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The DNA invertase Gin of phage Mu: formation of a covalent complex with DNA via a phosphoserine at amino acid position 9

Anke Klippel, Gabriele Mertens[1,2], Tilo Patschinsky[1,3] and Regine Kahmann

Institut für Genbiologische Forschung Berlin GmbH, D-1000 Berlin 33 and [1]Max-Planck-Institut für Molekulare Genetik, Otto-Warburg-Laboratorium, D-1000 Berlin 33, FRG

[2]Present address: Robert Koch Institut des Bundesgesundheitsamtes, D-1000 Berlin 65, FRG

[3]Present address: Heinrich Pette Institut, D-2000 Hamburg 20, FRG

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The DNA invertase Gin encoded by bacteriophage Mu catalyses efficient site-specific recombination between inverted repeat sequences (IR) *in vivo* and *in vitro* in the presence of the host factor FIS and the recombinational enhancer. We demonstrate that Gin alone is able to introduce single strand breaks into duplex DNA fragments which contain the IR sequence. Strand cleavage is site-specific and can occur on either strand within the IR. Cleaved molecules contain Gin covalently attached to DNA. The covalent complex is formed through linkage of Gin to the 5′ DNA phosphate at the site of the break via a phosphoserine. Extensive site-directed mutational analysis showed that all mutants altered at serine position 9 were completely recombination deficient *in vivo* and *in vitro*. The mutant proteins bind to DNA but lack topoisomerase activity and are unable to introduce nicks. This holds true even for a conservative amino acid substitution at position 9. We conclude that serine at position 9 is part of the catalytic domain of Gin. The intriguing finding that the DNA invertase Gin has the same catalytic center as the DNA resolvases that promote deletions without recombinational enhancer and host factor FIS is discussed.

Key words: bacteriophage Mu/site-specific recombination/covalent protein-DNA complex/site-directed mutagenesis

Introduction

DNA invertases catalyse site-specific recombination events that regulate the alternate expression of genes. The best characterized systems are flagellar phase variation in *Salmonella typhimurium* and host range switching in phage Mu and P1 (Zieg *et al.*, 1977; Kamp *et al.*, 1978; van de Putte *et al.*, 1980; Iida, 1984). Each invertible DNA segment is associated with its own recombinase, the respective genes map in close proximity to the recombination sites. The DNA invertase systems are closely related which is reflected by the exchangeability of the respective functions (Zieg and Simon, 1980; Kamp and Kahmann, 1981; Hiestand-Nauer and Iida, 1983). A property unique to this group is the existence of recombinational enhancer sequences that stimulate recombination relatively independent of orientation and distance to the crossover sites (Kahmann *et al.*, 1985; Johnson and Simon, 1985; Huber *et al.*, 1985). The recombinational enhancer sequence is recognized by a 12 kd host protein termed FIS (Koch and Kahmann, 1986). To account for the distance and orientation independent function of the enhancer we presume that protein-protein interactions between the DNA invertase bound to the crossover sequences and FIS bound to the enhancer are necessary for the formation of a productive synaptic complex (see also Johnson *et al.*, 1987). The DNA invertases catalyse inversion between sequences that are arranged as inverted repeats much more efficiently than deletion events between recombination sites organized as direct repeats (Plasterk *et al.*, 1983a). A more distant relationship (~30% homology on the amino acid level) exists between the DNA invertases and resolvases encoded by the Tn*3* family (see review by Grindley *et al.*, 1985). These resolvases differ from the DNA invertases, however, in that they preferentially promote deletions and do not necessitate an enhancer and host factor for efficient recombination. For all these recombinases *in vitro* assay systems have been developed which work efficiently in the presence of a supercoiled DNA substrate and buffer containing Mg^{2+} (Reed, 1981; Johnson *et al.*, 1984; Plasterk *et al.*, 1984; Mertens *et al.*, 1984). Since no high energy cofactors are required the energy of the phosphodiester bond must be conserved during cleavage and religation. For $\gamma\delta$ resolvase it has been shown that the protein is transiently linked to DNA through a 5′ phosphate. The linkage occurs via a phosphoserine near the N-terminus at position 10 (Reed and Moser, 1984). For one of the DNA invertases, the Gin protein of phage Mu, a similar analysis has now been carried out and is the subject of this communication. The Gin protein has a mol. wt of 21.7 kd (Plasterk *et al.*, 1983b) and binds specifically to the 34 bp recombination sites that flank the G segment as inverted repeats. During the course of our Gin footprinting analysis we have observed a cleavage product slightly shifted in mobility with respect to the MPE·Fe(II) induced fragments. We attributed this fragment to Gin induced DNA cleavage between the two Gin binding sites within the IR (Mertens *et al.*, see accompanying paper). In this communication we map the cleavage position, determine its polarity and report the presence and nature of Gin-DNA linkage.

Results

The central dinucleotide within the IR is the position of Gin cleavage

The position of Gin mediated strand cleavage was determined directly by nicking tests as described in Materials and methods. When purified Gin was incubated wih 5′ end-labelled DNA fragments containing the crossing-over sites IR-L or IR-R respectively specific breaks were introduced. No such products were obtained when Gin was omitted (Figure 1A, lanes 2-6; Figure 1B, lanes 2, 3 and 10).

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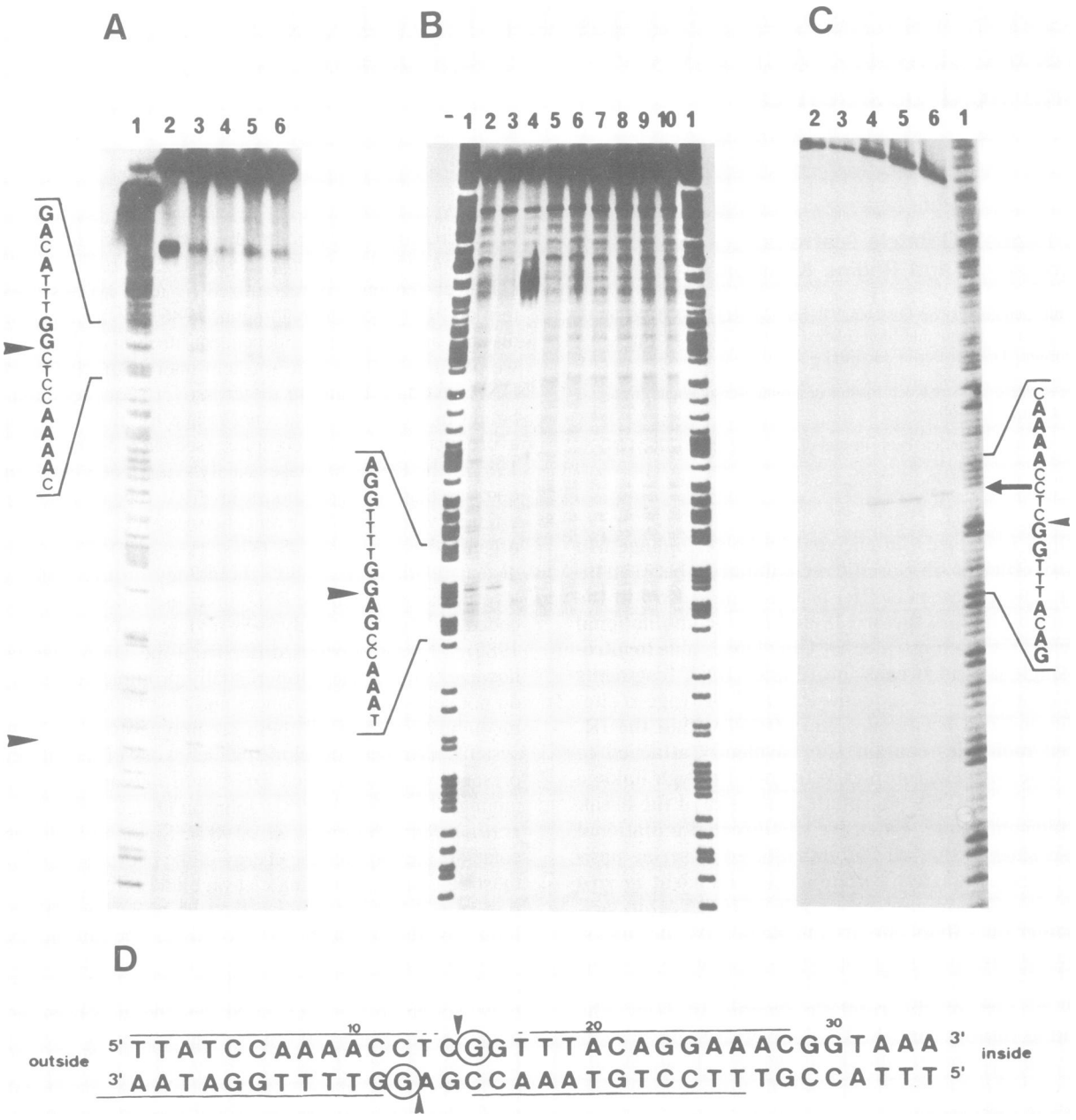

Fig. 1. Identification of position and polarity of Gin induced DNA cleavage. (**A**) Mapping of Gin induced DNA cleavage on the upper strand. The labelled 230-bp *Ssp*I fragment containing IR-R was assayed for Gin induced nicking as described in Materials and methods. **Lane 1** shows the Maxam–Gilbert G + A reaction of the 5′ labelled *Ssp*I fragment recleaved with *Bam*HI after labelling. The positions of the two Gin cleavage products are marked with arrowtips. The G + A reaction of the sequence indicated on the left corresponds to the larger of the two fragments generated by Gin cleavage of the upper strand shown in (**D**). Gin concentrations were 90 nM (**lane 2**), 30 nM (**lane 3**), 15 nM (**lane 4**), 9 nM (**lane 5**), no Gin was added (**lane 6**). The DNA concentration was 1 nM for all reactions. (**B**) Mapping of Gin induced DNA cleavage on the lower strand. The labelled 280-bp *Eco*RV–*Ssp*I fragment containing IR-L was used. **Lane 1** shows the G + A reaction of the slightly larger *Hin*dIII–*Ssp*I fragment 5′ labelled at the same *Ssp*I site as the 280-bp fragment. The Gin cleavage position is indicated by an arrowtip. The G + A sequence and the sequence indicated on the left correspond to the fragment generated through Gin cleavage of the lower strand shown in (**D**). **Lanes 2** and **3**: wild-type Gin; **lanes 4** and **5**: GinSA9; **lanes 6** and **7**: GinST9; **lanes 8** and **9**: GinT9S10. In **lane 10** protein was omitted. In lanes with even numbers the protein concentration was 20 nM, in lanes with odd numbers 10 nM. The DNA concentration was 1 nM in all reactions. The position of the second Gin cleavage product is not indicated, because it migrates in a region of high background. (**C**) Covalent attachment of Gin to 3′ labelled fragments. The 290-bp *Eco*RV–*Bam*HI fragment containing IR-L, 3′-labelled at the *Bam*HI end was incubated with Gin (**lanes 3–6**) and subsequently treated with proteinase K as described in Materials and methods. **Lane 2**: no protein was added, **lane 3**: proteinase K was omitted, **lane 4**: 10 μg/ml proteinase K, **lane 5**: 100 μg/ml proteinase K, **lane 6**: 200 μg/ml proteinase K. **Lane 1** is the Maxam–Gilbert G + A reaction of the same fragment. The expected cleavage position as determined in (**A**) is indicated by an arrowtip. Only the major cleavage product is marked with an arrow. (**D**) Summary of Gin cleavage positions within the IR sequence. The position of DNA breakage on both strands is marked with arrowtips. The G nucleotides which become attached to Gin after cleavage are shown in circles. Regions protected by Gin as determined in MPE·Fe(II) footprinting studies (see accompanying paper by Mertens *et al.*) are marked by lines.

Neither the presence of the recombinational enhancer on the fragment nor the addition of FIS to the reaction altered the cleavage pattern (data not shown). The mobility of the single-stranded products is slightly different from fragments obtained in the Maxam–Gilbert analysis. We attribute this difference to the presence of 3′-phosphate ends in fragments

generated by the Maxam–Gilbert sequencing reaction and the occurrence of 3′-hydroxyl ends in the products of Gin cleavage. Fragments carrying a free 3′-hydroxyl end migrate about one and a half nucleotides slower than respective fragments with a 3′-phosphate (Maxam and Gilbert, 1980; Reed and Grindley, 1981). Taking this into account we have determined the exact cleavage position on both DNA strands relative to the G + A Maxam–Gilbert sequencing reactions of appropriate fragments (Figure 1A,B). Gin cleavage occurs between nucleotides C and G at positions 14 and 15 of the IR on the upper strand and between position 12 (G) and 13 (A) on the lower strand as indicated in Figure 1D. We conclude further that Gin alone is sufficient to introduce nicks within both strands of the IR at comparable frequency. If the observed breaks within the IR arise in concerted fashion, a double-strand break leaving a 3′ overhang of two bases would result. To this end we analysed the reaction mixture after proteinase K treatment on non-denaturing gels. Even after prolonged exposure double-strand cleavage products could not be detected (data not shown), suggesting that the cleavage-activity of Gin on linear DNA is restricted to introducing single-strand nicks in either the upper or the lower strand of the IR.

Gin is covalently linked to the 5′ end of DNA at the position of cleavage

When Gin was assayed with fragments containing an IR labelled at one 3′ end and reaction products were analysed on a 6% denaturing gel (see Materials and methods) (Figure 1C, lane 3), no cleavage products appeared. Following proteinase K treatment one strong and several weak cleavage products were released (Figure 1C, lanes 4–6). All these fragments were larger than calculated from the cleavage position in 5′ labelled fragments. The finding that their appearance depends on proteinase K treatment suggests that Gin is covalently linked to the 5′ G at the site of the single-strand nick. The altered mobilities can be explained by assuming that one or more residual amino acids are still attached to DNA through a protease-resistant phosphoester linkage.

Gin is covalently linked to DNA via a phosphoserine

To characterize the covalent Gin–DNA complex we have isolated Gin which is specifically labelled via the covalently attached DNA. For this purpose an appropriate DNA substrate containing the IR, M13mp9-IR (for details see Materials and methods) was labelled *in vitro* with [α^{32}P]dGTP to high specific activity and incubated with Gin. The reaction mixture was subjected to *Bal*31 digestion as described by Reed and Moser (1984) and ^{32}P-labelled Gin protein was isolated after gel electrophoresis. To identify the amino acid residue involved in the covalent linkage a phosphoamino acid analysis was performed according to Bister *et al.* (1987). After two-dimensional separation together with unlabelled phosphoamino acid references, a labelled spot corresponding to phosphoserine was detected in the autoradiogram (Figure 2). The huge amount of free phosphate most likely arises by hydrolysis of nucleotides not removed by *Bal*31. The other spots observed do not migrate with any of the phosphoamino acid markers and might represent degradation products or derivatives of phosphoserine (Reed and Moser, 1984).

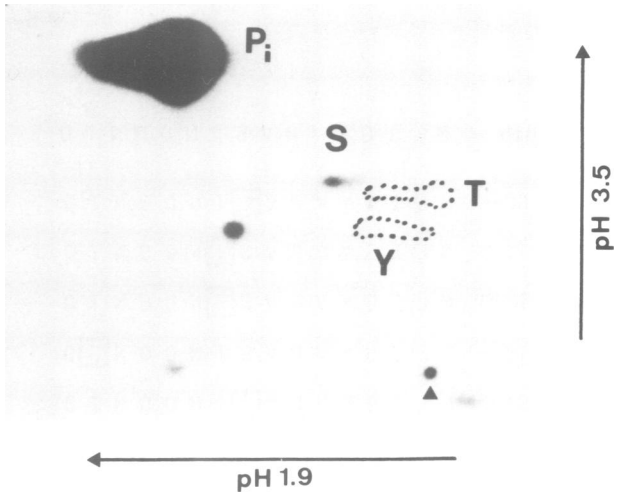

Fig. 2. Two-dimensional thin layer analysis of ^{32}P-labelled Gin–oligonucleotide complex. The experiment was carried out as described in Materials and methods. ^{32}P-labelled phosphoserine was identified after acid hydrolysis by comigration with internal standards of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y) stained with ninhydrin. The positions of unlabelled phosphoamino acids are indicated by dotted circles. P_i shows the location of free phosphate; a triangle marks the origin to which the mixture was applied.

Mutational analysis of Gin: the catalytic center

The finding that Gin is linked to DNA via a serine residue lends further support to a functional relatedness to resolvases. Gin protein contains serine at nine positions (Plasterk *et al.*, 1983b). Serine at position 9 is situated within the highly conserved N-terminal portion of invertases and resolvases (Grindley *et al.*, 1985). For γδ resolvase it has been shown by mutational analysis that Ser10 may be the catalytic site (Hatfull and Grindley, 1986). Therefore our mutational analysis concentrated on the N-terminal portion of Gin and in particular on serine at position 9. Using oligonucleotide-directed mutagenesis according to Stanssens *et al.* (in preparation) seven *gin* mutants were isolated (Figure 3). *gin* mutants were tested for their ability to catalyse recombination *in vivo* in a sensitive chromogenic assay as described in detail in Materials and methods. This experimental system has the advantage of reflecting both the recombinogenic activity and the binding capability of the respective mutant proteins. The *in vivo* assay for DNA binding revealed (see Materials and methods) that all mutant proteins still bind to the IR sequence when they are expressed in large amounts (Figure 3). All proteins altered at position 9, SL9 (Ser9 → Leu), SA9 (Ser9 → Ala), ST9 (Ser9 → Thr), T9S10 (Ser9 → Thr/Thr10 → Ser) are recombination deficient. Thr9 cannot replace Ser9, which shows that even minimal changes at this position abolish recombinational activity. The idea for construction of T9S10 was to try to compensate the deficiency of the ST9 mutant by introducing an additional mutation which exchanges Thr10 for serine (see Figure 3). However, this mutant too is recombination deficient. In the case of *gin*$^-$ mutant TI10 (Thr10 → Ile) we supposed that the large hydrophobic residue introduced by inserting isoleucine at position 10 causes structural disorders of the whole protein domain or might interfere with covalent complex formation at Ser9 through steric hindrance. To test this TS10 (Thr10 → Ser) was constructed which contains Ser

Properties of Gin - Mutants

	5 10 15	Inversion	Nicking-Activity	Binding at IR	Topoisomerase-Activity
wt Gin	M L I G Y V R V S T N D Q N T D	+++	+	+++	+
Gin SL9	M L I G Y V R V [L] T N D Q N T D	-	nt	(+)	nt
Gin TI10	M L I G Y V R V S [I] N D Q N T D	-	nt	(+)	nt
Gin TS10	M L I G Y V R V S [S] N D Q N T D	++	nt	(+)	nt
Gin TS15	M L I G Y V R V S T N D Q N [S] D	++	nt	(+)	nt
Gin SA9	M L I G Y V R V [A] T N D Q N T D	-	-	+	-
Gin ST9	M L I G Y V R V [T] T N D Q N T D	-	-	++	-
Gin T9S10	M L I G Y V R V [T S] N D Q N T D	-	-	++	-

Fig. 3. Summary of *in vivo* and *in vitro* properties of Gin mutants. The N-terminal amino acid sequence of wild-type (wt) and mutant Gin proteins is shown on the left. Amino acids exchanged with respect to wild-type Gin are boxed. Properties are shown on the right; the respective degree of activity is given in relation to values obtained for the wild-type protein. (+) indicates that DNA-binding was assayed *in vivo* as described in Materials and methods. nt: not tested.

instead of Thr at position 10. This mutant exhibited efficient inversion activity *in vivo* as does TS15 (Thr15 → Ser). Taking these data together we conclude that a Ser residue at position 9 is essential for recombinogenic activity of Gin. In a next step we have investigated which of the activities required for inversion are blocked by mutations at position 9 of Gin. Three proteins, SA9, ST9 and T9S10 harbouring conservative mutations at position 9 were purified after overproduction (see Materials and methods). Wild-type Gin protein and the three Gin mutant proteins were tested for relaxation of appropriate supercoiled DNA substrates in the presence of FIS (see Materials and methods). In contrast to wild-type Gin, mutant proteins were unable to relax DNA as tested on gels separating topoisomers (Figure 4). Since topoisomerase activity requires nicking and resealing of the ends we have analysed the mutant proteins for their ability to introduce single-strand breaks within the IR. All mutant proteins were unable to nick DNA (Figure 1B, lanes 4–9) which supports the idea that Ser9 is the centre of catalytic activity in Gin.

The binding of mutant Gin proteins to the IR in vitro

To rule out the possibility that the mutant Gin proteins are affected in binding to DNA we have investigated the binding activity of purified GinSA9, GinST9 and GinT9S10 to the IR in gel retardation experiments as described in Materials and methods. Compared to the wild-type Gin protein the binding affinity of all three mutant proteins was reduced (Figure 5). The mobility of protein–DNA complexes, however, was identical to the respective complexes observed with wild-type Gin. This indicates that the mutant proteins are folded correctly and that the complexes have the same geometry as those with wild-type Gin. The degree of binding, however, is affected by the amino acid which substitutes for Ser9. ST9 and T9S10 have nearly identical binding-affinities which were calculated to be 5×10^7/M. The average affinity of wild-type Gin protein was estimated to be 2.4×10^8/M. In contrast all preparations of SA9 exhibited a significantly decreased affinity to IR ($\approx 1 \times$

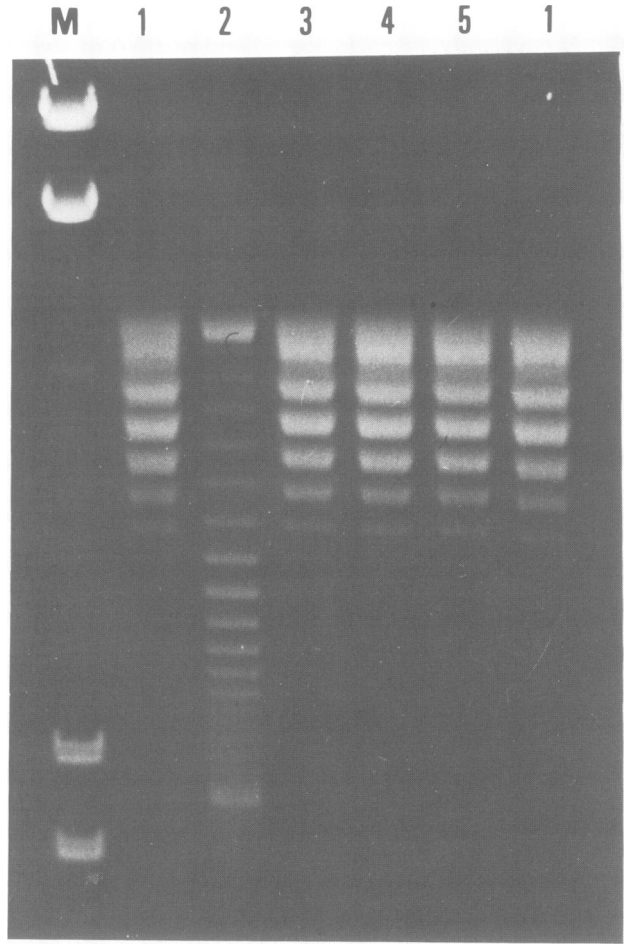

Fig. 4. Topoisomerase-activity of wild-type and mutant Gin proteins. Relaxation of pBRminiG(–) DNA was assayed under standard inversion conditions in the presence of FIS and wild-type or mutant Gin proteins. DNA was analysed on a gel containing 50 μg/ml chloroquine-phosphate as described in Materials and methods. At this chloroquine concentration relaxed forms have a higher mobility than the supercoil (Shure *et al.*, 1977). **Lane 1**: proteins were omitted, **lane 2**: wild-type Gin, **lane 3**: Gin SA9, **lane 4**: Gin ST9, **lane 5**: Gin T9S10. M is λ DNA digested with *Hin*dIII.

10^7/M). We cannot, of course, formally rule out the possibility that the inactivation of the protein happened during the purification, however, we consider this possibility unlikely since several different preparations behaved identically.

Inhibition of G inversion by mutant proteins in vitro

Since the linear fragments employed in gel retardation assay do not represent substrates for recombination we have analysed the properties of Gin mutant proteins SA9 and T9S10 in combination with wild-type Gin protein and a supercoiled recombination substrate. Purified proteins were incubated with pAK3 under standard inversion conditions. The inversion rate dropped when increasing amounts of mutant protein were added (Figure 6). Half maximal inversion was obtained at a mutant to wild-type protein ratio of ~2.5:1. In contrast to binding linear DNA fragments the two Gin mutants analysed exhibited no significant difference in inhibiting inversion by wild-type Gin protein. For a critical evaluation of these differences we refer to the Discussion.

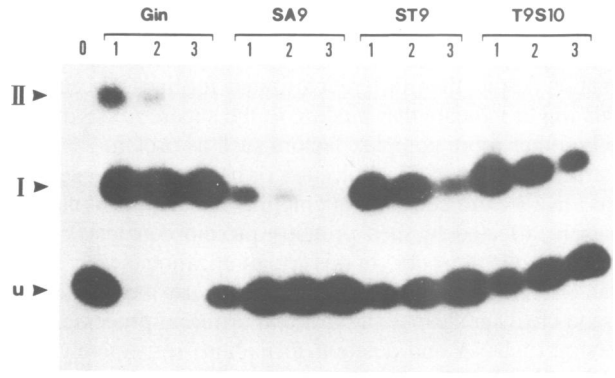

Fig. 5. Gel-retardation analysis with mutant Gin proteins. The 280-bp *Eco*RV–*Ssp*I fragment containing IR-L was incubated with Gin and mutant Gin proteins SA9, ST9 and T9S10 as indicated above the respective lanes. Complexes were analysed as described in Materials and methods and are designated I and II. The position of the unbound fragment is indicated by u. The following protein concentrations were used: 30 nM (**lanes 1**), 15 nM (**lanes 2**) and 6 nM (**lanes 3**); the DNA concentration was about 0.2 nM. **Lane 0** is a control in which protein was omitted.

Discussion

Gin introduces single-strand breaks within the IR on linear DNA fragments. The nicks on the upper and lower strand are staggered by 2 bp (Figure 1D). This mode of cleavage was predicted from an extensive genetic analysis of quasi recombination substrates for the DNA invertase Cin (Iida and Hiestand-Nauer, 1986, 1987). A free 3′ hydroxyl end is generated while Gin is covalently attached via a phosphoserine to the newly formed 5′-phosphoryl terminus. This reaction is a transesterification, a nucleophilic attack is made from the hydroxyl-group of the active site serine toward the phosphodiester bond in the DNA backbone. Gin alone is able to introduce these nicks, the presence of the enhancer on the same fragment and FIS in the reaction does not alter the observed cleavage pattern (data not shown). Nicks are introduced either on the upper or the lower strand, but breakage of the double strand has never been observed; for recombination, however, both strands have to be cut. It is possible that the artificial linear substrate employed in the Gin cleavage reaction, which is not recombinogenic, only allows the first step to occur. On the other hand the observed mode of nicking of just one strand may indicate that recombination takes place in two steps involving a Holliday intermediate (Holliday, 1964) in which only one strand of each parent has been exchanged. This structure would then be resolved by the second pair of cleavages on the second strand. On supercoiled DNA carrying two recombination sites in proper orientation the nicks within the two IR sequences would have to be introduced on opposite strands for the formation of a functional intermediate. Such a sequential type of mechanism has been proposed for Int mediated site specific recombination in phage λ (Nunes-Düby *et al.*, 1987; Nash *et al.*, 1987; Kitts and Nash, 1987). Recent experiments performed with supercoiled DNA substrates indicate that they are cleaved like linear fragments, however, the generation of single stranded nicks requires

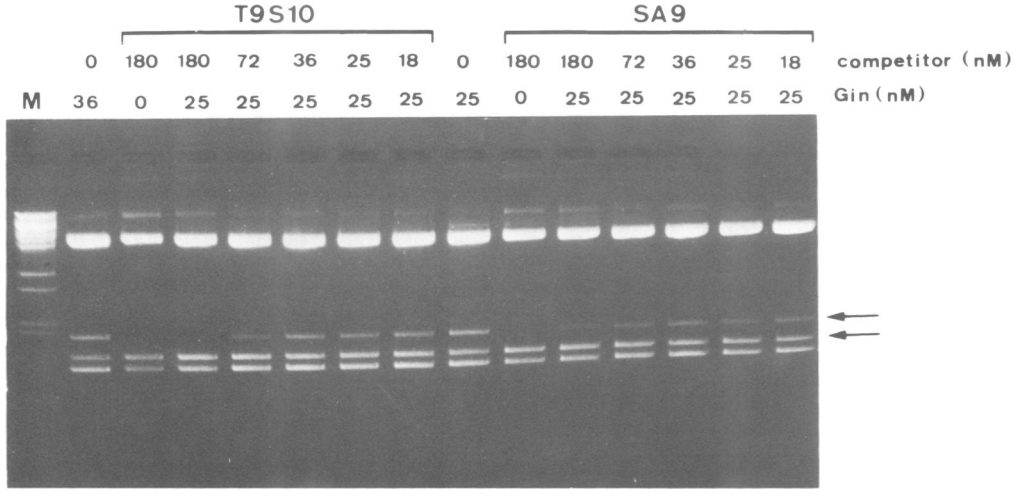

Fig. 6. Gin mediated DNA inversion in the presence of both wild-type and mutant Gin protein. pAK3 DNA was incubated with the indicated amounts of Gin and 2U FIS under standard inversion conditions. Concentrations of wild-type Gin and the respective mutant Gin proteins are shown above each line. DNA was cleaved with *Pst*I and analysed on a 2% agarose gel. Arrows mark bands that arise due to inversion. M is λ DNA cleaved with *Bst*EII.

the recombinational enhancer and FIS protein (A.Klippel, unpublished). This suggests that the enhancer and FIS might participate in the formation or selection of the proper synaptic structure in supercoiled DNA at a step that precedes nicking and recombination. It should be noted that in all experiments only a minute fraction of molecules can be isolated in nicked form as is expected for a transient intermediate.

Our results demonstrate that Gin and γδ resolvase have the same mode of cleaving DNA and both form a covalent complex with DNA via a phosphoserine. Covalent intermediates with DNA have been described for a number of enzymes (for review see Sadowski, 1986; Maxwell and Gellert, 1986). The phosphoserine attachment to DNA distinguishes invertases and resolvases from all other recombinases that have been analysed in this respect. For topoisomerases, λ integrase and the yeast FLP enzyme the reaction involves a phosphotyrosine linked intermediate. The tyrosine phosphoester bond has a relatively high free energy of hydrolysis compared to the aliphatic phosphoesters, phosphoserine and phosphothreonine (Holzer and Wohlhueter, 1972), which may indicate that different mechanisms are used for conserving the energy of the broken phosphodiester bonds. A mutational analysis has revealed that Ser at position 9 is essential for recombination. The amino acids neighbouring Ser9 on either side are highly conserved among DNA invertases and resolvases (Grindley *et al.*, 1985) suggesting the need for a special environment to expose the catalytic site. This is supported by two mutations in Thr10. When Thr10 is replaced by Ile which introduces a large hydrophobic residue a *gin*$^-$ mutant is generated whereas changing Thr10 to Ser does not affect the recombinogenic properties of Gin nor does a Thr15 to Ser change (Figure 3). Since the free energy of hydrolysis for both phosphoserine and phosphothreonine is comparable (Holzer and Wohlhueter, 1972) we anticipated that a Ser to Thr exchange at position 9 of Gin might result in a mutant protein retaining at least nicking activity. This, however, is clearly not the case. The analysis was extended even further by changing the order of Ser9 Thr10 to Thr9 Ser10 in mutant T9S10. This mutant, again, is unable to nick the IR which provides the most compelling evidence that a serine at position 9 is required for nicking and covalent complex formation. All Gin mutants analysed do not abolish binding of Gin to the IR sequences and form protein−DNA complexes that migrate like wild-type Gin−DNA complexes. This suggests that the mutations do not affect the correct folding of the protein or specific recognition of the IR. The DNA binding domains for resolvases and the Hin DNA invertase have been mapped to the C-terminal portions of the respective proteins by demonstrating that proteolytic or synthetic C-terminal fragments bind to DNA specifically (Abdel-Meguid *et al.*, 1984; Bruist *et al.*, 1987). For Gin the same conclusion has been reached by constructing a hybrid protein between Gin and Pin and analysing its inversion specificity (Plasterk and van de Putte, 1984). Our DNA binding data for different Ser9 mutants show that these mutant proteins have a lower affinity to linear DNA fragments than wild-type Gin. Ala at position 9 affects DNA binding more severely than Thr or the exchange Ser9Thr10 to Thr9Ser10 (Figure 5). This suggests that the catalytic site of Gin contributes to DNA binding, most likely through formation of hydrophilic contacts. This is supported by recent footprinting experiments with mutant Gin proteins which indicate that the unprotected region between Gin binding sites I and II (see accompanying paper by Mertens *et al.*) is enlarged (A.Klippel, unpublished). In the competition assay for recombination (Figure 6) both GinSA9 and GinT9S10 proteins inhibit DNA inversion by wild-type Gin. In a similar assay for γδ resolvase, Hatfull and Grindley (1986) have shown that in the presence of suboptimal amounts of wild-type resolvase resolution of co-integrates could be stimulated by the addition of Ser10 mutant γδ resolvase. This may reflect the varying degrees of complexity of the respective recombination substrates. Each *res* site is composed of 3 binding sites for γδ resolvase (Abdel-Meguid *et al.*, 1984). It is feasible that the mutant proteins can occupy sites II and III and make the necessary protein−protein interactions when the two halves of region I, where strand exchange takes place, are occupied by wild-type protein (Hatfull and Grindly, 1986). The IR sequence, in contrast, is composed of just two symmetrically arranged binding sites for Gin (see accompanying paper by Mertens *et al.*) and, functionally resembles binding site I of the *res* site. Binding of mutant Gin protein to either half of the IR would be expected to inhibit recombination. Both mutant proteins have the same inhibitory effect on recombination despite their different affinities for linear substrates. This might indicate that binding to the supercoil is governed by other more complex factors such as protein−protein interaction between Gin molecules or between Gin molecules and FIS. We are currently attempting to isolate Gin mutants that are affected in such protein−protein contacts.

The results presented in this communication clearly show that DNA invertases and resolvases share their mode of strand cleavage despite the fact that different processes (inversion of DNA and deletion formation, respectively) are catalysed by these two classes of enzymes and despite the fact that these reactions have different protein requirements. It is tempting to speculate that the divergencies manifest themselves at a step prior to strand exchange, perhaps involving the proper alignment of recombination sites in a productive synaptic complex. Once this has been accomplished through protein−protein contacts of resolvase bound to individual domains of the *res* sites or Gin and FIS bound to their respective sites within the IRs and the recombinational enhancer, the actual enzymatic step involving cleavage and religation of DNA may proceed by the same mechanism.

Materials and methods

Bacterial strains and phages

The *Escherichia coli* K12 strain CSH50 (Miller, 1972) is *pin*$^-$ (Kamp and Kahmann, 1981) and was used for the propagation of plasmids and for *in vivo* inversion assays. For selection of mutants constructed by the gapped-duplex DNA method (Kramer *et al.*, 1984) the su$^-$ strain WK6(Δ(*lac pro*), *gal*E, *str*A/F′*lac*I^q, *lac*ZΔ*M15*, *pro*A$^+$B$^+$) and its *mut*S derivative were used. They were lysogenized with λcI$^+$ when used for the propagation of expression vectors carrying λpL. These strains also served as hosts for superinfection with the helper phage f1IR1 (Dotto and Horiuchi, 1981) to prepare single-stranded DNA of plasmids carrying f1 *ori*.

Cloned DNA templates

The following inversion-test plasmids are described elsewhere: pAK3 (Mertens *et al.*, see accompanying paper) and pBRminiG(−) (Kahmann *et al.*, 1985).

M13mp9-IR carries one 34-bp IR on a 70-bp *Bam*HI−*Eco*RI fragment from pSR1neo34 (Mertens *et al.*, see accompanying paper) cloned into the respective sites of M13mp9.

pMA5-8 and pMC5-8 are plasmid vectors which allow efficient mutagenesis through alternate strand selection (Stanssens *et al.*, in preparation).

Next to a multi-cloning site two fd terminators and the f1 origin of replication are inserted. This region is followed by the pBR322 origin of replication and the coding regions for *cat* and *bla*. pMA5-8 contains an amber mutation in the *cat* gene, pMC5-8 contains an amber mutation in the *bla* gene. The amber mutations are suppressed in *sup*E and *sup*F strains. pMA5-8pL and pMC5-8pL were constructed by inserting a 260 bp *Xho*II fragment from pLMugin-0163 (Mertens *et al.*, 1984) which carries λpL, a deletion 5′ to the *gin* gene and an altered Shine−Dalgarno region ensuring overexpression of Gin, into the *Bam*HI site of pMA5-8 and pMC5-8 respectively. For construction of pLMA5-8gin the gene for the DNA invertase Gin was isolated from pLMugin-0163 on a *Sty*I−*Hin*dIII fragment and cloned into the corresponding sites of pMA5-8pL.

The inversion test plasmid pMD3lacZ is a derivative of pACYC184 (Chang and Cohen, 1978). The 1100-bp *Pst*I−*Bgl*II fragment of phage λ containing the λcI857 repressor under control of p_{rm} was made blunt-ended using T4-DNA polymerase and inserted into the *Hin*dII site at position 3200 of pACYC184. A 3000-bp *Fsp*I−*Eco*RI fragment containing the Mu G segment (deleted for the internal *Hpa*I fragment) and flanking regions α and β was isolated from pMuG3(−)ΔH (Mertens *et al.*, 1984). After converting the *Fsp*I end to an *Eco*RI end, this fragment was inserted into the unique *Eco*RI site of pACYC184. The product containing the insert in an orientation where α is located near the Cm promoter was selected. To use this plasmid for G orientation dependent expression of an indicator gene a truncated 3120-bp fragment containing the coding region of β-galactosidase except for the first 5 codons was inserted. This fragment was isolated as a *Sma*I−*Dra*I fragment from pMLB1034 (Silhavy *et al.*, 1984) and cloned into the unique *Hpa*I-site within the invertible region. In pMD3lacZ the *lac*Z portion is fused in frame to the variable part of gene *S* of Mu (S_v, Kahmann and Kamp, 1987). Since the invertible segment is in the (−) orientation, expression of β-galactosidase requires recombination to fuse the S_v″*lac*Z portion to S_c and the Cm promoter. A similar plasmid has been constructed by Plasterk *et al.* (1983a).

DNA manipulation

Plasmid DNA isolation, cleavage of DNA with restriction endonucleases, converting recessed DNA ends to blunt ends, alkaline phosphatase treatment, *Bal*31 digestion, ligation and transformation of *E.coli* were done according to standard methods (Maniatis *et al.*, 1982). DNA fragments were labelled at their 5′ or 3′ termini, isolated and purified from agarose or polyacrylamide gels as described by Maniatis *et al.* (1982). DNA sequence analysis was performed according to Maxam and Gilbert (1980) or by the dideoxy method of Sanger *et al.* (1977). Labelling by primer extension was done according to the Sanger protocol without adding chain terminators.

Oligonucleotides

Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA Synthesizer by the solid-phase phosphoramidite method (Matteucci and Caruthers, 1981). After cleavage from the support and deprotection in ammonium hydroxide the products were purified by reversed phase HPLC on an ODS column (5μ particle size) (Fritz *et al.*, 1978). Detritylation was performed by treatment with 80% acetic acid. After drying in a speed-vac concentrator and ethanol precipitation, 200 pmol of oligonucleotide was kinased according to standard protocols (Maniatis *et al.*, 1982).

The following oligonucleotides were used for mutagenesis. Mutant positions are underlined.

SL9	5′ CATTTGTTAACACCCTTAC 3′
TI10	5′ GTCATTTATCGATACCCCTTAC 3′
TS10	5′ GTATTCTGGTCATTCGATGATACCCTT 3′
TS15	5′ AGGTCTGAATTCTGG 3′
SA9	5′ CTGGTCATTTGTTGCTACCCTTACA 3′
ST9	5′ CTGGTCATTTGTTGTTACCCTTACA 3′
T9S10	5′ GGTCATTCGATGTTACCCTTACA 3′

The following oligonucleotides served as sequencing primers:

gin590	5′ ATAATCGATCTGTATTACC 3′
gin414	5′ TTAGTTTAGGTGGTCGC 3′
gin228	5′ CTCGTAATTCCCCTACG 3′
gin71	5′ TTGTTCACATCCTGCAC 3′

Isolation of gin mutants

Oligonucleotide directed construction of mutations within the *gin* gene were performed by the gapped-duplex DNA method (Kramer *et al.*, 1984) using the pMAC plasmid vectors developed by Stanssens *et al.* (in preparation). For mutagenesis gapped-duplex molecules are formed between single stranded pLMA5-8gin and pMC5-8pL cleaved with *Hin*dIII and *Sty*I. To ensure efficient resealing of the 700-bp gap the respective mutagenic oligonucleotide was mixed with the sequencing oligonucleotides for the fill-in reaction. A typical reaction contained 15 fmol gapped-duplex DNA substrate, 5 pmol mutagenic oligonucleotide and 30 fmol of each primer. After transformation into the su^- strain WK6*mut*S :: Tn10(λcI$^+$) plasmid DNA was isolated and re-transformed into WK6(λcI$^+$). Transformants were grown in the presence of Cm which selects for the mutagenized strand. All gin mutants except T9S10 were derived from a gapped-duplex DNA mixture containing pLMA5-8gin single-stranded DNA and pMC5-8pL cut with *Sty*I−*Hin*dIII. T9S10 was produced by a second round of mutagenesis starting from *gin*-mutant TS10. A gapped-duplex DNA mixture of single stranded pLMC5-8ginTS10 and pMA5-8pL digested with *Sty*I and *Hin*dIII was used. *gin* mutants were identified either by restriction analysis in cases where the mutation had created a new restriction site or by sequence analysis. The mutation frequency ranged between 50 and 80%. The mutant genotype was verified by sequencing the entire *gin* coding region to ensure that no additional mutations had occurred during the fill-in reaction.

In vivo test system for DNA inversion

Mutant Gin proteins were assayed for their ability to catalyse DNA inversion after transforming the respective plasmids into CSH50 harbouring the compatible inversion-test plasmid pMD3lacZ. Gin expression on pLMAC5-8 derived plasmids is under the control of the λpL promoter. At 28°C the λcI857 repressor encoded by pMD3lacZ blocks transcription from λpL and colonies remain white on X-Gal plates. Overexpression of Gin is induced at 42°C. Such colonies are blue on X-Gal plates if active Gin protein is produced. Strains harbouring both plasmids were grown in the presence of the appropriate antibiotics at 28°C. 10 μl from various dilutions were spotted on selective plates containing X-Gal (70 μg/ml). After 10 h at 28°C Gin expression was induced by shifting the temperature to 42°C for 5 h. Recombinational activity is proportional to the intensity of the blue colour. Colonies not producing Gin protein turn light blue during prolonged incubation. We attribute the pale blue colour under gin^-pin^- conditions to the action of a yet unknown host function which is able to complement Gin very weakly. If high amounts of mutant Gin protein are produced inversion attributed to the host function is reduced and such colonies remain white. We presume that mutant Gin protein that is able to bind to the IR blocks access to the IR by the host function in a manner similar to that for blocking wild-type Gin action *in vitro* (Figure 4). This indirect assay allows the ability of mutant Gin protein to bind to the IR sequence to be assessed *in vivo*.

Purification of wild-type and mutant proteins

Wild-type Gin protein and the host factor FIS were purified according to published procedures (Mertens *et al.*, 1986; Koch and Kahmann, 1986). Mutant Gin proteins were isolated from CSH50 harbouring pMD3lacZ and the respective plasmids expressing mutant Gin proteins. Cells were grown in 600 ml dYT (Miller, 1972) to a density of 8×10^8 cells/ml. Overexpression of Gin was achieved by shifting the temperature to 42°C for 1 h. Purification of proteins from crude cell extract was done by hydrophobic chromatography on a 12 ml Sepharose Cl-6B column as described previously (Mertens *et al.*, 1986). Fractions were pooled and dialysed against Gin-storage buffer. This purification step yields Gin preparations which are about 90% homogeneous and lack FIS activity (Mertens *et al.*, 1986).

DNA inversion in vitro

DNA inversion with purified proteins was performed as described previously (Mertens *et al.*, 1986). The inversion-rate was quantitated physically after restriction enzyme analysis or in a biological assay (Kahmann *et al.*, 1985).

Test for topoisomerase activity

Supercoil relaxation was assayed by incubating mutant or wild-type Gin-protein and pBRminiG(−) DNA in the standard *in vitro* DNA inversion reaction in the presence of FIS for 90 min. Topoisomers were separated on a 1% agarose slab gel (12.5 × 21.5 cm) containing chloroquine phosphate (50 μg/ml) according to Shure *et al.* (1977). Electrophoresis was carried out at 150 V for 16 h at 4°C. Bands were visualized after staining with ethidium bromide.

Gel retardation assay

Appropriate DNA fragments containing IR-L or IR-R were isolated from pBRminiG(−) DNA. A 230 bp fragment containing IR-R flanked by 50 bp of the G segment and 143 bp of β was isolated after *Ssp*I digestion. A 280-bp fragment containing 172 bp of α, IR-L and 72 bp of G was isolated after *Eco*RV−*Ssp*I digestion. Both fragments were 5′ end labelled. Protein preparations were assayed for DNA binding in a total volume of 20 μl con-

taining 25 mM Tris−HCl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 100 mM NaCl, 500 μg/ml BSA, 100 ng sonicated calf thymus DNA and 0.2 nM 5′-labelled DNA fragment (3000−5000 c.p.m.). After addition of Gin or mutant Gin proteins at concentrations indicated in each experiment samples were incubated for 20 min at 25°C and analysed electrophoretically according to Bushman *et al.* (1984). The relative amounts of bound and unbound fragment were calculated using a LKB Ultroscan XL laser densitometer.

Assay for DNA-nicking activity and covalent protein−DNA complexes

Gin and mutant Gin proteins were tested for their ability to introduce nicks within the IR sequence. Purified proteins were incubated with 1 nM 5′-^{32}P-labelled DNA fragments (60 000 c.p.m.) containing the IR under standard inversion conditions (Mertens *et al.*, 1986) for 5 min. 1/10 volume of 10 mM DTT was added and after another 5 min at 37°C the samples were placed on ice. Following treatment with proteinase K (600 μg/ml) in 1% SDS for 30 min at 37°C, the reaction mixtures were extracted with phenol/chloroform (1:1) twice and ethanol-precipitated. 10 000 c.p.m. per reaction were loaded on a 6% sequencing gel. For the visualization of the covalent attachment of Gin to DNA a 290-bp *Eco*RV−*Bam*HI fragment containing 172 bp of α, IR-L and 81 bp of G was isolated from pBRminG(−). This fragment was ^{32}P-labelled at the 3′ *Bam*HI end. Following incubation with Gin under standard conditions samples were left untreated or incubated with various amounts of proteinase K respectively, extracted with phenol/chloroform and ethanol precipitated. For analysis samples were dissolved in formamide loading dye and applied to a 6% sequencing gel.

Phosphoamino acid analysis

To identify the amino acid involved in formation of the covalent Gin−DNA linkage a partially double-stranded molecule labelled to high specific activity was constructed by primer-extension using M13mp9-IR. The reaction followed the Sanger sequencing protocol (1977). Single-stranded M13mp9-IR DNA served as template, the 17mer oligonucleotide of the M13 system was used as labelling primer, [α^{32}P]dGTP was employed as label. The reaction was stopped by phenol extraction. After removing the free nucleotides on a Sephadex G-50 column (Maniatis *et al.*, 1982) and ethanol precipitation, 100 ng of DNA was incubated with Gin in a final volume of 250 μl under standard inversion conditions (Mertens *et al.*, 1986), FIS was omitted from the reaction. After 3 min the reaction was stopped in 0.5% SDS on ice. The mixture was ethanol-precipitated, incubated at 100°C for 2 min in 400 μl 0.5% SDS and treated with 30 U *Bal*31 in 800 μl *Bal*31 buffer (Maniatis *et al.*, 1982; Reed and Moser, 1984). The protein was concentrated by TCA precipitation, washed twice with ice-cold acetone, denatured by boiling for 3 min in cracking buffer and loaded on a 15% SDS−polyacrylamide-gel (Laemmli, 1970). A portion of gel containing purified Gin protein was stained with Coomassie Brilliant Blue G-250 and destained according to Weber and Osborn (1969) to determine the gel position of Gin. The remainder of the gel was autoradiographed to visualize ^{32}P-labelled Gin protein. Phosphoamino acid analysis was done essentially as described in detail by Bister *et al.* (1987). Briefly, gel purified protein was extracted from homogenized gel strips into 50 mM NH_4HCO_3, 0.1% SDS, 5% mercaptoethanol and concentrated by precipitation with TCA. Hydrolysis was performed in 50 μl 5.7 N HCl for 2 h at 110°C. After evaporation of the acid, hydrolysation products were electrophoretically separated into two dimensions on cellulose-coated thin-layer plates. The first dimension was run for 20 min at 2300 V in pH 1.9 buffer consisting of formic acid/acetic acid/water (15:52:600, v/v), followed by electrophoresis in the second dimension for 16 min at 1500 V in pH 3.5 buffer containing pyridine/acetic acid/water (1:10:189, v/v) (Cooper *et al.*, 1983). Internal phosphoamino acid markers were visualized by staining with 0.25% ninhydrin in acetone, ^{32}P-labelled amino acids were detected by autoradiography using intensifying screens at −70°C and identified by co-migration with stained marker amino acids.

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