

# Stimulation of DNA inversion by FIS: evidence for enhancer-independent contacts with the Gin–gix complex

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## ABSTRACT

**Efficient DNA inversion catalysed by the invertase Gin requires the *cis*-acting recombinational enhancer and the *Escherichia coli* FIS protein. Binding of FIS bends the enhancer DNA and, on a negatively supercoiled DNA inversion substrate, facilitates the formation of a synaptic complex with specific topology. Previous studies have indicated that FIS-independent Gin mutants can be isolated which have lost the topological constraints imposed on the inversion reaction yet remain sensitive to the stimulatory effect of FIS. Whether the effect of FIS is purely architectural, or whether in addition direct protein contacts between Gin and FIS are required for efficient catalysis has remained an unresolved question. Here we show that FIS mutants impaired in DNA binding are capable of either positively or negatively affecting the inversion reaction both *in vivo* and *in vitro*. We further demonstrate that the mutant protein FIS K25E/V66A/M67T dramatically enhances the cleavage of recombination sites by FIS-independent Gin in an enhancer-independent manner. Our observations suggest that FIS plays a dual role in the inversion reaction and stimulates both the assembly of the synaptic complex as well as DNA strand cleavage.**

## INTRODUCTION

The *Escherichia coli* protein FIS was originally discovered as a host factor required for stimulation of DNA inversion which is catalysed by DNA invertases Gin, Hin and Cin (1–3). Inversion switches the expression of alternate sets of genes serving adaptive purposes in phage and bacteria (for review see 4). Subsequent studies showed that FIS is involved in a variety of cellular processes, including transcriptional regulation of stable RNA promoters (5,6), chromosomal replication (7,8), regulation of transposition frequency (9,10) and maintenance of phage lysogeny (11–13).

FIS consists of 98 amino acids and exists as a homodimer in solution (14,15). The crystal structure of FIS reveals three domains with apparently distinct functions. The C-terminal  $\alpha$ C and  $\alpha$ D helices form a helix–turn–helix (HTH) motif responsible for DNA binding. The central part of FIS comprises two  $\alpha$  helices,  $\alpha$ A and  $\alpha$ B, involved in the dimerization of FIS. The N-terminal 24 amino acids show no electron density in the crystal structure and are thus assumed to be flexible (16–18). The N-terminal domain is absolutely required for stimulation of DNA inversion but is dispensable for other functions of FIS such as specific DNA binding and bending (19,20).

The stimulatory effect of FIS on DNA inversion has been studied in much detail both in the Gin and Hin systems. The reaction steps leading to inversion require formation of a complex nucleoprotein assembly of unique topology, the synaptic complex, in which two negative supercoils are trapped between the recombination sites (21,22). In this complex the two inversely oriented recombination sites each bound by a dimer of invertase are thought to be held together by protein contacts. Depending on the system FIS binds DNA at two or three specific sites within the recombinational enhancer (20,23,24). In turn the enhancer adopts a specific configuration due to strong bends introduced by FIS at each site (25–28). The enhancer with bound FIS becomes physically associated with the paired recombination sites at a branch point in supercoiled DNA (21,22). The invertase dimers are then proposed to undergo a conformational transition followed by the introduction of concerted 2 bp staggered cuts within both crossover sites, rotation of the DNA strands by 180° and their religation in the recombinant configuration (4).

Different models have been proposed to explain the stimulatory function of FIS and the enhancer. The enhancer could work in a ‘hit-and-run’ fashion being required only during the assembly of the synapse, but being dispensable during strand rotation and rejoining (29). In this model the enhancer is released after synapse assembly. In an alternative model the enhancer is thought to remain associated with the recombination sites during strand exchange and restricts strand rotation to a single round; upon premature release of the enhancer multiple rounds of strand rotation would occur resulting in the formation of complex knots (30). Although there is ample evidence that FIS and enhancer

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facilitate the assembly of a topologically unique synapse it is still unclear whether the stimulatory effect of FIS is direct and involves specific protein contacts between FIS and the invertase, or indirect, i.e. mediated solely by the specific geometry of the enhancer which in turn specifies the proper topology of the synaptic complex.

The isolation and biochemical characterisation of FIS-independent Gin mutant proteins (31,32) provided valuable information on reaction steps possibly affected by FIS and enhancer. In particular, the FIS-independent mutant Gin M114V is capable of unwinding the recombination (*gix*) sites suggesting that FIS may induce a similar conformational change in the wild-type Gin-*gix* complex (32). Investigations in the Hin system also indicate that FIS bound at the enhancer triggers conformational adjustments of Hin subunits important for the concerted strand cleavage within both crossover sites (33). It is conceivable that these conformational transitions in the synaptic complex are at least in part mediated via direct interactions between FIS and the invertase. In the Hin system experimental evidence for the close proximity of FIS and Hin within the 'invertosome' has been obtained (22). Furthermore, the combination of the mutation M114V with a second mutation in another domain of Gin leads to a protein (Gin M114V/G44E) that requires FIS but does not require the enhancer for recombination (34).

In contrast to wild-type Gin and FIS where one specific synapse is assembled, the FIS-independent mutant Gin M114V has lost this selectivity and assembles a broad spectrum of topologically distinct complexes in which different numbers of negative supercoils are entrapped between the *gix* sites (35). However, FIS is still able to stimulate DNA inversion by this mutant protein (31) suggesting that the putative contact site for FIS remains intact. We used this property of Gin M114V to design a genetic screen for *fis* mutants that inhibit the activity of Gin M114V. In this study we describe isolation of a mutant FIS protein which affects the cleavage reaction catalysed by Gin M114V in an enhancer-independent manner.

## MATERIALS AND METHODS

### Bacterial strains and plasmids

Bacterial strains used in this study were CSH50*fis*::Kan (15), CSH50Δ*fis*::Cm (36), WK6 mut( $\lambda$ cI<sup>+</sup>) S215::Tn10 (37). The plasmids used in this study were pAK3 (38), pIR1, pIR2 (31) and pLMC5-8gin (37), pGP1-2 and pT7-5 (39), pLMC*fis*2 and pIQ (20), pMAK700 (40), pSP72 (Promega, EMBL accession no. X65332), pSL1180 (41), pUC4K (42,43); pUHE25-2 is an expression plasmid containing the tightly regulated *lac* promoter pA1-04/03 (44,45).

The inversion test strain AD1 is a derivative of CSH50*fis*::Kan and was constructed as follows. The DNA fragment containing the *lacZ* gene flanked by *gix* sites, the recombinational enhancer and the Cm promoter was excised as *Hind*III-*Xmn*I fragment from pMD3*lacZ* (37), the *Hind*III ends were blunted and the fragment was cloned in the *Msc*I site of the *oxyR* gene carried by the plasmid pBRarg (46). From this plasmid the *lacZ* gene flanked by *oxyR* sequences (1100 and 1200 bp) was excised as a 8800 bp *Bam*HI-*Eco*RI fragment and cloned into the respective sites of pSP72. From there the same fragment was reisolated as a *Hind*III/*Bgl*II fragment and cloned in *Hind*III/*Bam*HI sites of pMAK700 (40). The respective plasmid was introduced in CSH50*fis*::Kan and derivatives where the *lacZ* gene was inserted

in the *oxyR* locus by homologous recombination were isolated as described (46). The *lacI*<sup>q</sup> gene was obtained as an *Eco*RI fragment from pIQ, the ends were blunted and the fragment was cloned into the *Sma*I site of the kanamycin resistance gene of pUC4K, reisolated from pUC4K as a *Bam*HI fragment, cloned into the respective site of pMAK700 and inserted via homologous recombination into the kanamycin resistance gene of CSH50*fis*::Kan *oxyR*::*lacZ*.

To construct pAD*fis* the Cm resistance gene of pUHE25-2 was inactivated by deletion of a *Pvu*II-*Msc*I fragment. Into this plasmid (pUHE25-2ΔCm) the wild-type *fis* gene with an altered initiation region (20) was cloned as *Eco*RI-*Hind*III fragment into the respective sites. To construct the plasmids pMD3gin and pMD3gin M114V the respective genes were excised from pLMC5-8gin (37) and pLMC5-8ginMV (32) together with the upstream  $\lambda$ P<sub>L</sub> promoter as *Eco*RI-*Hind*III fragments, the *Hind*III site was filled up, and the fragments were then cloned in *Eco*RI-*Xmn*I sites of pMD3*lacZ*. This cloning procedure deletes the *lacZ* gene of pMD3*lacZ*. To overproduce FIS and the FIS mutant proteins the corresponding genes were excised as *Eco*RI-*Hind*III fragments from pAD*fis* and cloned into the respective sites of pT7-5. To construct pAD*fis* R85C, *fis* R85C was excised from pLMC*fis*2 as an *Eco*RI-*Hind*III fragment and cloned into the respective sites of pAD*fis*. The *fis*-*myc* construct pT7-5*fis*-*myc* was obtained by means of PCR by fusing the sequence coding for an 11 amino acid peptide (EQKLISEEDLN) from human *c-myc* protooncogene to the second amino acid of the N-terminus of *fis*. The primer used for the fusion of the *myc* tag carries the methionine codon (underlined) and has the following sequence: 5'-GGTGACAGATCTATGGAACAGAACTGATCTCCGAAGAAGACCTGAACTTCGAACAACGC-3'. The PCR product was digested with *Bgl*II and *Hind*III and ligated into respective sites of pAD*fis*. From pAD*fis*, the *myc*-tagged *fis* gene was isolated as *Eco*RI-*Hind*III fragment and ligated into the respective sites of pT7-5 for overexpression.

### Mutagenesis and isolation of *fis* mutants that inhibit the activity of Gin M114V

Mutagenesis of the *fis* gene was done by error prone PCR as described (47).  $2 \times 10^6$  independent clones were generated and 40% of these carried defective *fis* genes as assayed in conjunction with pMD3gin in strain AD1. Specific mutations were introduced into the *fis* gene by targeted mutagenesis using synthetic oligonucleotides (48,49), their sequences being available on request.

In AD1 the *lacZ* gene flanked by inversely oriented *gix* sites is placed in 'off' orientation with respect to the P<sub>Cm</sub> promoter. A productive inversion event places the *lacZ* gene in 'on' orientation allowing expression of  $\beta$ -galactosidase from the P<sub>Cm</sub> promoter. A pool of plasmids containing the mutagenised *fis* gene (pAD*fis*\*) was introduced in AD1. Into this pool of transformants then the plasmid pMD3gin M114V was introduced. The effect of *fis* mutants on Gin M114V mediated recombination was analysed by growing the transformants in the presence of appropriate antibiotics at 37°C on X-Gal plates containing 10  $\mu$ M IPTG. The recombinational activity was evaluated by the intensity of the blue colour of colonies. The colonies remaining white with pMD3gin M114V after 24 h were scored as those containing *fis* mutants being able to suppress the activity of Gin M114V. The *fis* mutant phenotypes were verified by retransformation of isolated plasmids.

## Chemicals and enzymes

Chemicals and enzymes used in this work were obtained from commercial sources. Wild-type Gin and Gin M114V were purified as previously described by Mertens *et al.* (52). FIS and mutant FIS proteins were purified as described by Koch and Kahmann (53). Polyclonal anti-Gin antibodies were kindly provided by Nora Goosen. Monoclonal anti-Myc antibodies were purchased from Dianova (Calbiochem).

## In vitro recombination reactions

*In vitro* inversion reactions were performed as described previously except that DTT (to 10  $\mu$ M) was included in the incubation mixtures (50). Typically 1  $\mu$ g supercoiled substrate DNA was incubated with different amounts of Gin and FIS as indicated in the figure legends. Reactions were terminated by heating the samples to 80°C for 10 min. The DNA was restricted with *Eco*RI and *Bam*HI to reveal fragments indicative of productive recombination. After electrophoresis the DNA was either stained by ethidium bromide or transferred to a membrane, hybridised with a 44 bp <sup>32</sup>P-end-labelled DNA fragment carrying the *gix* site (51) and visualised by autoradiography.

## DNA procedures

The 190 bp fragment harbouring the recombinational enhancer was derived from pAK3 by restriction with *Bam*HI. The 44 bp oligonucleotide, containing the 34 bp *gix* site has the following sequence: top strand: 5'-GGGATCCCATTTATCCAAAACCTC-GGTTTACAGGAAACGGTTCGAC-3'; bottom strand: 3'-CCC-TAGGGTAATAGGTTTTGGAGCCAAATGTCCTTTGCC-AGCTG-5'. The 30 bp fragment carrying the FIS binding site III from the UAS of the *tyr*T promoter has the sequence: top strand: 5'-AATCGAACGATTATTCCTTTAATCGCCAGCA-3'; bottom strand: 3'-TTAGCTTGCTAATAAGAAATTAGCGGTCGT-5'. The obtained mutations in *fis* were identified by sequence analysis.

## Gel retardation assay

Gel retardation experiments (54) were carried out in 20  $\mu$ l volume in a buffer containing 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 mg/ml BSA, 10  $\mu$ g/ml sonicated salmon sperm DNA, 10% glycerol, 2–5 nM DNA fragments end labelled using [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase, and proteins as indicated. After incubation for 20 min at 37°C, 2.2  $\mu$ l of 50% glycerol containing 0.25% bromophenol blue was added and the samples loaded on the running gel at 100 V. Gel electrophoresis was in polyacrylamide gels using 0.25 $\times$  TBE as the electrophoresis buffer. The complexes were visualised either by autoradiography or by phosphorimaging using the Storm 860 PhosphorImager (Molecular Dynamics).

Gel retardation experiments with supercoiled inversion DNA substrate were carried out in 20  $\mu$ l volume in a buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 10% glycerol and proteins as indicated. After incubation the samples were loaded on 1% agarose gels using 0.5 $\times$  TBE as electrophoresis buffer. The gels were stained with ethidium bromide and photographed under UV light.

## Dimerization assay

FIS-Myc alone or in combination with wild-type FIS or mutant FIS proteins were incubated at 95°C for 10 min in a buffer containing 20 mM Tris-HCl, pH 8, 100 mM NaCl, and allowed to cool to room temperature. Afterwards the 30 bp <sup>32</sup>P-end-labelled DNA fragment carrying the FIS binding site III from the UAS of the *tyr*T promoter was added and incubation continued for 5 min at 37°C. The samples were loaded on a 10% acrylamide gel running at 100 V. The complexes were visualised as described above.

## Molecular dynamics (MD) simulations

MD simulations were calculated under the AMBER4.1 force field (55) and analysed with the SYBYL package (Tripos, St Louis, USA). Atom coordinates of the FIS protein were from the crystal structure analysis by Kostrewa *et al.* (16) with hydrogen atoms added by SYBYL. A model of the mutant FIS V66A/M67T was constructed by removing the amino acid side-chains at positions 66 and 67 from the crystal structure of wild-type FIS and placing the exchanged side-chains in a favourable orientation directed by the interaction energy with the surrounding residues. Both models, of wild-type and mutant FIS, were subjected to 5000 steps of conjugate gradient energy minimisation before MD simulations. The simulations were performed in vacuum with a distant dependent dielectric constant and a 16 Å cut-off applied on the non-bonded interactions. A time step of 1 fs was used. The final simulation temperature of 300 K was reached by heating in steps of 50 K starting at 1 or 10 K with typically 3 ps equilibration at each temperature followed by 300 ps of productive MD simulation at 300 K. Several trajectories with slight differences in the heating protocol were calculated for both FIS models. The simulation data for the wild-type and mutant FIS proteins were compared with respect to conformational rearrangements and changes in distances between interacting side-chains, especially those changes indicating broken or newly formed hydrogen bonds.

## RESULTS

### Isolation of a *fis* mutant that inhibits the activity of Gin M114V

To design a test system for screening *fis* mutants affected in their interaction with Gin we made use of the FIS-independent *gin* mutant M114V which still responds to FIS (31,32). The rationale of this approach was that, provided the contact site for FIS is present, it should be possible to isolate *fis* mutants which inhibit, rather than stimulate recombination. To establish an assay for inversion the *lacZ* gene flanked by inversely oriented *gix* sites was introduced into the chromosome of the *E.coli* strain CSH50 *fis*::Kan in 'off' orientation with respect to the P<sub>Cm</sub> promoter. In addition, this strain AD1 also carries a *lacI<sup>q</sup>* repressor gene on its chromosome (see Materials and Methods for details). A productive inversion event places the gene in 'on' orientation under the control of the P<sub>Cm</sub> promoter allowing expression of  $\beta$ -galactosidase. Afterwards a pool of plasmids containing the mutagenised *fis* gene under the control of an IPTG-inducible promoter (pAD*fis*\*) was introduced into AD1. Cells carrying the pAD*fis*\* plasmids were then transformed with plasmid pMD3*gin* M114V carrying *gin* M114V under the control of the  $\lambda$ P<sub>L</sub> promoter. Cells containing pMD3*gin* M114V and pAD*fis* invert their *lacZ* gene efficiently and form dark blue colonies on X-gal indicator plates (Table 3). Cells containing inhibitory *fis* mutants were expected

to remain white or light blue. Out of 750 000 screened colonies from the mutant pool only one showed this phenotype. The phenotype was confirmed by retransformation of the pADfis\* plasmid. Sequencing revealed that the isolated *fis* mutant carried three mutations leading to amino acid substitutions at positions 25 (K25E), 66 (V66A) and 67 (M67T).

**Table 3.** DNA inversion activity in the presence of mutant FIS proteins *in vivo*

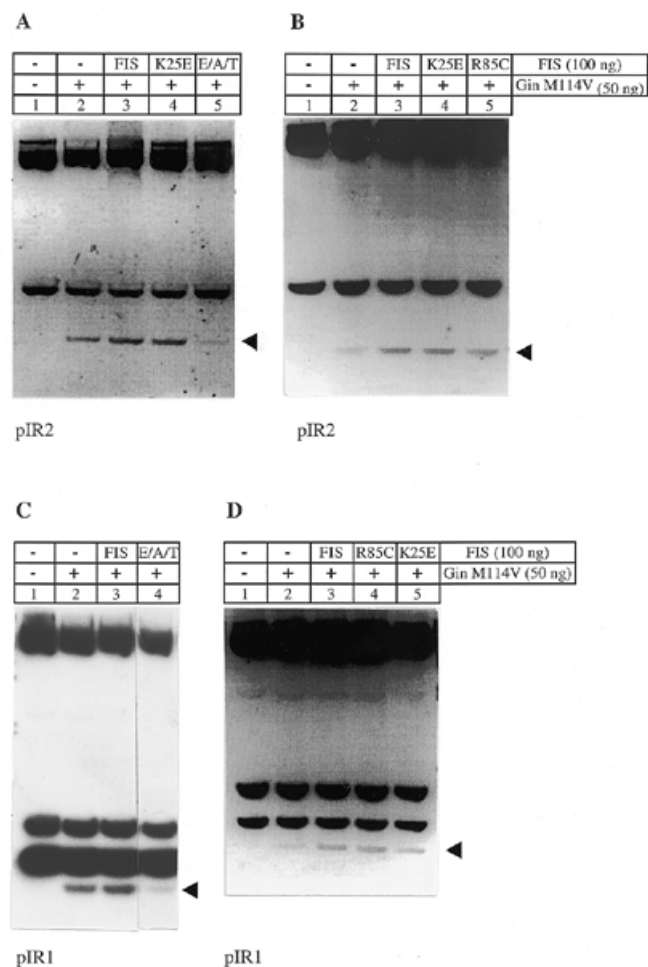
	Gin M114V (36°C, 24 h)	Gin wt (36°C, 36 h)
Without FIS	+++	-
FIS wt	+++	+++
FIS K25E	+++	++
FIS V66A	+++	+++
FIS M67T	+++	+++
FIS K25E/V66A	+++	++
FIS K25E/M67T	+++	++
FIS V66A/M67T	+++	+++
FIS K25E/V66A/M67T	(+)	+
FIS R85C	+++	+

Recombinational activity was determined as described in Materials and Methods. -, No activity detectable; (+), barely detectable activity; +, low activity; ++, intermediate activity; +++, full activity.

To investigate which of these mutations in *fis* K25E/V66A/M67T were necessary to cause the inhibition of Gin M114V, individual mutations were separated and tested alone and also in pairwise combinations. We found that all three mutations are required to observe inhibition of recombination by Gin M114V (Table 3). The inhibitory effect was not specific for *gin* M114V; DNA inversion by FIS-independent mutants *gin* M114I and *gin* M114L was also inhibited in the presence of *fis* K25E/V66A/M67T (data not shown). Furthermore, *fis* K25E/V66A/M67T was able to stimulate inversion by wild-type *gin*, albeit weakly (Table 3).

#### Effect of FIS K25E/V66A/M67T on DNA recombination *in vitro*

We next investigated whether the inhibitory effect of FIS K25E/V66A/M67T on inversion catalysed by Gin M114V *in vivo* can also be observed *in vitro*. Using the supercoiled inversion DNA substrate pIR2 containing the enhancer, we observed that purified FIS K25E/V66A/M67T, but not the mutant protein FIS K25E, inhibited inversion by Gin M114V (Fig. 1A). Unexpectedly FIS K25E/V66A/M67T also inhibited inversion when plasmid pIR1 lacking the enhancer was used as a substrate (Fig. 1C). This result demonstrates that the inhibitory effect of FIS K25E/V66A/M67T on recombination by Gin M114V does not depend on FIS binding to the enhancer sequence. To test the possibility that FIS can affect the recombination reaction without binding to the enhancer we used the previously characterised DNA binding deficient mutant FIS R85C which carries a mutation in the recognition helix of the HTH motif (19,20) and tested its effect on inversion both *in vivo* and *in vitro*. Under the *in vivo* conditions used here, where FIS is strongly overproduced, we observe a weak stimulatory effect of FIS R85C on inversion frequency by wild-type Gin (Table 3). This weak stimulatory effect was not detected previously, presumably because a different



**Figure 1.** *In vitro* DNA inversion mediated by Gin M114V in the presence of mutant FIS proteins. E/A/T indicates FIS K25E/V66A/M67T and R85C is FIS R85C. DNA fragments generated by inversion are indicated by an arrow. (A and B) The supercoiled DNA inversion substrate pIR2 was incubated with Gin M114V and mutant FIS proteins as indicated. (A) Lane 1, free DNA. Wild-type FIS and FIS K25E stimulate the inversion by Gin M114V (compare lane 2 with lanes 3 and 4), whereas FIS K25E/V66A/M67T inhibits recombination by Gin M114V (compare lanes 2 and 5). (B) Lane 1, free DNA. FIS R85C stimulates the inversion by Gin M114V similar to wild-type FIS and FIS K25E (compare lane 2 with lanes 3, 4 and 5). (C and D) The supercoiled DNA inversion substrate pIR1 lacking the recombinational enhancer was used. (C) Lane 1, free DNA. Wild-type FIS weakly stimulates recombination (compare lanes 2 and 3) whereas FIS K25E/V66A/M67T inhibits inversion by Gin M114V (lane 4). (D) Lane 1, free DNA. FIS R85C weakly stimulates recombination (compare lanes 2 and 4) and FIS K25E exerts no noticeable effect (compare lanes 2 and 5). The plasmid DNA was restricted with *Bam*HI and *Eco*RI and analysed on a 2% agarose gel. The DNA fragments in (C) were visualised by Southern blotting and autoradiography, whereas (A), (B) and (D) represent scans of ethidium bromide stained gels.

expression vector and less sensitive screening procedures were used (19,20). Subsequent *in vitro* analysis confirmed that FIS R85C slightly stimulates DNA inversion and that this effect is enhancer independent (Fig. 1B and D).

#### Molecular dynamics simulations

The need for three distinct amino acid substitutions to observe the inhibitory effect of FIS K25E/V66A/M67T suggested a cooperative effect of the mutations. To gain insight into the possible alterations

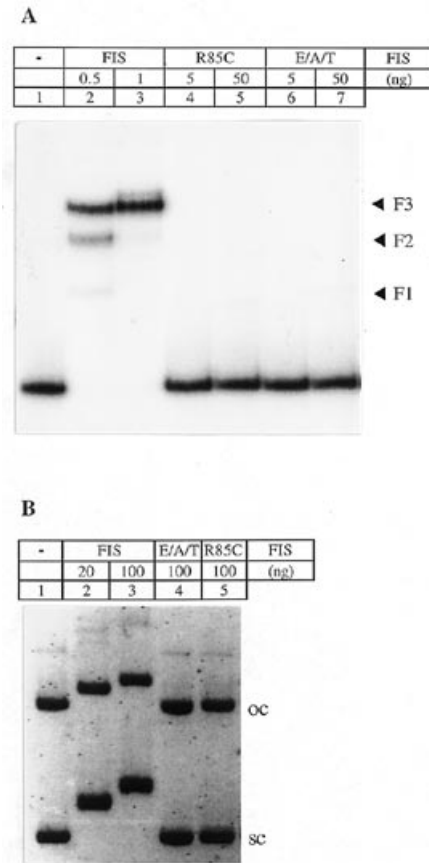
caused by the substitutions comparative molecular dynamics (MD) simulations of wild-type and mutant FIS proteins were performed. Since lysine 25 in the N-terminus of FIS is the last residue resolved by crystallographic analysis (16–18) the mutation K25E could not be included in calculations. The mutations V66A and M67T are both located in the  $\alpha$ B helix and affect amino acids which are important both for the structure of the monomer and for the dimerization of FIS. Valine 66 is situated in the dimerization surface of the monomers and interacts with a hydrophobic cleft formed between the  $\alpha$ A and  $\alpha$ B helices of the opposite monomer. These interactions involve van der Waals contacts between amino acid side chains without participation of water molecules. The substitution of the smaller alanine for valine at this position introduces a gap in the hydrophobic core of the protein that, as indicated by the MD simulations, causes either compaction or destabilisation of the FIS dimer. The threonine/methionine substitution at position 67 (M67T) could allow the formation of hydrogen bonds between the threonine hydroxyl group to carbonyl groups of neighbouring amino acids (either Leu63 or Asp64). These alternate additional hydrogen bonds would increase the stiffness of the  $\alpha$ B helix. In addition the simulations indicate that Thr67 could form a hydrogen bond to the last C-terminal amino acid of FIS (Asn98) creating an additional bond between the  $\alpha$ B and  $\alpha$ D helices. This could restrict the flexibility of the HTH domain. Since the HTH domains in FIS are separated by significantly less than 34 Å (the distance from one major groove to the next along the helix axis) (16,17), binding of FIS to two adjacent major grooves is likely to require a certain flexibility of the HTH domains. Thus, the mutations V66A and M67T could potentially alter both the dimerization and DNA-binding properties of FIS K25E/V66A/M67T.

### DNA binding and dimerization properties of FIS K25E/V66A/M67T

To test the predictions of molecular modelling we carried out DNA binding studies with the purified mutant protein FIS K25E/V66A/M67T by using different DNA substrates. DNA binding was analysed by gel-retardation assay (54). First we used a radiolabeled 190 bp DNA fragment containing the *Gin* recombinational enhancer with three specific FIS binding sites. While wild-type FIS formed three distinct complexes with this fragment in a concentration-dependent manner, neither the DNA binding deficient FIS mutant R85C nor FIS K25E/V66A/M67T bound this fragment even if used in 100-fold excess over the concentration at which three complexes were formed by wild-type FIS (Fig. 2A). Thus, although none of the three mutations in FIS K25E/V66A/M67T is located in the DNA binding domain it appears that these mutations in combination strongly impair the binding of mutant FIS protein to DNA.

We then tested the binding of FIS K25E/V66A/M67T to pIR2, a supercoiled DNA inversion substrate containing the recombinational enhancer. Even at high concentrations neither FIS K25E/V66A/M67T nor FIS R85C were able to bind the supercoiled DNA substrate, whereas wild-type FIS retarded the migration of supercoiled DNA in the gel in a concentration-dependent manner (Fig. 2B).

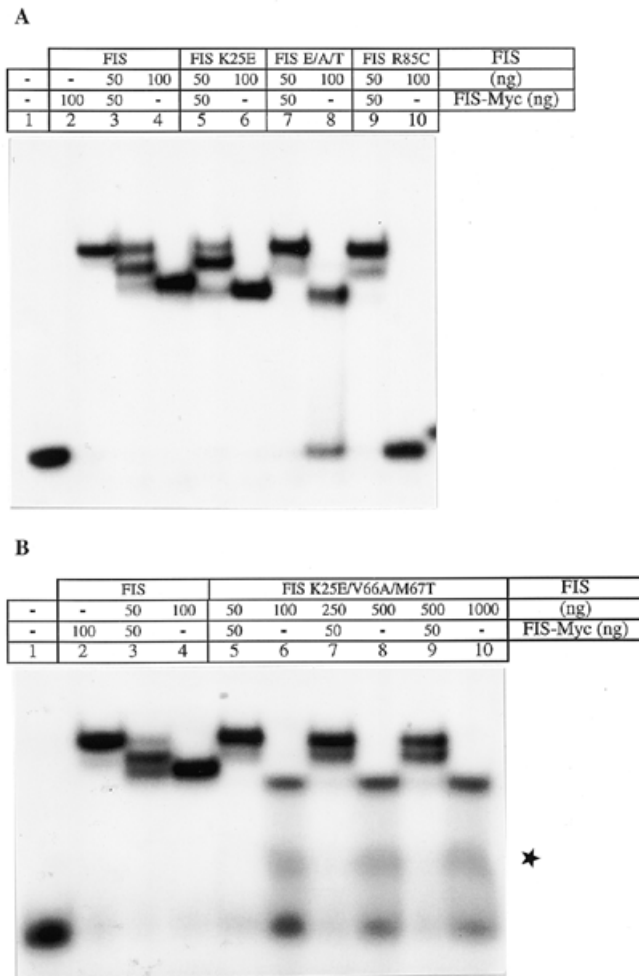
Nevertheless, FIS K25E/V66A/M67T showed weak binding to a 30 bp DNA fragment containing the FIS binding site III from the upstream activating sequence (UAS) of the *tyrT* promoter (56) (Fig. 3A, lane 8). However, FIS K25E/V66A/M67T was



**Figure 2.** Analysis of binding of mutant FIS proteins to DNA containing the *Mu* recombinational enhancer. The proteins are denoted as in the legend to Figure 1. (A) The indicated concentrations of FIS proteins were incubated with the 190 bp  $^{32}$ P-end-labeled enhancer fragment. In lane 1 free DNA is displayed. Wild-type FIS forms three specific complexes, F1, F2 and F3, corresponding to sequential occupation of three binding sites in the enhancer. The complexes were separated on a 5% native acrylamide gel and visualized by autoradiography. (B) Binding of FIS to the supercoiled DNA inversion substrate pIR2. The denotations are as in (A). Lane 1, free DNA. The open circular form (oc) as well as supercoiled plasmid (sc) are indicated. The complexes were separated on a 1% agarose gel and visualized by ethidium bromide staining. A scan of an ethidium bromide stained gel is shown.

unable to bind the very same site when it was located on a longer (200 bp) DNA fragment containing the whole UAS region (data not shown). This indicates that binding of FIS K25E/V66A/M67T to this particular site is affected by sequence context.

We used the capacity of FIS K25E/V66A/M67T to bind the 30 bp fragment to test the dimerization properties of this mutant protein. For this purpose we established a novel assay for dimerization which relies on the property of FIS monomers to renature and fully regain DNA binding capacity after heat denaturation. To conduct the assay the FIS protein of interest is mixed with an equal amount of wild-type FIS carrying an 11 amino acid extension at the N-terminus which does not interfere with its biological activity (c-Myc tag, see Materials and Methods). After heat denaturation the proteins were renatured and were then assayed for binding to the radiolabeled 30 bp *tyrT* DNA fragment. When wild-type FIS is mixed with FIS-Myc three complexes with different mobilities are observed in the gel. The upper and lower complexes represent DNA bound by the homodimeric



**Figure 3.** Dimer formation between FIS-Myc and different FIS proteins. The preformed 'heterodimers' (see text for details) were mixed with the  $^{32}\text{P}$ -end-labeled 30 bp fragment containing FIS site III from *tyrT* UAS. Protein concentrations are indicated and the notations are as in the legend to Figure 1. The complexes were separated on a 10% neutral polyacrylamide gel and visualised by autoradiography. (A) Lane 1, free DNA. The mobility of both FIS K25E homodimers and FIS K25E/FIS-Myc 'heterodimers' differs from that of wild-type FIS, presumably due to the differences in protein net charge. (B) Lane 1, free DNA. Note that FIS K25E/V66A/M67T forms 'heterodimers' only when used in high excess relative to FIS-Myc. The putative FIS K25E/V66A/M67T monomer-DNA complexes are indicated by an asterisk.

species and the middle complex is formed by the FIS/FIS-Myc 'heterodimers' (Fig. 3A, lanes 2-4). The 'heterodimeric' complex is ~2-fold more abundant than the two homodimeric complexes, as expected from the molar ratios of the two proteins used. The mutant K25E carrying only the N-terminal substitution (but having an intact DNA binding domain) was capable of 'heterodimer' formation with an efficiency comparable to that of wild-type FIS (Fig. 3A, lanes 5 and 6). When FIS R85C is mixed with FIS-Myc we observe the absence of the lower molecular weight complex and only a low level of 'heterodimeric' complexes. This indicates that FIS R85C is impaired in formation of DNA binding proficient 'heterodimers' with FIS-Myc (Fig. 3A, lanes 9 and 10) presumably because the presence of only one intact DNA binding domain in these 'heterodimers' strongly affects stable binding. In comparison to FIS R85C the FIS

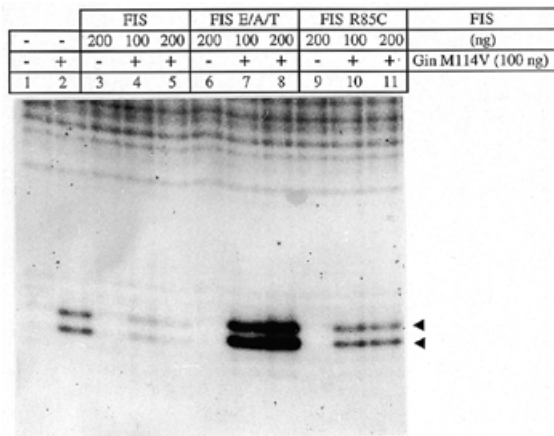
K25E/V66A/M67T homodimer binds the 30 bp DNA fragment reasonably well (Fig. 3A, compare lanes 8 and 10). Nevertheless, at equal protein ratios the DNA complexes containing FIS K25E/V66A/M67T-FIS Myc 'heterodimers' did not form more efficiently than with FIS R85C-FIS-Myc 'heterodimers' (Fig. 3A, compare lanes 7 and 9). A titration experiment using different concentrations of FIS K25E/V66A/M67T in the presence of a fixed amount of FIS-Myc showed that only when the concentration of FIS K25E/V66A/M67T was raised relative to FIS-Myc an increase in the amount of the 'heterodimeric' complex (and also the homodimeric FIS K25E/V66A/M67T complex) became visible (Fig. 3B, lanes 7 and 9). This could reflect an impaired ability of dimer formation between FIS K25E/V66A/M67T and FIS-Myc. Alternatively the monomers in these 'heterodimers' might be arranged in a way which precludes efficient DNA binding. In addition we observed a faster migrating complex when FIS K25E/V66A/M67T was used alone (Fig. 3B, lanes 6, 8 and 10) which suggests that a significant amount of monomeric molecules is present in FIS K25E/V66A/M67T preparations. Taken together these results are consistent with the molecular modelling data and imply that the triple mutant has suffered a conformational alteration, most probably at the dimerization interface, which affects its ability to bind DNA. Since the mutation in the N-terminus (K25E) does not significantly impair either the DNA binding or the dimerization properties of FIS (Fig. 3A, lanes 5 and 6), the substitutions in the  $\alpha\text{B}$  helix of FIS (V66A/M67T) must be responsible for this effect.

#### Effect of FIS K25E/V66A/M67T on the DNA cleavage activity of Gin M114V

Next we addressed the question which step in the recombination reaction is affected by FIS K25E/V66A/M67T. Gin M114V is known to efficiently cleave linear DNA fragments containing a *gix* site (37,50,57). We therefore analysed the cleavage of the 44 bp DNA fragment containing the 34 bp *gix* sequence by Gin M114V in the presence of mutant FIS proteins. As expected Gin M114V alone cleaved the 44 bp *gix* sequence within the 2 bp spacer region (Fig. 4, lane 2). In the presence of wild-type FIS cleavage by Gin M114V was noticeably reduced (Fig. 4, compare lane 2 with lanes 4 and 5) whereas the DNA binding deficient protein FIS R85C exerted no inhibitory effect (Fig. 4, lanes 10 and 11). Unexpectedly, addition of FIS K25E/V66A/M67T dramatically increased the cleavage by Gin M114V (Fig. 4A, lanes 7 and 8). The observation that FIS K25E/V66A/M67T activates cleavage by Gin M114V suggests the existence of direct contacts between FIS and the Gin-*gix* complex leading to an accumulation of cleaved *gix* sites.

#### DISCUSSION

Using a genetic screen we have isolated a triple *fis* mutant which inhibits recombination catalysed by Gin M114V in an enhancer-independent manner. This mutant FIS protein proved to be impaired in its ability to bind the enhancer sequence which suggests that the inhibitory effect on recombination is mediated by direct contacts between FIS and the Gin-*gix* complex. In this context it is interesting that a recent report describes mutant forms of the enhancer-binding protein NtrC which have lost their ability to bind the enhancer but retain residual capacity to activate transcription (58). It is thus likely that in the DNA inversion reaction FIS functions both as an architectural factor responsible



**Figure 4.** Cleavage of linear *gix* fragments by Gin M114V in the presence of FIS proteins. The proteins are denoted as in the legend to Figure 1. The <sup>32</sup>P-end-labeled 44 bp DNA fragment containing the 34 bp *gix* site was incubated with Gin M114V and FIS proteins as indicated and after digestion with proteinase K (45 min, 45°C) separated on a 10% denaturing polyacrylamide gel. The two products resulting from 2 bp staggered cleavage within the crossover region (indicated by arrows) were visualized by autoradiography.

for the proper shaping of the DNA through binding to the enhancer sequence and also as an activator of the catalytic function of invertase.

#### The mechanism of recombination inhibition by FIS K25E/V66A/M67T

The inhibitory effect of FIS K25E/V66A/M67T was specifically observed in the reactions catalysed by FIS-independent Gin mutants but not with wild-type Gin protein. On the contrary, in the *in vivo* experiments we observe a weak stimulation of wild-type Gin by FIS K25E/V66A/M67T comparable to the effect of the DNA binding deficient FIS mutant R85C. It has been shown previously that recombination by wild-type Gin and FIS proceeds exclusively through the -2 synaptic complex (21,29,35). Although FIS and enhancer are required for the formation of this complex (29), such complexes also assemble in the absence of FIS, although with low efficiency (35). The observation that FIS K25E/V66A/M67T as well as the DNA binding deficient FIS mutant protein R85C weakly stimulate the inversion reaction by wild-type Gin suggests a separate effect of FIS on the inversion reaction which is independent of binding and bending of the enhancer. We propose that it is the low proportion of formed -2 synaptic complexes which become activated by FIS R85C and FIS K25E/V66A/M67T, since only in these complexes the requirement for the architectural function of FIS and enhancer is bypassed.

The mutation M114V is located in the  $\alpha$  helix of Gin which, when modelled on the crystal structure of the closely related  $\gamma\delta$  resolvase, constitutes the dimer interface in solution (59,60). The FIS-independent phenotype of mutant Gin M114V is associated with a conformational change of the Gin-*gix* complex which facilitates the unwinding of DNA within the crossover sites and strand cleavage (32). Unlike wild-type Gin, which cleaves linear substrates inefficiently (50), Gin M114V can efficiently cleave linear *gix* sites presumably because the conformational change activates the cleavage function without the need for a proper synapse

(32). We have shown in this study that FIS K25E/V66A/M67T further enhances the cleavage by Gin M114V, whereas it does not significantly affect the cleavage of linear *gix* sites by wild-type Gin dimer (A.Deufel, unpublished data). Thus the structural alteration present in Gin M114V is required for the cleavage enhancement by FIS K25E/V66A/M67T. In contrast to Gin M114V efficient cleavage by wild-type Gin requires the complete synaptic complex (including the enhancer and FIS) (32). Moreover, for productive recombination both strands of recombination sites must be cleaved in concert by DNA resolvases and invertases (61,62). It is conceivable that in the synapse FIS adopts a specific 'active' conformation required for the stimulation of strand cleavage by Gin and that the conformation of FIS K25E/V66A/M67T mimics this active state. The ability of Gin M114V to cleave DNA when bound to a single *gix* site in conjunction with the specific structural alteration present in FIS K25E/V66A/M67T may thus imitate the interactions between wild-type FIS and Gin in the synapse. To explain how the enhanced cleavage activity observed on linear *gix* sites relates to the inhibitory effect of FIS K25E/V66A/M67T on DNA inversion by Gin M114V we assume that the accumulation of cleaved *gix* sites counteracts the coordinated cleavage steps required for recombination.

At the present stage we cannot discriminate whether FIS K25E/V66A/M67T enhances DNA cleavage by directly contacting the invertase, or exerts its effect via interactions with the *gix* site DNA. It is possible that K25E/V66A/M67T oscillates rapidly between DNA bound and unbound states and this could affect the cleavage function of the Gin M114V-*gix* complex. Alternatively, the observed effects could be caused by direct protein-protein interactions between FIS and Gin. This possibility is supported by our finding that the DNA-binding deficient mutant FIS R85C is able to stimulate inversion in the absence of the enhancer *in vitro*. It is thus likely that also the effect of FIS K25E/V66A/M67T is, at least in part, mediated via direct protein contacts with Gin. We note that activation of transcription by FIS also involves both DNA-mediated effects and direct protein interactions between FIS and RNA polymerase (63). The dual function of FIS in DNA inversion has been suggested also by the ability of the double mutant Gin M114V/G44E to catalyze inversion in the absence of the enhancer (34). However, these authors could not exclude the possibility that in the absence of the enhancer FIS stimulated inversion by occupying fortuitous sites in DNA (31).

We also show in this study that in contrast to FIS K25E/V66A/M67T neither wild-type FIS nor FIS R85C increase the cleavage of linear *gix* fragments by Gin M114V. This is at variance with the stimulatory effect of wild-type FIS on cleavage of supercoiled DNA inversion substrates by Gin (20). One possible explanation for this discrepancy is that wild-type FIS stabilises Gin M114V dimer bound at the 44 bp *gix* fragment in a conformation which prevents the activation of cleavage function. Alternatively, the Gin M114V dimer may not be competent to respond with enhanced cleavage to direct protein interactions either with wild-type FIS or FIS R85C but may acquire this property during synapse on a negatively supercoiled DNA molecule.

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## REFERENCES

- 1 Kahmann,R., Rudt,F., Koch,C. and Mertens,G. (1985) *Cell*, **41**, 771–780.
- 2 Johnson,R.C. and Simon,M.I. (1985) *Cell*, **41**, 781–791.
- 3 Huber,H.E., Iida,S., Arber,W. and Bickle,T.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3776–3780.
- 4 van de Putte,P. and Goosen,N. (1992) *Trends Genet.*, **8**, 457–462.
- 5 Nilsson,L., Vanet,A., Vijgenboom,E. and Bosch, L. (1990) *EMBO J.*, **9**, 727–734.
- 6 Ross,W., Thompson,J.F., Newlands,J.T. and Gourse,R.L. (1990) *EMBO J.*, **9**, 3733–3742.
- 7 Gille,H., Egan,J.B., Roth,A. and Messer,W. (1991) *Nucleic Acids Res.*, **19**, 4167–4172.
- 8 Cassler,M.R., Grimwade,J.E. and Leonard,A.C. (1995) *EMBO J.*, **14**, 5833–5841.
- 9 Weinreich,M.D. and Reznikoff,W.S. (1992) *J. Bacteriol.*, **174**, 4530–4537.
- 10 van Drunen,C.M., van Zuylen,C., Mientjes,E.J., Goosen,N. and van de Putte,P. (1993) *Mol. Microbiol.*, **10**, 293–298.
- 11 Thompson,J.F., Moitoso de Vargas,L., Koch,C., Kahmann,R. and Landy,A. (1987) *Cell*, **50**, 901–908.
- 12 Ball,C.A. and Johnson,R.C. (1991) *J. Bacteriol.*, **173**, 4032–4038.
- 13 Betermier,M., Poquet,I., Alazard,R. and Chandler,M. (1993) *J. Bacteriol.*, **175**, 3798–3811.
- 14 Johnson,R.C., Ball,C.A. and Simon,M.I. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 3484–3488.
- 15 Koch,C., Vanderkerckhove,J. and Kahmann,R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 4237–4241.
- 16 Kostrewa,D., Granzin,J., Koch,C., Choe,H.-W., Raghunathan,S., Wolf,W., Labahn,J., Kahmann,R. and Saenger,W. (1991) *Nature*, **349**, 178–180.
- 17 Yuan,H.S., Finkel,S.E., Feng,J.-A., Kraczor-Grzeskowiak,M., Johnson,R.C. and Dickerson,E. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9558–9562.
- 18 Kostrewa,D., Granzin,J., Stock,D., Choe,H.-W., Labahn,J. and Saenger,W. (1992) *Mol. Biol.*, **226**, 209–226.
- 19 Osuna,R., Finkel,S.E. and Johnson,R.C. (1991) *EMBO J.*, **10**, 1593–1603.
- 20 Koch,C., Ninnemann,O., Fuss,H. and Kahmann,R. (1991) *Nucleic Acids Res.*, **19**, 5915–5922.
- 21 Kanaar,R., van de Putte,P. and Cozzarelli,N.R. (1989) *Cell*, **58**, 147–159.
- 22 Heichmann,K.A. and Johnson,R.C. (1990) *Science*, **249**, 511–517.
- 23 Bruist,M.F., Glasgow,A.C., Johnson,R.C. and Simon,M.I. (1987) *Genes Dev.*, **1**, 762–772.
- 24 Kahmann,R., Mertens,G., Klippel,A., Brauer,B., Rudt,F., and Koch,C. (1987) In McMacken,R. and Kelly,T.J. (eds), *DNA Replication and Recombination, UCLA Symposia on Molecular and Cellular Biology*, New series, **47**, Alan R. Liss, NY, pp. 681–690.
- 25 Thompson,J.F. and Landy,A. (1988) *Nucleic Acids Res.*, **16**, 9687–9705.
- 26 Hübner,P., Haffter,P., Iida,S. and Arber,W. (1989) *J. Mol. Biol.*, **205**, 493–500.
- 27 Pan,C.Q., Feng,J.-A., Finkel,S.E., Landgraf,R., Sigman,D. and Johnson,R.C. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 1721–1725.
- 28 Pan,C.Q., Finkel,S.E., Crampton,S.E., Feng,J.-A., Sigman,D.S. and Johnson,R.C. (1996) *J. Mol. Biol.*, **264**, 675–695.
- 29 Kanaar,R., Klippel,A., Shekhtman,E., Dungan,J.M., Kahmann,R. and Cozzarelli,N.R. (1990) *Cell*, **62**, 353–366.
- 30 Heichmann,K.A., Moskowitz,I.P.G. and Johnson,R.C. (1991) *Genes Dev.*, **5**, 1622–1634.
- 31 Klippel,A., Cloppenburg,K. and Kahmann,R. (1988b) *EMBO J.*, **7**, 3983–3989.
- 32 Klippel,A., Kanaar,R., Kahmann,R. and Cozzarelli,N.R. (1993) *EMBO J.*, **12**, 1047–1057.
- 33 Haykinson,M.J., Johnson,L.M., Soong,J. and Johnson,R.C. (1996) *Curr. Biol.*, **6**, 163–176.
- 34 Spaeny-Dekking,E.H.A. (1995) Ph.D. thesis, Leiden University, The Netherlands.
- 35 Crisona,N.J., Kanaar,R., Gonzalez,T.N., Zechiedrich,E.L., Klippel,A. and Cozzarelli,N.R. (1994) *J. Mol. Biol.*, **243**, 437–457.
- 36 Gonzalez-Gil,G., Bringmann,P. and Kahmann,R. (1996) *Mol. Microbiol.*, **22**, 21–29.
- 37 Klippel,A., Mertens,G., Patschinsky,T. and Kahmann,R. (1988) *EMBO J.*, **7**, 1229–1237.
- 38 Mertens,G., Klippel,A., Fuss,H., Blöcker,H., Frank,R. and Kahmann,R. (1988) *EMBO J.*, **7**, 1219–1227.
- 39 Tabor,S. and Richardson,C.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1074–1078.
- 40 Hamilton,C.M., Aldea,M., Washburn,B.K., Babitzke,P. and Kushner,S.R. (1989) *J. Bacteriol.*, **171**, 4617–4622.
- 41 Brosius,J. (1989) *DNA*, **8**, 759–777.
- 42 Oka,A., Sugisaki,H. and Takunami,M. (1981) *J. Mol. Biol.*, **147**, 217–226.
- 43 Vieira,J. and Messing,J. (1982) *Gene*, **19**, 259–268.
- 44 Bujard,H., Gentz,R., Lancer,M., Stüber,D., Müller,M., Ibrahim,I., Häuptle,M.T. and Dobberstein,B. (1987) *Methods Enzymol.*, **155**, 416–433.
- 45 Lancer,M. and Bujard,H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 8973–8977.
- 46 Bölker,M. and Kahmann,R. (1989) *EMBO J.*, **8**, 2403–2410.
- 47 Spee,J.H., de Vos,W.M. and Kuipers,O.P. (1993) *Nucleic Acids Res.*, **21**, 777–778.
- 48 Landt,O., Grunert,H.-P. and Hahn,U. (1990) *Gene*, **96**, 125–128.
- 49 Kuipers,O.P., Boot,H.J. and de Vos,W.M. (1991) *Nucleic Acids Res.*, **19**, 4558.
- 50 Merker,P., Muskhelishvili,G., Deufel,A., Rusch,K. and Kahmann,R. (1993) *Cold Spring Harbour Symposium on Quant. Biol.*, **58**, 505–513.
- 51 Southern,E. M. (1975) *J. Mol. Biol.*, **98**, 503–517.
- 52 Mertens,G., Fuss,H. and Kahmann,R. (1986) *J. Biol. Chem.*, **261**, 15668–15672.
- 53 Koch,C. and Kahmann,R. (1986) *J. Biol. Chem.*, **261**, 15673–15678.
- 54 Fried,M. and Crothers,D. (1981) *Nucleic Acids Res.*, **9**, 6505–6525.
- 55 Weiner,S.J., Kollmann,P.A., Case,D.A., Singh,U.C., Ghio,C., Alagona,G., Profeta,S. and Weiner,P. (1984) *J. Am. Chem. Soc.*, **106**, 765–784.
- 56 Lazarus,L. and Travers,A.A. (1993) *EMBO J.*, **12**, 2483–2494.
- 57 Klippel,A. (1990) Ph.D. thesis, Fachbereich Chemie, Freie Universität Berlin.
- 58 North,A.K. and Kustu,S. (1997) *J. Mol. Biol.*, **267**, 17–36.
- 59 Hughes,R.E., Hatfull,G.H., Rice,P., Steitz, T.A. and Grindley,N.D.F. (1990) *Cell*, **63**, 1331–1338.
- 60 Sanderson,M.R., Freemont,P.S., Rice,P.A., Goldman,A., Hatfull,G.F., Grindley,N.D.F. and Steitz,T.A. (1990) *Cell*, **63**, 1323–1329.
- 61 Reed,R.R. and Grindley,N.D.F. (1981) *Cell*, **25**, 721–728.
- 62 Johnson,R.C. and Bruist,M.F. (1989) *EMBO J.*, **8**, 1581–1590.
- 63 Muskhelishvili,G., Travers,A.A., Heumann,H. and Kahmann,R. (1995) *EMBO J.*, **14**, 1446–1452.