Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 2689–2692, May 1984
Biochemistry

A genetic switch *in vitro*: DNA inversion by Gin protein of phage Mu

(site-specific recombination/phase variation/protein overproduction)

RONALD H. A. PLASTERK, ROLAND KANAAR, AND PIETER VAN DE PUTTE

Laboratory of Molecular Genetics, Department of Biochemistry, State University of Leiden, Wassenaarseweg 64, 2333 AL Leiden, The Netherlands

Communicated by Allan Campbell, January 11, 1984

ABSTRACT **Inversion of the G segment in the DNA of *Escherichia coli* phage Mu depends on the Mu Gin protein and alters the host range of the phage. The frequency of the inversion reaction is low both in the lysogenic state and during lytic growth. A sensitive assay was developed to detect low levels of G inversion: the *E. coli lac* operon was inserted within the invertible G segment in such a way that the *lac* operon was expressed only by G(−) clones. As a result Gin-catalyzed inversion from G(+) to G(−) can be monitored as a lactose-negative to lactose-utilizing switch. Using a crude extract from a Gin-overproducing strain and this assay plasmid, we could detect a low level of G inversion *in vitro* (1% in 30 min). The reaction depends on Mg^{2+} and a supercoiled substrate. Under optimized reaction conditions over 15% of the plasmids had the G segment inverted after incubation with Gin *in vitro*. The inversion was then visualized by agarose gel analysis of plasmid DNA digested by restriction endonucleases. The Gin protein retains its catalytic properties upon partial purification. The mechanism of this genetic switch can now be studied *in vitro*.**

Phase variation is the reversible switch between two relatively stable genotypes. Several examples of phase variation by DNA inversion in Enterobacteriaceae have been described: host-range variation in phage Mu and related phage P1 by inversion of the G or C segment (1–3); flagellar variation of *Salmonella typhimurium* (4, 5); and P inversion in the *Escherichia coli* chromosomal element e14 (6, 7), for which the function is still unknown. The proteins responsible for the DNA inversions are closely related: they complement each other (8, 9) and are about 60–70% homologous in their amino acid sequence (3, 10).

The genetic organization of the invertible G region of Mu is shown in Fig. 1. Two sets of genes are alternatively expressed: *S* and *U* by G(+) phages, *S'* and *U'* by G(−) phages. The frequency of the switch between the two genotypes [G(+) and G(−)] is low: 1% G inversion per infection cycle in lytically grown Mu (12). This low rate of variation seems essential to avoid mixing of the two types, which would probably lead to instability in the phenotype [both Mu tail fibers and *Salmonella* flagella are polymers of one or a few protein subunits (13)]. We wanted to visualize *in vitro* the inversion reaction, which proceeds at only a low rate *in vivo*. Therefore we took the approach of both overproducing the recombination-catalyzing protein Gin and developing a sensitive assay for the reaction. We previously described the construction of a plasmid that serves to monitor the inversion of a cloned G segment *in vivo* as a switch from lactose-negative (Lac^-) to lactose-utilizing (Lac^+) (10). This plasmid was now used for *in vitro* detection of G inversion. After the incubation with Gin extract the plasmid DNA can be used to transform a Lac^- strain of *E. coli*, and very low levels of Lac^+ transformants are expected to be detectable.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

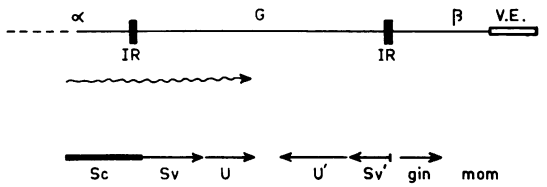

FIG. 1. Genetic organization of the Mu G region. The Mu G region is here shown in the (+) orientation. Thus the genes *S* and *U* are expressed. The G(−) gene *S'* shares a common region (*Sc*) with the *S* gene in the noninverting DNA. The exact functions of the two sets of genes are not yet known, but it is certain that the proteins play a role in the adsorption to the cell wall of the host; they are probably part of the Mu tail. The inverted repeats (IRs) flanking the G region are shown by blocks; these are the recombination sites for the Gin-catalyzed inversion. The genetic organization of G has been described previously (11). V.E., variable end DNA.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The strains used during this study were: KMBL1164(λ) (*pin*, Δ*lac–pro111*, λ; our laboratory), KMBL1060 (*endA*) and NF1 (λ c*I*857; E. Remaut; ref. 14). The plasmids used were pCI857 and pCP3 (from E. Remaut) (14) and pGP204Δ66 (6) and pGP231 (10).

Recombinant DNA Techniques. These were as described previously (6). The proteins coded by the Gin overproducer were analyzed essentially as described (14); cells containing the appropriate plasmid were pulse-labeled for 5 min at various times after thermoinduction, using 5 μCi of [^{35}S]methionine (New England Nuclear; 1 Ci = 37 GBq). The labeled proteins were separated by electrophoresis on a sodium dodecyl sulfate/15% polyacrylamide gel, and autoradiography was done overnight at −80°C.

Extraction of Gin from Overproducer. The strain KMBL1060 containing the plasmid pCI857 and pGP241 was grown at 28°C until OD_{703} = 0.2. The culture was then shifted to 42°C and grown for another 3 hr. The cells were spun down and suspended in 20 mM Tris·HCl, pH 8.0/20 mM glucose/2 mg of lysozyme per ml and kept on ice for 10 min; cells from a 1-liter culture were suspended in 1 ml. Cells were disrupted by grinding in the presence of Alcoa alumina, and debris was spun down at 10,000 rpm in a Sorvall centrifuge with a SS34 rotor for 10 min. Gin in the crude extract or after several purification steps was stable at 4°C for weeks.

G Inversion *in Vitro*. A mixture of 40 μl of pGP231 DNA (5 μg), 105 μl of H_2O, 6 μl of reaction buffer (60 mM Tris·HCl, pH 7.5/60 mM $MgCl_2$/60 mM 2-mercaptoethanol/500 mM NaCl), and 5 μl of Gin extract was incubated at 37°C for the indicated time. After the incubation the DNA was extracted with phenol and precipitated by the addition of cold ethanol.

Partial Purification of Gin Activity. To 2 ml of crude extract we added 105 μl of a solution of 10% (vol/vol) Polymin P (Pfaltz and Bauer) in 20 mM Tris·HCl, pH 8.0/10 mM

Abbreviations: Lac^+ and Lac^-, lactose-utilizing and nonutilizing, respectively; IR, inverted repeat.

$MgCl_2$/10 mM 2-mercaptoethanol. After 10 min on ice the precipitate was sedimented (10 min at 12,000 rpm in the Sorvall) and dissolved in 2 ml of 20 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/1 M NaCl. After 10 min on ice the remaining debris was sedimented. The supernatant was dialyzed against standard buffer (20 mM Tris·HCl, pH 7.6/10 mM $MgCl_2$/10 mM 2-mercaptoethanol/0.1 M NaCl) for 2 hr at 4°C. The precipitate was spun down; the Gin activity was in the supernatant. The initial purification steps are largely the same as described by Reed (15). As for TnpR, at this stage the Gin activity could be removed from the solution by dialysis against lower salt concentrations, but, unlike TnpR, Gin activity could not be recovered after dissolving the precipitate. Here, as in other purification steps, a precipitate of Gin seems insoluble once it has been formed (results not shown). The next step therefore was gel filtration (Sephadex G-100, 30-ml bed volume, elution in standard buffer). The activity in the collected fractions was assayed by the standard test (as described above). The active fractions were pooled and form the partially purified Gin extract mentioned in the text.

RESULTS

The low rate of G inversion is at least partially due to low Gin expression in a Mu lysogen or during the lytic life cycle. When Gin is overproduced the inversion rate rises considerably (10). However, fusion of the *gin* gene to a strong promoter on a multicopy plasmid is not sufficient to visualize the gene product in crude cellular extracts (10). To further enhance Gin production we inserted the λ p_L promoter closer to the *gin* gene and used a plasmid with a "runaway replication" origin (Fig. 2). When this plasmid (pGP241) is maintained in a strain containing the thermosensitive λ repressor encoded by c*I*857, thermoinduction will result in amplification of the plasmid DNA in the cell and in high levels of the transcript made from this promoter (14). As shown in Fig. 3, thermoinduction of a strain containing pGP241 leads to high rates of synthesis of a protein that in all probability is Gin. In fact this is the major protein synthesized 30 min after induction.

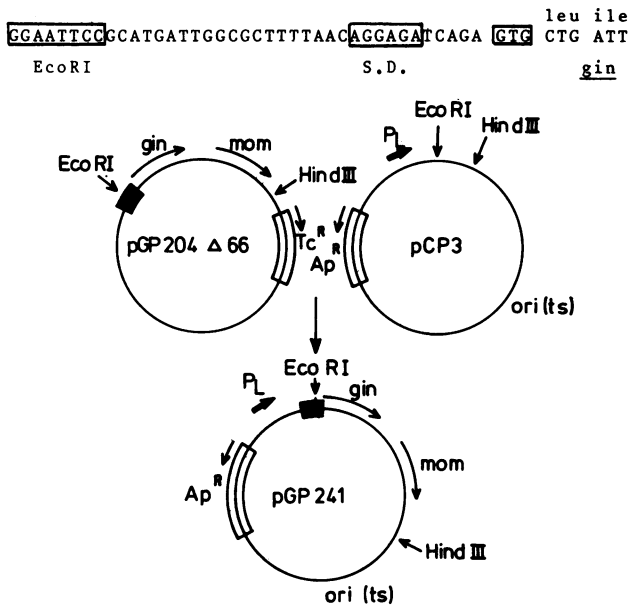

FIG. 2. Construction of a Gin overproducer. (*Upper*) To construct a Gin overproducer we inserted a linker close before the *gin* gene. The details of this and the entire sequence of *gin* were described previously (6). The sequence of the NH_2-terminal part of *gin* in the plasmid pGP204 Δ66 is shown here. The Shine–Dalgarno sequence and initiation triplet of *gin* are boxed. (*Lower*) pCP3 is a plasmid containing the λ p_L promoter and a runaway origin [ori(ts), which means that at elevated temperature the copy number of the plasmid increases (14)]. Plasmid pGP204 Δ66 and pCP3 were digested with *Eco*RI and *Hin*dIII and the appropriate fragments were purified from agarose gels as described (16). After ligation ampicillin-resistant transformants of KMBL1164(λ) were selected. Tc^R and Ap^R, tetracycline and ampicillin resistance.

To detect low rates of G inversion we constructed a plasmid, the essentials of which are summarized in Fig. 4: the *lac* operon devoid of transcription initiation signals and of the translation start of *lacZ* was inserted into the G region on a plasmid (10). This was done in such a way that the plasmid carrying the G region in the (−) orientation contained a functional fusion of *lacZ* to the *S′* gene of Mu, whereas hosts carrying the G(+) form of the plasmid are Lac^-. pGP231 in the Lac^- [i.e., G(+)] form is a sensitive indicator for Gin activity. It was used *in vivo* to estimate the rate of G inversion in different genetic backgrounds (10).

We used this plasmid to test whether an extract from the constructed Gin-overproducing strain would work *in vitro*. An extract was made from pGP241-containing strain 3 hr after thermoinduction. The extract was incubated with pGP231 DNA for 15–30 min, and the DNA was subsequently

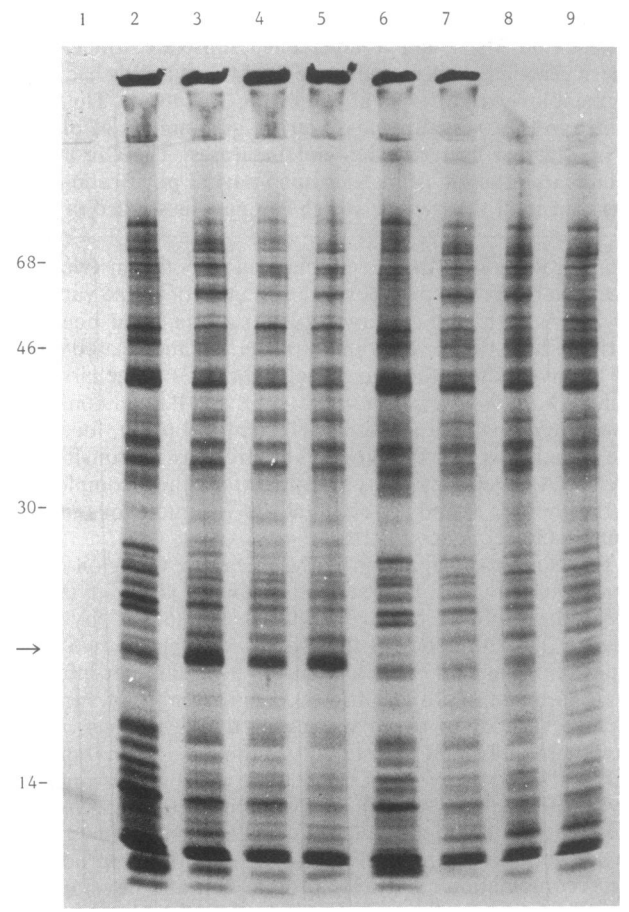

FIG. 3. Overproduction of Gin. Cells containing either the plasmid pCP3 (the vector) or the plasmid pGP241 (containing *gin*) were pulse labeled with 5 μCi of [^{35}S]methionine for 5 min at various times after thermoinduction. The labeled proteins were separated by electrophoresis on a sodium dodecyl sulfate/15% polyacrylamide gel, and autoradiography was done overnight at −80°C. Details of gel electrophoresis were described elsewhere (10). Lane 1, protein size markers; lanes 2–5, pGP241 at, respectively, 0, 30, 60, and 120 min after thermoinduction; lanes 6–9, pCP3 at, respectively, 0, 30, 60, and 120 min after thermoinduction. A prominent band appears after thermoinduction of the pGP241-containing strain. This band is of the size of Gin (21 kilodaltons) (indicated by an arrow) (6, 17).

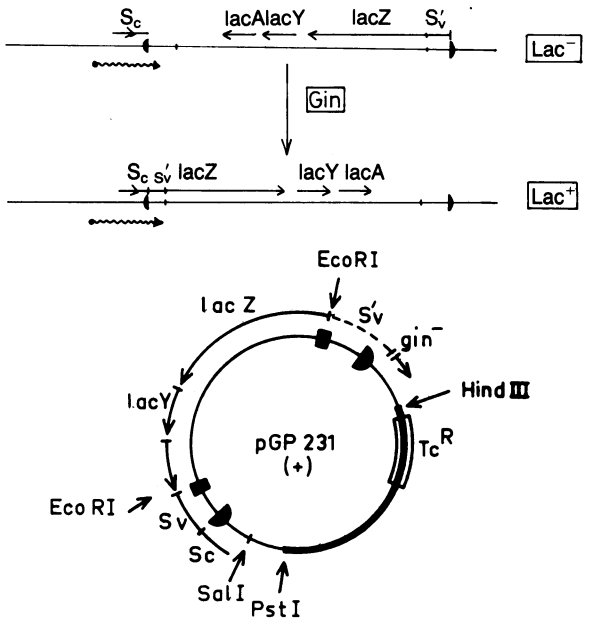

FIG. 4. (*Upper*) Linear presentation of the relevant part of the plasmid pGP231 (10). The *lacZ* gene of *E. coli* has been fused to the domain of the *S'* gene located within the invertible G segment of Mu. Whereas normal G inversion constitutes a switch between the expression of the genes *S* and *S'* (and similarly of *U* and *U'*), in this clone the switch is between expression of the *S'–lacZ* fusion gene and no expression of this gene. In a *gin*$^-$ background the plasmid is perfectly stable, and the frequency of a switch from Lac$^-$ to Lac$^+$ is lower than 10^{-8} (also in a *rec*$^+$ background) (10). When the plasmid is maintained in a *gin*$^+$ background, inversions from Lac$^-$ to Lac$^+$ can be seen (e.g., by plating cells on a plate containing an indicator for β-galactosidase activity). (*Lower*) The circular restriction map of pGP231 (10) shows the restriction fragments that can be expected after *Eco*RI/*Hin*dIII digestion. The sizes of the fragments are 6.2 kilobase pairs for the *lac* fragment, 7152 and 7452 base pairs for, respectively, the G(+) and G(−) forms of the larger fragment, and 2740 and 2440 base pairs for, respectively, the smaller fragment in G(+) and G(−) form. The *Eco*RI site into which the *lac* operon has been inserted is 600 base pairs from the IRL and 900 base pairs from the IRR. The easiest way to determine the orientation of the G segment in a plasmid is therefore to see if the smaller *Eco*RI/*Hin*dIII fragment is 2740 or 2440 base pairs.

recovered and used to transform a Lac$^-$ strain. Tetracycline-resistant colonies were selected on plates containing 5-chloro-4-bromo-3-indolyl β-D-galactoside. The blue colonies were indicators of G inversion. As a control the DNA of pGP231 was incubated 0 min with the Gin extract (phenol was added immediately after addition of Gin). In fact this control resulted also in a low percentage of blue colonies. These were thought to result from simultaneous transformation of a cell with pGP231 and the Gin-producing plasmid pGP241. Apparently *gin* was expressed after transformation of the cell with the two plasmids. Therefore we used a λ-immune strain as recipient for the transformation (in such a strain the p_L promoter before *gin* in pGP241 will be repressed, and *gin* will not be expressed). Indeed, this led to a considerably lower background of inversions *in vivo*. The inversion frequency obtained after 30-min incubation was 1–2% (the background was lower than 0.05%).

Using this test, we could optimize the conditions to achieve higher rates of G inversion. One of the major improvements was to dilute the crude extracts 1:30 in the reaction buffer. Apparently the high concentration of proteins in the undiluted extract inhibits the reaction. The reaction conditions are presented in *Materials and Methods*. Under these conditions about 15% of the pGP231 molecules will have their G regions inverted after 1-hr incubation. This can be visualized by restriction analysis: two *Eco*RI restriction sites are located asymmetrically in the invertible region; a *Hin*dIII site is in the vector DNA (see Fig. 4). As shown in Fig. 5, *Eco*RI/*Hin*dIII restriction of both forms of pGP231 DNA

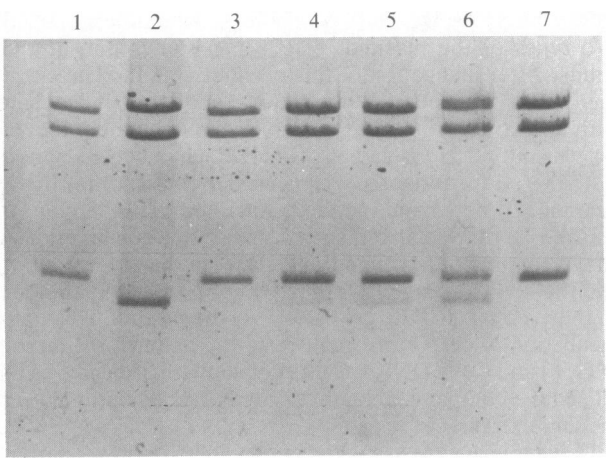

FIG. 5. *In vitro* G inversion visualized on a gel. A negative print of the ethidium bromide-stained gel is shown. On digestion with *Eco*RI and *Hin*dIII, pGP231 shows three bands after gel electrophoresis when the G region is fixed in one orientation. The other orientation of G results in two other bands (and one identical band) (see Fig. 4). The larger of the bands of G(+) and G(−) almost comigrate on 1% agarose gels, but the smaller ones are easily distinguishable (10). Lane 1, pGP231(+) digested with *Eco*RI and *Hin*dIII; lane 2, pGP231(+) (which is Lac$^-$); lanes 3–6, plasmid pGP231(+) after 0, 0.5, 1, and 16 hr of incubation with partially purified Gin. As can be seen, the band corresponding to pGP231(−) appears. Lane 7, reaction product obtained when Mg^{2+} is left out of the reaction mixture.

Table 1. Requirement for Mg^{2+} and a supercoiled substrate

Substrate and buffer	(Blue/total colonies) × 100	Total colonies
pGP213 supercoiled, complete buffer	16.7	10,000
pGP213 relaxed, complete buffer	0.2	2,000
pGP213 supercoiled, buffer without Mg^{2+}	0.1	1,000
pGP213 supercoiled, complete buffer, 0-min incubation	<0.05	3,000
pGP213 supercoiled, complete buffer, incubated with extract from KMBL1060(pCP3, pCI857)	<0.05	5,000

The *in vitro* Gin test was carried out as described in *Materials and Methods*. The transformation test for inversion of pGP231 was described in ref. 10. Competent cells (100 μl) of a *lac* strain were transformed with 1/10th of the DNA from a standard incubation. After 1 hr of growth in nonselective medium the cells were spread on two minimal medium plates containing the antibiotic tetracycline and the indicator 5-chloro-4-bromo-3-indolyl β-D-galactoside. After 24 hr the blue and white colonies were counted. The plasmid DNA was isolated from several blue and white colonies to demonstrate that they indeed contained G(−) or G(+) DNA. This was confirmed by restriction analysis. The total number of transformants was between 1000 and 10,000 per test; among different tests there was no significant difference in the number of transformants obtained after the different types of incubation. The values for the ratio of blue to white colonies showed a difference of a factor <3 among independent experiments. pGP231 was made relaxed by digestion with *Hin*dIII and subsequent ligation with T4 DNA ligase. The supercoiled substrate was treated similarly, but without the *Hin*dIII, to rule out possible influences of the digestion or ligation buffer. The fourth line shows the control ("*in vivo*" inversions, explained in the text). In this experiment the control showed no blue colonies among 3000 white colonies. The bottom line shows the results when the reaction proceeded in the presence of an extract from an isogeneic strain containing the vector plasmid pCP3 instead of pGP241: no blue colonies were found.

will result in easily distinguishable bands on gels from the two types of the plasmid. pGP231(+) shows only the (+) bands. After incubation of this plasmid with the Gin extract the (−) bands appear. When the plasmid DNA is incubated with an extract from an isogeneic strain containing the vector plasmid pCP3 instead of pGP241 the band corresponding to the G(−) form does not appear. To confirm that the appearance of new bands in the gel is due to G inversion, the plasmid DNA was also digested with other combinations of enzymes that cut asymmetrically in both the inverting DNA and the noninverting DNA (*Pst* I/*Eco*RI and *Bam*HI alone). In these cases also the gel pattern of the plasmid DNA after incubation *in vitro* corresponded to the pattern of a mixture of G(+) and G(−) DNA (results not shown). This proves that indeed the complete DNA inversion is carried out *in vitro* by the Gin extract.

Using this *in vitro* assay to detect Gin activity, we partially purified the Gin protein from a crude extract. The data in Fig. 5 were obtained by using partially purified Gin.

When Mg^{2+} is left out of the incubation buffer no inversions are found (see Table 1 and Fig. 5). The substrate requirements of Gin were tested by presenting pGP231 DNA in relaxed and supercoiled form. As shown in Table 1, only supercoiled DNA is a good substrate for Gin.

DISCUSSION

We here describe a system of *in vitro* recombination that is responsible for a switch between the expression of alternative genes.

Several site-specific recombination systems in prokaryotes have been described; some of these have recently been analyzed *in vitro*: phage λ integration/excision (18), TnpR-catalyzed resolution of Tn*3* (15), and phage P1 resolution by Cre (19). The DNA invertases constitute a separate class of recombination-catalyzing enzymes: they complement each other and are not complemented by any of the other recombinases tested, they preferentially catalyze inversions (10, 20), and the reaction is carried out at a low rate *in vivo* (4, 10). There is some evolutionary relation between the different classes however: it has been shown that TnpR and Hin are partially homologous on the amino acid level (4). This raises the question of how the difference in preference for either inversions or deletions can be mechanistically explained. This can be studied in detail now that the inversion can be carried out *in vitro* at a reasonably high rate.

Because the Gin activity can be at least partially purified from a crude extract it seems highly probable that no other proteins are involved in the reaction, and the complete inversion is carried out by Gin alone.

We show that the reaction is independent of the addition of ATP. For TnpR it has been suggested that, like many topoisomerases, it retains the high energy of the phosphate bond in the DNA backbone in a covalent bond between the phosphate group and a specific tyrosine residue (15); we previously pointed out two tyrosines in Gin that are conserved among all DNA invertases and resolvases (10). It is possible that DNA binds specifically to one of these tyrosines.

The substrate DNA has to be supercoiled for an efficient inversion reaction. This resembles the requirements of TnpR, contrary to, e.g., Cre of P1. A question that can now be investigated is what is the topology of the G-inversion reaction: does Gin recognize its target sequences by tracking along the DNA, and what is the primary product of the inversion reaction?

We thank Dr. M. Giphart-Gassler for critical reading of the manuscript and Mr. L. Welmers for the drawings.

1. Kamp, D., Kahmann, R., Zipser, D., Broker, T. R. & Chow, L. T. (1978) *Nature (London)* **271,** 577–580.
2. van de Putte, P., Cramer, S. & Giphart-Gassler, M. (1980) *Nature (London)* **286,** 218–222.
3. Iida, S., Meyer, J., Kennedy, K. E. & Arber, W. (1982) *EMBO J.* **1,** 1445–1453.
4. Zieg, J. & Simon, M. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4196–4201.
5. Silverman, M. & Simon, M. (1980) *Cell* **19,** 845–854.
6. Plasterk, R. H. A., Brinkman, A. & van de Putte, P. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5355–5358.
7. van de Putte, P., Plasterk, R. H. A. & Kuyper, A. (1984) *J. Bacteriol.*, in press.
8. Kutsukake, K. & Iino, T. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7338–7341.
9. Kahmann, R. & Kamp, D. (1981) *Mol. Gen. Genet.* **184,** 564–566.
10. Plasterk, R. H. A., Ilmer, T. A. M. & van de Putte, P. (1983) *Virology* **127,** 24–36.
11. Giphart-Gassler, M., Plasterk, R. H. A. & van de Putte, P. (1982) *Nature (London)* **297,** 339–342.
12. Daniel, E., Abelson, J. K. & Davidson, N. (1973) *Virology* **51,** 237–239.
13. Symonds, N. (1982) *Nature (London)* **297,** 288.
14. Remaut, E., Tsao, H. & Fiers, W. (1983) *Gene* **22,** 103–113.
15. Reed, R. R. (1981) *Cell* **25,** 713–719.
16. Plasterk, R. H. A., Vrieling, H. & van de Putte, P. (1983) *Nature (London)* **301,** 344–347.
17. Kwoh, D. Y. & Zipser, D. (1981) *Virology* **114,** 291–296.
18. Nash, H., Mizuuchi, K., Enquist, L. W. & Weisberg, R. A. (1981) *Cold Spring Harbor Symp. Quant. Biol.* **45,** 417–418.
19. Abremski, K., Hoess, R. & Sternberg, N. (1983) *Cell* **32,** 1301–1311.
20. Scott, T. N. & Simon, M. I. (1982) *Mol. Gen. Genet.* **188,** 313–321.