .
CAGGATGTGAATGACCTCTATGAGCTGGTACTGGCTGAAGTAGAACAGCCCCTGTTGGAC
GlnAspValAsnAspLeuTyrGluLeuValLeuAlaGluValGluGlnProLeuLeuAsp

       200                 220                 240
        .          .          .          .          .          .
ATGGTGATGCAATACACCCGTGGTAACCAGACCCGTGCTGCGCTGATGATGGGCATCAAC
MetValMetGlnTyrThrArgGlyAsnGlnThrArgAlaAlaLeuMetMetGlyIleAsn

       260                 280                 300
        .          .          .     XmnI .          .
CGTGGTACGCTGCGTAAAAAATTGAAAAAATACGGCATGAACTAATTCAGGTTAGCTAAA
ArgGlyThrLeuArgLysLysLeuLysLysTyrGlyMetAsn

       320                 340                 360
        .          .          .          .          .
TGCTTGATTAAAAAGGCGCTACTCGGCATGGGGAAGCGCCTTTTTTATAGGTGTCA
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FIG. 2. DNA sequence of the *fis* gene. The coding strand of the *fis* gene, including some upstream and downstream sequences, is shown. The numbering starts with the first nucleotide of the *fis* ORF; every 10th nucleotide is indicated by a small dot above. The amino acid sequence is written below the ORF nucleotide sequence. The amino acid sequence determined experimentally is underlined. Relevant restriction sites are marked. Symmetrical sequences are indicated by arrows. Large dots under the nucleotide sequence represent the potential ribosome binding site.

a polypeptide that comigrates with the purified Fis protein (lanes 4–7). This polypeptide is not detected in cells harboring the vector plasmid (lanes 1–3). Furthermore, extracts prepared from cells expressing this protein stimulated G inversion *in vitro* efficiently (Fig. 3*B*), whereas extracts from the control cells were unable to stimulate inversion under the assay conditions used. These results unambiguously prove that we have cloned the *fis* gene.

Location of the *fis* Gene on the *Es. coli* Chromosome. The establishment of a restriction map for the whole *Es. coli* chromosome of strain W3110 (16) offered the possibility to directly map the *fis* gene without further genetic manipulations. The cloned *fis* gene was hybridized to a set of 480 λ clones from strain W3110 encompassing the whole chromosome on a single nitrocellulose filter (kindly provided by K. Isono, Kobe University, Japan). A single clone, 2C2 (16), hybridized to the *fis* gene (data not shown). The restriction map around that region is in good agreement with the map obtained with the cloned gene as a probe (Fig. 4), except for the position of a single *Bam*HI site. This analysis places the *fis* gene at ≈71.5 min between *mdh* and *rrnE* (24) in strain W3110 (Fig. 4). Since strain W3110 carries an inversion between *rrnE* and *rrnD* (16), *fis* should be genetically linked to *rrnD* in *Es. coli* K12 derivatives that do not contain the inversion. The physical mapping was verified by phage P1 transductions in crosses involving genes *aroE*, *rpsL*, and the mutant *fis* gene described below (data not shown).

Isolation of a *fis* Mutant. To study the *in vivo* effects of Fis on site-specific recombination, a mutant in *fis* was constructed by marker exchange. To this end, plasmid pCF444 was constructed by inserting the 1.3-kilobase kanamycin resistance gene *kan* from pUC4-K into the unique *Hin*cII site

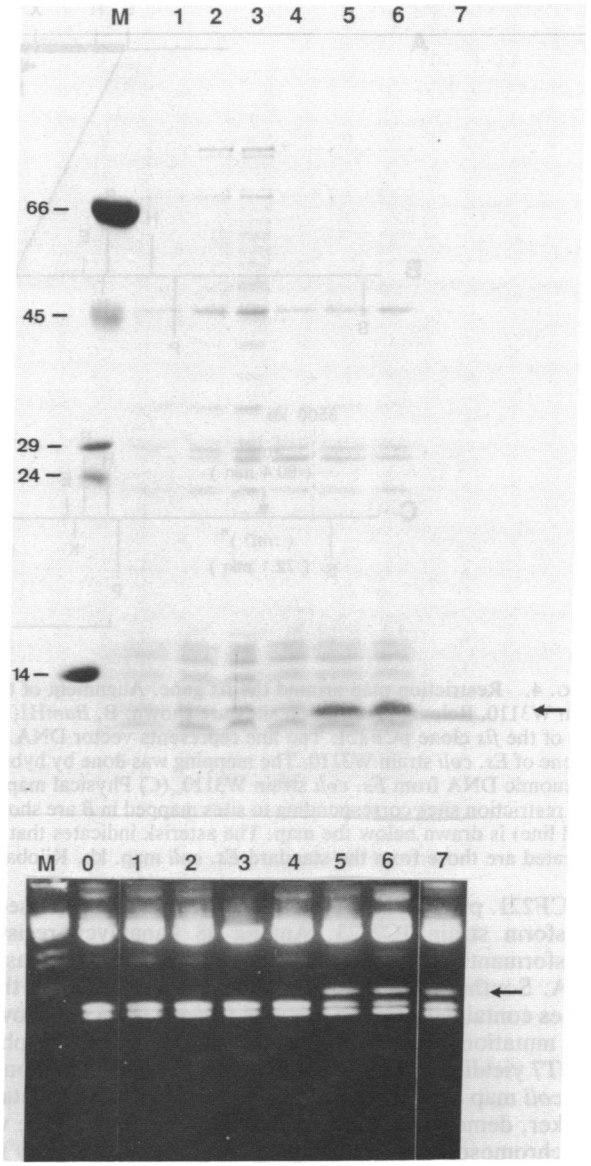

FIG. 3. Expression of the *fis* gene by T7 RNA polymerase *in vivo*. *Es. coli* strains JM83[pGP1-2,pT7-5] (lanes 1–3) and JM83[pGP1-2,pCF351] (lanes 4–6) were grown at 28°C in dYT to early logarithmic phase. After taking samples (uninduced, lanes 1 and 4), cultures were shifted to 42°C for 20 min (induced, lanes 2 and 5) and then grown at 37°C for another 2 hr (induced, lanes 3 and 6). (*Upper*) $NaDodSO_4$/polyacrylamide gel electrophoresis of protein extracts. After adjusting the cell concentration, protein extracts from each sample were made and boiled in sample buffer. Extracts were analyzed on a 17.5% $NaDodSO_4$/polyacrylamide gel. The gel was stained with Coomassie brilliant blue. Numbers to the left denote the molecular mass in kDa of the size markers (lane M). The arrow indicates the position of the Fis protein. Lane 7 contains purified Fis. (*Lower*) Fis activity in crude extracts. Extracts from the same samples as in *Upper* were analyzed for their stimulating effect on G inversion *in vitro*. For the preparation of crude Fis, the extracts were adjusted to 50 mM NaCl, boiled for 5 min, and centrifuged. The supernatant was used as a Fis preparation. Each extract (50 ng) was added to a standard recombination reaction mixture (9) containing pAK3 DNA and Gin protein (lanes 1–6). Lane 0 had no added Fis. Lane 7 contained 20 ng of purified Fis. After completion of the recombination reaction the substrate DNA was digested with *Pst* I and analyzed on a 2% agarose gel. The arrow marks a restriction fragment characteristic for molecules that have undergone inversion.

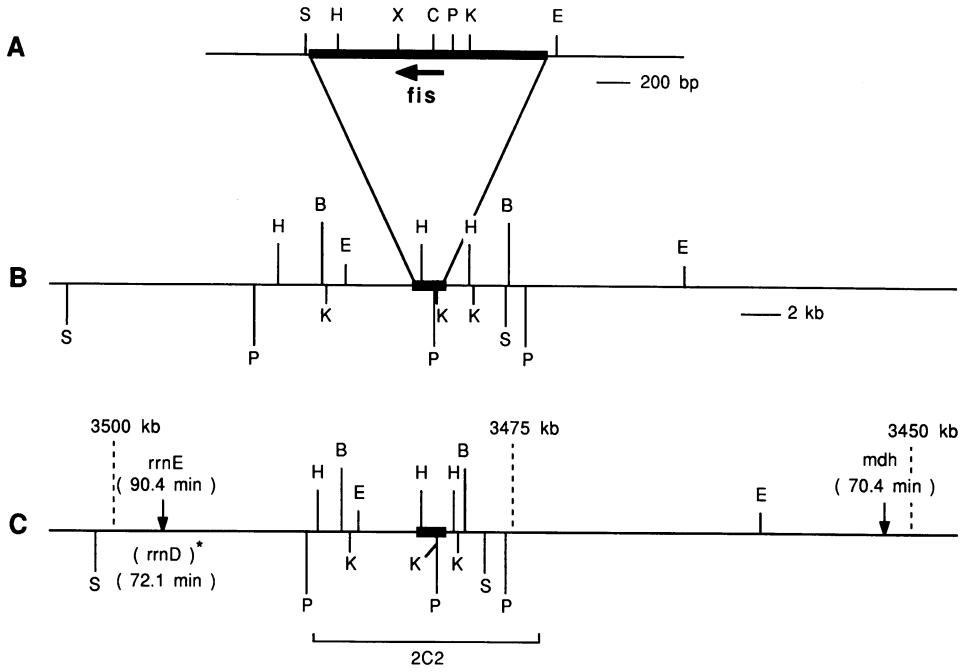

FIG. 4. Restriction map around the *fis* gene. Alignment of the restriction map of *fis* with the physical map of the *Es. coli* chromosome of strain W3110. Relevant restriction sites are shown: B, *Bam*HI; C, *Hin*cII; E, *Eco*RI; H, *Hin*dIII; K, *Kpn* I; P, *Pvu* II; S, *Pst* I; X, *Xmn* I. (*A*) Map of the *fis* clone pCF221. The line represents vector DNA. The Fis ORF is marked with an arrow. (*B*) Partial restriction map around the *fis* gene of *Es. coli* strain W3110. The mapping was done by hybridizing the *Eco*RI–*Pst* I fragment of pCF221 (bold line) to restriction fragments of genomic DNA from *Es. coli* strain W3110. (*C*) Physical map of the *Es. coli* chromosome. The data are taken from Kohara *et al.* (16), and only restriction sites corresponding to sites mapped in *B* are shown. The λ clone 2C2 from the *Es. coli* library (16) that hybridizes to the *fis* gene (bold line) is drawn below the map. The asterisk indicates that strain W3110 carries an inversion between *rrnD* and *rrnE*. The map positions indicated are those from the standard *Es. coli* map. kb, Kilobases.

of pCF221. pCF444 DNA linearized with *Eco*RI was used to transform strain JC7623. Among 38 kanamycin-resistant transformants, 3 were shown not to contain any plasmid DNA. Southern blot hybridization proved that these three clones contained only the mutated *fis* gene (data not shown). The mutation was then transduced into CSH50 with phage T4GT7 yielding strain CSH50*fis*::Kan. *rpsL* at 73 min on the *Es. coli* map (24) could be cotransduced with the resistance marker, demonstrating the association of the *kan* gene with the chromosome. Cells carrying the *fis* mutation grew normally, showing that *fis* is a nonessential gene.

***fis* Mutants Do Not Support Inversion of the G Segment.** We next analyzed whether inversion of the G segment was affected in the *fis* mutant. In phage Mu, G inversion leads to a change in host range that can be quantified by determining the plating efficiencies of phage lysates on the G(+) host CSH50 and on the G(−) host *En. cloacae*. CSH50*fis*::Kan and CSH50 were freshly lysogenized with Mucts62. The *fis* mutation had no obvious effect on lysogenization and subsequent phage development. Phage lysates were obtained after induction of the prophage. For lysates from the mutant and the wild-type strains, G(+) phage had titers of 4×10^9 plaque-forming units/ml as measured on CSH50 indicators. On *En. cloacae*, G(−) phage, obtained from the lysate of the wild-type strain, had a titer of 2×10^6 plaque-forming units/ml. The reduced titer is accounted for by restriction in *En. cloacae*. The phage lysate obtained from CSH50*fis*::Kan had a titer of $<2 \times 10^2$ plaque-forming units/ml on *En. cloacae*. Thus G inversion in the *fis* mutant was reduced by at least four orders of magnitude, suggesting that *fis* is essential for inverting the G segment in phage Mu *in vivo*.

DISCUSSION

In this paper we report the isolation and characterization of the gene encoding Fis, the *Es. coli* host protein required for site-specific DNA inversion *in vitro*. The gene, which we designate *fis*, was characterized by DNA sequence analysis, its location on the *Es. coli* chromosome was mapped, and a mutant was constructed.

Since we did not use genetic criteria for the isolation of the *fis* gene, we inspected the region on the *Es. coli* chromosome to which we mapped the gene for known genetic markers to find out if *fis* had been described or if it was part of a larger transcription unit. *fis* was mapped between 71 and 72 min on the *Es. coli* map. This region of the map is not very well characterized (24), and, considering the phenotypes of mutants in that region, it is unlikely that any of them correspond to *fis*. Inspection of DNA sequences upstream of the Fis coding region did not reveal strong homologies to *Es. coli* promoter elements. This might indicate that the *fis* promoter does not conform to the *Es. coli* consensus promoter sequence; alternatively *fis* might be part of a larger operon. In the T7 expression experiments where a fragment containing 700 bp upstream and 600 bp downstream of *fis* was analyzed, there was no indication for the expression of genes other than *fis* as judged by $NaDodSO_4$/polyacrylamide gel electrophoresis. Sequences immediately downstream of *fis* resemble a ρ-independent transcription terminator. Its function *in vivo* remains to be tested.

To further test whether *fis* is related to other genes, sequence comparisons on the DNA level and on the amino acid level were performed. We have not found any relevant DNA homologies to entries in the GenBank or EMBO sequence libraries.** To investigate whether Fis is structurally related to integration host factor and HU, we compared the amino acid sequence of Fis with sequences of these and other type II DNA-binding proteins. Alignments of the amino acid sequences with the Bestfit program from the UWGCG

**EMBL/GenBank Genetic Sequence Database (1987) GenBank (IntelliGenetics, Mountain View, CA), Tape Release 54.0.

sequence analysis software package (University of Wisconsin) showed no statistically significant similarity between Fis and these DNA-binding proteins. This was somewhat unexpected, because these three proteins serve similar functions in site-specific recombination and have comparable physical properties. Since Fis is known to bind to DNA specifically, Fis was analyzed for homology to the helix–turn–helix motif found in the DNA-binding domains of many DNA-binding proteins, such as Cro and catabolite activator protein (25). We have used the method of Dodd and Egan (26), which determines the similarity of a given sequence to a master set of Cro-like sequences. This method estimates the probability of the Fis carboxyl terminus being Cro-like as 60%. The alignment of this part of the Fis sequence with sequences from proteins known to adopt such a conformation is shown in Fig. 5. Within this structural motif, the most conserved positions that appear to be crucial for determining the α-helical structure (25) are also conserved in the carboxyl-terminal part of the Fis protein. Thus the carboxyl terminus of the Fis protein could potentially represent a DNA-binding domain. Determination of the structure of HU (27) has shown that it does not contain a helix–turn–helix motif but interacts with DNA through two symmetrical arms. Thus Fis probably belongs to a different class of proteins than HU and integration host factor.

The reduction of G inversion *in vivo* by at least four orders of magnitude in the *fis* mutant demonstrates that Fis is an essential component for the inversion reaction *in vivo*. *In vitro* experiments addressing this question failed to yield clear-cut results (19), presumably because Gin isolated from *fis*$^+$ strains still contained trace amounts of Fis. The availability of a *fis* mutant now permits use of a genetic approach to understand how and at which stage in site-specific recombination the enhancer and Fis participate. Fis is not required for *Es. coli* growth, as cells lacking Fis protein have no obvious phenotype. However, there are several arguments for an involvement of Fis in regulatory processes other than site-specific recombination: Fis is a specific DNA-binding protein that recognizes sites other than those involved in stimulating DNA inversion, e.g., in insertion sequence 5 (IS5) and in the *lac* promoter region (C.K., unpublished data). The protein is not very abundant and the intracellular concentrations vary with growth conditions (14). The *fis* mutant should be of invaluable help for all approaches to define the genuine function of Fis in *Es. coli*.

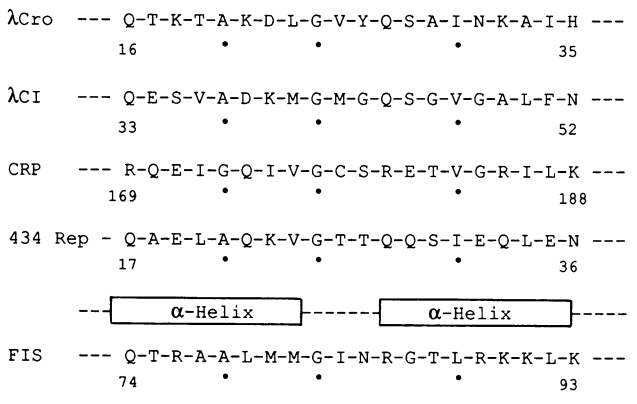

FIG. 5. The putative DNA-binding domain of Fis. Alignment of the carboxyl-terminal amino acid sequence of Fis with the helix–turn–helix motif characteristic for the DNA-binding domains of λ Cro, catabolite activator protein, and λ cI repressor. The highly conserved positions of this motif are marked by dots. Sequence data are taken from Pabo and Sauer (25).

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