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Bacteriophage T4 *gol* Site: Sequence Analysis and Effects of the Site on Plasmid Transformation†

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The *Escherichia coli lit* gene product is required for the multiplication of bacteriophage T4 at temperatures below 34°C. After infection of a *lit* mutant host, early gene product synthesis is normal, as is T4 DNA replication; however, the late gene products never appear, and early gene product synthesis eventually ceases. Consequently, at late times, there is no protein synthesis of any kind (W. Cooley, K. Sirotkin, R. Green, and L. Snyder, J. Bacteriol. **140**:83–91, 1979; W. Champness and L. Snyder, J. Mol. Biol. **155**:395–407, 1982), and no phage are produced. We have isolated T4 mutants which can multiply in *lit* mutant hosts. The responsible T4 mutations (called *gol* mutations) completely overcome the block to T4 gene expression (Cooley et al., J. Bacteriol. **140**:83–91). We have proposed that *gol* mutations alter a *cis*-acting regulatory site on T4 DNA rather than a diffusible gene product and that the wild-type form of the *gol* site (*gol*$^+$) somehow interferes with gene expression late in infection (Champness and Snyder, J. Mol. Biol. **155**:395–409). In this communication, we report the sequence of the *gol* region of the T4 genome from five different *gol* mutants. The *gol* mutations are all single-base-pair transitions within 40 base pairs of DNA. Therefore, the *gol* site is at least 40 base pairs long. The sequence data confirm that the *gol* phenotype is not due to an altered protein. We also report that the *gol*$^+$ site in plasmids prevents transformation of Lit$^-$ but not Lit$^+$ *E. coli*. Thus, the *gol* site is at least partially active in the absence of the T4 genome.

Bacteriophage T4 is one of the larger viruses, and the regulation of its gene expression is predictably complex, involving many regulatory gene products. Some of these gene products bind to the host RNA polymerase and thereby, presumably, alter its specificity (5, 14, 23, 28). Others alter the T4 DNA template in ways which are poorly understood but which are required for the optimal activation of many T4 promoters (cf. references 12 and 15). It was demonstrated recently that some correct initiation of late transcription occurs in vitro with RNA polymerase purified from infected cells (8). But so far, relatively high levels of transcription have been achieved only with relatively impure systems prepared from lysates of T4-infected cells in which the template is left relatively intact (13, 26). Apparently, T4 DNA undergoes changes in the cell, and some of these changes are required to render it competent for the activation of the later-used promoters.

In addition to the plethora of T4 genes, there are host genes whose products are involved in T4 late gene expression. One of these is the *Escherichia coli lit* gene at 25 min on the *E. coli* K-12 genetic map. Mutations in this gene can prevent the expression of all T4 genes late in infection at temperatures below 34°C. At least some of this defect is at the level of transcription, although effects on translation are not excluded (4). The defect is specifically on gene expression; T4 DNA replication is not significantly affected, nor is the T4 genome degraded.

This communication is concerned not with the *lit* gene of *E. coli*, per se, but with T4 mutations which overcome the defect in T4 gene expression in a *lit* mutant host. We have proposed that these mutations, called *gol* mutations, for they grow on *lit*, define a *cis*-acting regulatory site rather than a diffusible gene product. Our evidence included the observation that, in mixed infections of a *lit*$^-$ host by *gol*$^+$ and *gol* mutant phage, the only late genes expressed are on the *gol* mutant DNA (3). We speculated that the *gol* site plays a role in the template processing required for the activation of the late promoters. But, because of the complexity of T4 DNA organization after infection, it is difficult to determine the fate of the *gol* site in intracellular T4 DNA. However, in this communication we report that the *gol* site in a plasmid affects transformation by the plasmid in a way which mimics its effect on T4 transcription. We also report the DNA sequence for some *gol* mutants, as a beginning to the molecular characterization of the *gol* site.

MATERIALS AND METHODS

Bacterial and phage strains. The strains used, their relevant characteristics, and the source or a reference are listed in Table 1.

Mutagenesis. Hydroxylamine, nitrosoguanidine, and 2-aminopurine mutagenesis were by the methods of Tessman (25), Adelberg et al. (1), and Miller (11), respectively.

Preparing cytosine-containing T4 DNA with a *gol* mutation. The DNA of T4 contains glucosylated 5-hydroxymethylcytosine, in place of cytosine, which makes it insensitive to most restriction nucleases. However, T4 with the appropriate combination of four or more mutations will replicate with cytosine, replacing most of the hydroxymethylcytosine in its DNA (22). Such a mutant phage is strain Dec8 (Table 1). To combine a *gol* mutation with the four mutations in Dec8 required for cytosine DNA, we mutagenized Dec8 and plated the mutagenized phage on the *lit* mutant *E. coli* MPH7, to select *gol* mutants directly. Sometimes we were interested in a particular *gol* mutant. We then crossed the mutant with T4 Dec8, and the progeny which retained the four mutations of Dec8 were selected from among the myriad of recombinant types by plating the progeny of the

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TABLE 1. Bacterial and phage strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
K803	*SupE* r_K^- m_K^-	27
B834	r_B^- m_B^- *rgl*$^+$ Su^0	27
B834 *galU56*	B834 with *galU* mutation	16
BKL403	B834 with *lit6* mutation; B834 *lit-403*	3
MPH7	AB2495 with *lit7* mutation	4
JM103	Δ*lac pro* r_K^- m_K^+ F′ *traD35 proAB lacI*^{q} *ZΔM15*	Bethesda Research Laboratories
Bacteriophage T4		
Dec8	56$^-$ (*amE*51) *denA*$^-$ (*nd*28) *denB*$^-$ (rIIH23) *alc8*	Our laboratory
6B	*gol*, spontaneous	3
HA1	*gol*, hydroxylamine mutagenesis of Dec8	
HA2	*gol*, hydroxylamine mutagenesis of Dec8	
AP-13	*gol*, 2-aminopurine mutagenesis of Dec8	
Plasmid		
pBR322	Tc^r and Ap^r, single *Eco*RI and *Hin*dIII sites, *Hin*dIII site in Tc^r	24
pACYC184	*Eco*RI site in Cm^r, *Hin*dIII in Tc^r	24
pLA1	T4 DNA insert from gene 23 region cloned in *Eco*RI site of pBR322	6
pLA3	Same as pLA1 but opposite orientation	6
pLA4	Same as pLA1 but one *Hin*dIII fragment deleted	6
pLA5	Same as pLA1 but two *Hin*dIII fragments deleted	6
pLA3Δ2	Deletion mutant of pLA3	6
pA67	T4 *Eco*RI fragment from pLA1 cloned in *Eco*RI site of pACYC184 Ap^r Cm^s	
pG6B	Same as pA67 but clone from 6B mutant containing *gol* mutation	
pGHA1	Same as pA67 but clone from HA1 mutant containing *gol* mutation	
pGHA2	Same as pA67 but clone from HA2 mutant containing *gol* mutation	
pG2AP-13	Same as pA67 but clone from 2AP-13 mutant containing *gol* mutation	
pA83	1.1-kb *Hin*dIII fragment from Dec8 cloned in *Hin*dIII site of pACYC184	

cross on *E. coli* B834 *galU56*, which is selective for cytosine DNA (16). As expected, about one-third of these also had the *gol* mutation, as evidenced by their ability to multiply in *E. coli* MPH7.

Cytosine-containing phage with a *gol* mutation were propagated on strain B834 *galU56* and purified on a CsCl step gradient as described previously (21). The DNA was phenol extracted in 0.1 M phosphate buffer (pH 7) and dialyzed against 1 M NaCl and then water.

Plasmid preparation. Plasmids were prepared by a modification of a procedure suggested by Lynna Hereford. About 500 ml of cells was grown to mid-log phase, and chloramphenicol was added to 250 μg/ml (or spectinomycin to 700 μg/ml, if the plasmid carried chloramphenicol resistance). After overnight incubation with aeration, the cells were pelleted at 8,000 × *g* and resuspended in 3 ml of cold 25% sucrose–50 mM Tris (pH 8.0) before 0.6 ml of 10-mg/ml lysozyme in 0.25 M Tris (pH 8) was added with gentle stirring. After 10 min on ice, 1.5 ml of 0.25 M EDTA (pH 8) was added, and after 10 more min, 4.8 ml of 1% Brij 35–0.4% deoxycholate–60 mM EDTA–50 mM Tris (pH 8) was added. In 10 min, the solution began to clear, and the debris was pelleted at 70,000 × *g* for 30 min. The supernatant was extracted once with phenol in water, and NaCl was added to 1 M along with 2 volumes of ethanol. After chilling overnight at −20°C, the precipitate was centrifuged and washed with 70% ethanol before being resuspended in 0.1 M Tris (pH 8). RNase A was added to 40 μg/ml for 30 min at 37°C, and the DNA was reextracted three times with phenol and reprecipitated with ethanol, as above, before being resuspended in water for storage at −20°C.

Cloning T4 DNA restriction fragments. Restriction enzymes were used according to the recommendations of the supplier. The digestion conditions for restriction enzymes were as follows: for *Acc*I, 6 mM Tris-hydrochloride (pH 7.5)–6 mM $MgCl_2$–6 mM NaCl–6 mM 2-mercaptoethanol–100 μg of bovine serum albumin per ml; for *Eco*RI, 100 mM Tris-hydrochloride (pH 7.2)–5 mM $MgCl_2$–2 mM 2-mercaptoethanol–50 mM NaCl; for *Hpa*II, 20 mM Tris-hydrochloride (pH 7.4)–7 mM $MgCl_2$–1 mM dithiothreitol; and for *Hin*dIII, 20 mM Tris-hydrochloride (pH 7.4)–7 mM $MgCl_2$–60 mM NaCl. All digestions were for 2 h at 37°C with about 1 U of enzyme per μg of DNA. DNA restriction fragments were separated on a 0.7% (wt/vol) agarose gel in 0.09 M Tris-borate (pH 8.3), the gels were stained with ethidium bromide, and the desired fragment was recovered by electrophoresis onto filter paper backed by dialysis tubing. The DNA was eluted by centrifugation in a syringe with repeated washings with electrophoresis buffer and finally was precipitated twice with ethanol in 0.1 M NaCl. The T4 restriction fragments were ligated to plasmid DNA at an equimolar ratio of plasmid to restriction fragment, at a total DNA concentration of 40 μg/ml, and incubated overnight at 18°C. The ligation conditions were 66 mM Tris-hydrochloride (pH 7.5), 6.6 mM $MgCl_2$, 66 μM ATP, 10 mM dithiothreitol, and ca. 1 U of T4 DNA ligase per μg of DNA.

To clone into M13 bacteriophage, the procedures were essentially as described in the Bethesda Research Laboratories M13 product manual, using the M13mp7 vector of Messing et al. (10). To ligate *Acc*I-cleaved M13mp7 DNA to *Hpa*II-cleaved T4 restriction fragments, the DNAs were mixed in a 1 to 3 molar ratio at a concentration of 12 ng/5 nl and incubated for 12 h at 9°C. For transfection, exponentially growing strain JM103 was pelleted by centrifugation at 7,000 × *g* for 5 min, resuspended in one-half of the growth volume in 50 mM $CaCl_2$, and then incubated on ice for 20 min. The cells were centrifuged again and resuspended in 0.1 of the growth volume in 50 mM $CaCl_2$. A 0.3-ml portion of cells was then added to 12 ng of a ligated mixture of M13mp7–T4 DNA, incubated on ice for 40 min, and heat shocked at 42°C for 2 min. Immediately, 0.2 ml of exponentially growing strain JM103, 3 ml of YT soft agar, isopropyl-β-D-thiogalactopyranoside (final concentration, 0.3 mM),

and 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (final concentration of 0.03%) were added, and the mixture was plated on YT agar plates. (YT agar contains (per liter): 8 g of tryptone, 5 g of NaCl, 5 g of yeast extract, and 18 g of agar.) After overnight incubation at 37°C, the colorless plaques (putative recombinants) were plaque purified on strain JM103, and an individual plaque was picked and suspended in 0.5 ml of 0.85% saline for marker rescue tests (see below).

Isolation of DNA for sequencing. Recombinant plaques which tested positively by marker rescue for the presence of a *gol* mutation were plaque purified as above, and a single colorless plaque was transferred with a sterile toothpick into 5 ml of YT medium. A 50-μl portion of an exponentially growing culture of strain JM103 was added, and the culture was incubated at 37°C with shaking for 7 h. A 1-ml amount was centrifuged at 14,000 × *g* for 10 min, the supernatant was poured off into another tube, and the virus was precipitated by incubation in 4% polyethylene glycol 6000–0.5 M NaCl at room temperature for 30 min. After centrifuging at 14,000 × *g* for 5 min, the supernatant was discarded, the inside walls of the tubes were wiped clean, and the virus was resuspended in TES buffer (20 mM Tris [pH 7.5], 10 mM NaCl, 1 mM Na_2-EDTA). The DNA was then extracted with phenol saturated with 10 mM Tris-hydrochloride (pH 8.0)–1 mM Na_2-EDTA for 5 min. After being centrifuged for 5 min, the aqueous layer was removed, and the DNA was precipitated by addition of sodium acetate to 0.1 M and 2 volumes of ethanol and overnight incubation at −20°C. The precipitated viral DNA was collected by centrifuging for 10 min, washing with cold ethanol, and then centrifuging again. After being dried, the DNA was resuspended in 25 μl of TES buffer. This procedure routinely gave enough DNA for 10 sets of sequencing reactions.

Identification of clones by marker rescue. (i) Plasmids. The details of the method used were somewhat different for clones in plasmids and those in M13 phage. First, exponentially growing MPH7 cells were plated with top agar. Approximately 10^6 cells of a culture of B834 cells containing the putative recombinant plasmid were then spotted onto the plate. Finally, about 10^7 wild-type T4 cells were spotted directly onto the first spot, and plates were incubated overnight at 28°C. Under these conditions, wild-type T4, when spotted alone or spotted onto cells containing only the plasmid cloning vector, gave very few plaques within the spot. If the cells contain a plasmid with a T4 insert with a *gol* mutation, many discrete plaques were produced within the spot due to *gol* mutant recombinants.

(ii) M13 clones. Exponentially growing MPH7 cells were plated as above. Spotting was done in the following order: first, a loopful (about 1 μl) of about 10^6 cells of a culture of *E. coli* JM103 were spotted onto the MPH7 lawn, followed by about 10^8 of the recombinant M13 phage suspension, and then a loopful containing about 10^7 wild-type T4 cells. After overnight incubation at 28°C, the spots were examined for plaque formation. Under these conditions spots of strain M13 on strain JM103 gave no plaques; spots with wild-type T4 spotted onto nonrecombinant M13 plus JM103 gave a background of no more than 1 to 5 plaques; and spots in which marker rescue occurred gave discrete plaques at a level at least 10-fold over background.

Transformation. Transformation by plasmid T4 DNA-ligated mixtures was by the method of Selzer et al. (20). Cells were spread on tryptone plates (10 g of tryptone, 20 g of agar, 5 g of NaCl, 10 g of Casamino Acids per liter) with (per ml) 25 μg of ampicillin, 10 μg of tetracycline, or 20 μg of chloramphenicol.

Sequencing reactions. The method was taken from the procedures of Sanger et al. (18), as adapted for M13 (19). A 1-μl portion of M13 template DNA and 5 ng of primer (a 26-base-pair fragment which is complementary to the *lac* DNA immediately adjacent to the *Eco*RI site) were annealed at 90°C for 5 min in 70 mM Tris-hydrochloride (pH 7.5)–70 mM $MgCl_2$–500 mM NaCl in a volume of 0.015 ml and then slowly cooled to room temperature. Deoxynucleoside triphosphates and dideoxynucleoside triphosphates were added to the concentrations given by Sanger et al. (18). To start the reaction, [α-^{32}P]dATP, 1 U of DNA *Pol*I large fragment, and 5 mM dithiothreitol were added, and the mixture was incubated for 15 min at 30°C. Afterward, 0.025 mM dATP was added, and the incubation continued for another 15 min. Reactions were stopped by addition of 10 μl of formamide dye mix–0.1% (wt/vol) xylene cyanol FF–0.1% (wt/vol) bromophenol blue–10 mM Na_2-EDTA–95% (vol/vol) deionized formamide. Sequencing gels (6%) were prepared as described by Sanger and Coulson (17). Usually, there were no ambiguities in the sequence. Otherwise, a clone of the same *gol* mutation was sequenced in the opposite orientation. Such confirmation proved necessary for *gol*2AP-13, since it was difficult to determine which T in a string of three had mutated.

Complementation by T4 gene 23 in plasmids. The complementation method was essentially that of Jacobs et al. (6). *E. coli* B834 containing the plasmid was grown overnight in LB broth plus 20 μg of ampicillin per ml, at 37°C. This was diluted 1:100 into the same medium, grown with shaking to an optical density of 0.4 at 625 nm, and infected with T4 *amB*17 at a multiplicity of infection of 0.1. After 3 min, they were diluted 1:100 into LB without ampicillin, and after 90 min, they were treated with $CHCl_3$, diluted further, and plated with amber-suppressing indicator bacteria to measure the phage yield. The number of infected bacteria was taken to be the number of input viruses.

Materials. [α-^{32}P]dATP was from New England Nuclear Corp. (400 Ci/mmol) or Amersham Corp. (800 Ci/mmol). Deoxynucleoside triphosphates, dideoxynucleoside triphosphates, M13mp7 replicative form DNA, and 26-bp primer were all from Bethesda Research Laboratories. Tetracycline, chloramphenicol, and the sodium salt of ampicillin were from Sigma Chemical Co. *Eco*RI, *Hin*dIII, *Hpa*II, DNA polymerase I large fragment (Klenow fragment), and T4 DNA ligase were from Bethesda Research Laboratories. *Acc*I was from New England Biolabs, Inc., and RNase A was the 5 times crystallized product of Sigma.

RESULTS

Sequencing DNA from *gol* mutants. According to genetic mapping data, *gol* mutations lie in gene 23 close to the amber mutation *B*17. A partial restriction map of this region is shown in Fig. 1. The entire region is carried on a 3.5-kilobase (kb) *Eco*RI fragment, which includes all of gene 23 (6, 9). The plasmid pLA1 (Fig. 1) has this T4 fragment cloned into the *Eco*RI site of plasmid pBR322 (6). Treatment of the T4 insert with *Hin*dIII will give rise to four fragments. One of these is about 1.1 kb and contains the N terminus of gene 23, so it should also contain the region of *gol* mutations. This entire *Hin*dIII fragment has been sequenced (A. Christensen, and E. T. Young, personal communication) so that the *Hpa*II recognition sites can be positioned as can the probable location of some gene 23 amber mutations. Digestion with *Hpa*II should generate five fragments of 460, 180, 75, 270, and 155 bp. The amber mutation *B*17 probably occurred

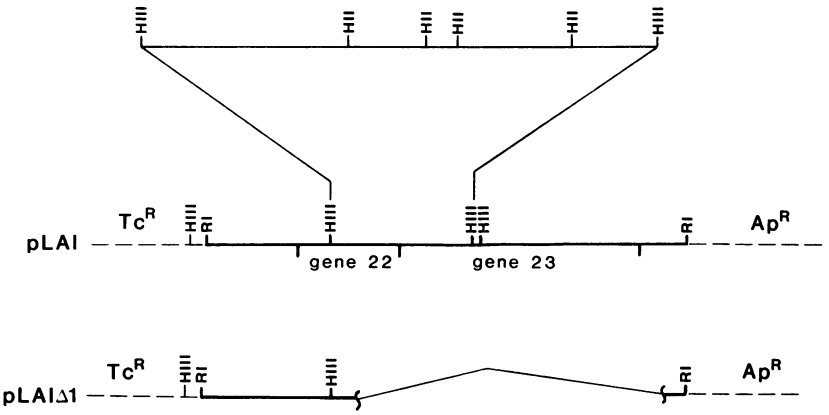

FIG. 1. Restriction map of the *gol* region of the T4 genome and the site of insertion into pBR322 of the T4 insert in plasmid PLA1. The region deleted in the plasmid pLAΔ1 is also shown. The *Hin*dIII restriction fragment harboring *gol* mutations has been expanded to show the relative positions of *Hpa*II sites. The sizes of the fragments after digestion with *Hpa*II are, from left to right, ca. 460, 180, 75, 270, and 155 bp (6; A. Christensen, personal communication). Abbreviations: HIII, *Hin*dIII; RI, *Eco*RI; HII, *Hpa*II; Ap^r, ampicillin resistance; Tc^r, tetracycline resistance; and ⌒, deleted region.

in the 270-bp fragment (A. Christensen, personal communication), and since *gol* mutations are very closely linked to *amB*17, they may also be in this fragment. But, rather than try to clone this *Hpa*II fragment directly, we used marker rescue (see above) to lead us to the correct *Hpa*II fragment, in case the genetic mapping data was deceptive.

To clone the *gol* region from a number of *gol* mutants, we first prepared cytosine-containing T4 DNA from *gol* mutants (see above), restricted the DNA with *Eco*RI, and separated the fragments on agarose gels. As a marker, we coelectrophoresed the plasmid pLA1, also treated with *Eco*RI. The fragments of about 3.5 kb from the *gol* mutant DNA were eluted and ligated into the *Eco*RI site of pACYC184. The ligated plasmids were transformed into *E. coli* B834, first selecting for tetracycline resistance (Tc^r), and cells containing plasmids with inserts were identified by chloramphenicol sensitivity (Cm^s). About one in four of the plasmids with inserts had the *gol* mutation, as determined by marker rescue. The remainder were presumably clones of T4 DNA fragments which comigrated with the 3.5-kb fragment and so were discarded. The plasmids from the *gol* mutant clones were then prepared as above.

To purify the 1.1-kb *Hin*dIII fragment, the plasmids were treated with *Eco*RI and *Hin*dIII, and the 1.1-kb fragments were electroeluted from agarose gels. The fragments were further digested with *Hpa*II and subcloned into the *Acc*I site of M13mp7. Recombinant (colorless) plaques were purified, and those with the *gol* mutation were identified with marker rescue tests. Generally, about one in five of the recombinant phage had the T4 insert with the *gol* mutation. The rest were discarded.

The DNA from the recombinant phage was sequenced as above (Fig. 2). All five *gol* mutations had occurred in the 270-bp *Hpa*II fragment, as predicted. Furthermore, in all five cases, there was only 1-bp difference between the *gol* mutant sequence and the wild-type sequence of Christensen and Young. Counting from the end of the 270-bp fragment closest to the N terminus of gene 23, the spontaneous *gol* mutation in strain 6B had changed the T-A at position 207 to C-G; the 2-aminopurine-induced mutation had changed the T-A at position 189 to C-G; and the two hydroxylamine-induced mutations, as well as the mutation induced in a plasmid with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (to be discussed below), all changed the C-G at position 167 to T-A. Thus, all five *gol* mutations lie within 40 bp of each other, and three of them have the same base-pair transition. We have determined the amino acid changes inflicted on the gene 23 protein by the *gol* mutations, as well as the changes which would be inflicted in an open reading frame in register with gene 23 on the other strand. It is important for further discussion that some of the base pair changes are silent in this other reading frame, changing a serine codon to another serine codon. Note that each of the base pair transitions shown, can, by itself, confer the *gol* phenotype on bacteriophage T4, since each is the only change in the 270-bp fragment and *gol* mutants can arise by

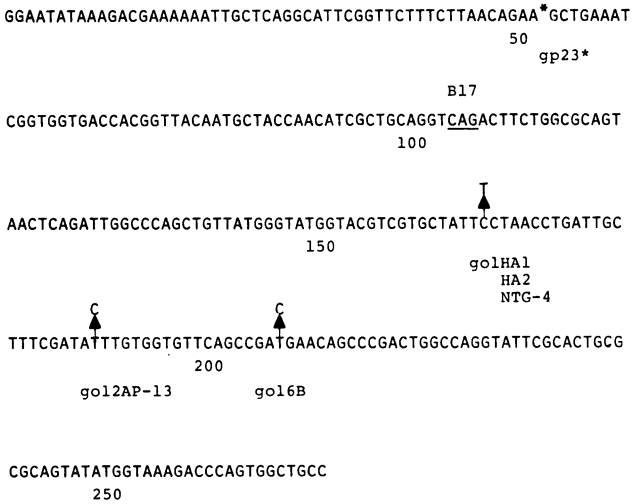

FIG. 2. DNA sequence of the 270-bp *Hpa*II fragment, written as the sequence of the gene 23 message and the changes due to five *gol* mutations. As landmarks, we have indicated the site on DNA that encodes the amino acids between which gp23 is cleaved to give gp23* during phage maturation and the probable site at which *amB*17 occurred.

recombination between each mutant fragment and wild-type T4.

***gol* region can affect plasmid transformation.** Since we had recombinant plasmids containing the *gol* region from T4 bacteriophage, it was a simple matter to try transforming such plasmids into *lit*$^-$ recipient cells. The rationale for the experiments was that since the *gol*$^+$ site can prevent T4 development in a *lit*$^-$ cell, it might also prevent transformation of a *lit*$^-$ cell by a plasmid containing the site. Happily, this expectation was realized. For example, when we used the plasmid pLA1, which, as discussed above, contains the wild-type 3.5-kb *Eco*RI fragment from the *gol* region, for transformation of *E. coli* B834 and the *lit*$^-$ mutant BLK403, thousands of transformants appeared with the *lit*$^+$ strain, but only a few, at most, appeared with the *lit*$^-$ strain (see Table 2). The number of transformants of BLK403 per μg of pLA1 DNA varied greatly from experiment to experiment and from plasmid preparation to plasmid preparation but was always at least 1,000-fold less than the number of transformants of B834. We analyzed six of the transformants of BLK403, and in two of them the plasmid had been deleted in the *gol* region. The other four were phenotypically Lit$^+$. One of the deleted plasmids (pLA1Δ1) is shown in Fig. 1. An analysis of the restriction sites remaining in the plasmid showed that it had suffered a deletion which had removed a substantial part of the T4 DNA insert, including the region of *gol* mutations (data not shown). The other class of transformants, those which were phenotypically Lit$^+$, were presumably due to a few apparent Lit$^+$ revertants in the population of BLK403 cells. The variation in the actual number of the few transformants of BLK403 is thus explained. Plasmid preparations will vary greatly in the percentage of the plasmids which have deletions, and populations of BLK403 cells will vary greatly in the percentage of Lit$^+$ revertants.

To further define the region which prevents transformation, experiments were done with subclones of the 3.5-kb *Eco*RI fragment. A series of plasmids which are derived from pLA1 have been constructed and described previously (16). These were tested to see if they could transform *lit*$^-$ *E. coli* (Table 2). The plasmid pLA4, which contains the 1.1-kb *Hin*dIII fragment and therefore also contains the region of *gol* mutations, did not transform BLK403 cells. In contrast, pLA5, which contains only the 1.4-kb *Hin*dIII-*Eco*RI fragment to the right of *gol* mutations and therefore does not include the *gol* region, was capable of transforming BLK403 cells. Thus, there is a correlation between the presence of the 1.1-kb *Hin*dIII fragment and the inability to transform *lit*$^-$ cells. To determine whether all of the necessary sequences are contained in the 1.1-kb fragment, we cloned this fragment from T4 Dec8 into the *Hin*dIII sites of pACYC184 and pBR325. In either of these plasmids, the fragment was able to prevent transformation of BLK403 cells. Thus, the *Hin*dIII fragment has all the sequences necessary to prevent plasmid transformation.

However, the behavior of plasmid pLA3Δ2 suggests that, even though the *Hin*dIII fragment is necessary to prevent transformation, it is not always sufficient, and sequences outside this region can influence the action of the *gol* site. The plasmid pLA3, from which pLA3Δ2 was derived, has the *Eco*RI fragment cloned in the inverse orientation from pLA1 and, as expected, does not transform BKL403 cells. However, pLA3Δ2, which has had a large segment of the carboxy terminus of gene 23 removed by exonuclease III digestion but still has the 1.1-kb *Hin*dIII fragment from the *gol* region (6), does transform BKL403 cells (Table 2). It was possible that pLA3Δ2 had a sequence change in the *gol* region. However, when we subcloned the 1.1-kb *Hin*dIII fragment from pLA3Δ2 into the *Hin*dIII site of pBR325 or pACYC184, it was indistinguishable from the same fragment from wild-type T4 in that it prevented transformation of BKL403 cells. We conclude that the *gol* region in pLA3Δ2 is not altered, but rather that the deletion of sequences outside the 1.1-kb fragment in pLA3Δ2 prevents the effect of the *gol* region on plasmid transformation.

TABLE 2. Ability of plasmids to transform *E. coli* BKL403 and B834[a]

Plasmid	*Hin*dIII fragment (1.1 kb)	*E. coli* B834	*E. coli* BKL403
pBR322	−	+	+
pLA1	+	+	−
pLA3	+	+	−
pLA1Δ1	−	+	+
pLA4	+	+	−
pLA5	−	+	+
pLA3Δ2	+	+	+
pACYC184	−	+	+
pA67	+	+	−
pG6B	+, but with *gol* mutation	+	+
pGHA1	+, but with *gol* mutation	+	+
pGHA2	+, but with *gol* mutation	+	+
pG2AP-13	+, but with *gol* mutation	+	+
pA83	+	+	−

[a] Transformation was carried out at 30°C as described in the text. The antibiotic resistance selected depended upon the plasmid used and did not affect the result. If transformation occurred normally, there were always >1,000 transformants per μg of plasmid DNA and a plus is assigned. If transformation could not occur without a genetic rearrangement of some sort, the number varied from plasmid to plasmid and from experiment to experiment but was always less than 10 transformants per μg, and a minus is assigned. + or − after the plasmid refers to whether the plasmid has the 1.1-kb *Hin*dIII fragment from the *gol* region of T4 (see Table 1).

If the effect of the *gol* region on plasmid transformation is analogous to its effect on T4 gene expression, we might not expect *gol* mutant clones to have the same effect. Accordingly, we investigated the transformation ability of pACYC184 recombinant plasmids into which had been cloned the 3.5-kb *Eco*RI fragments from *gol* mutant T4. The plasmids which have the *gol* mutant inserts, referred to as pG6B, pGHA1, pGHA2, and pGAP-13 in Table 2, were able to transform BKL403 cells, in contrast to a plasmid, pA67, which has the same insert, in the same orientation, but which was derived from wild-type (*gol*$^+$) T4. Thus, the same base-pair changes which permit T4 late gene expression in a *lit*$^-$ host, also allow a plasmid to transform *lit*$^-$ recipients, leaving little room for doubt that it is the *gol* site itself which is preventing transformation.

Inducing *gol* mutations in plasmids. We reasoned that since *gol* mutations permit transformation of *lit*$^-$ cells by plasmids containing the *gol* region, the converse may also be true; mutations induced in the plasmid which permit transformation may be *gol* mutations. We anticipated one important difference. When *gol* mutations are selected in the phage for permitting multiplication in a *lit*$^-$ host, they must, by necessity, leave the function of the gene 23 protein intact since this protein is required for phage development. There is no such restriction on mutations selected in plasmids, so they might define a broader class of *gol* mutations.

To induce point mutations in the *gol* region, we mutagen-

TABLE 3. Complementation of an amber mutation in gene 23[a]

Plasmid	Phage produced	Phage per infected cell
pBR325	10^7	0.2
pLA3-mutant 1	5×10^7	1.2
pLA3-mutant 2	10^7	0.2
pLA3-mutant 3	10^7	0.2
pLA3	4.8×10^8	12.0

[a] Experimental procedures are described in the text. Mutant 3 has a short deletion of about 100 bp, which removes the *gol* site and presumably inactivates gene 23. The phage yield after complementation by pLA3 is low, in agreement with the results of Jacobs et al. (6).

ized cells containing the plasmid pA83 with NTG for three cycles, and the plasmids were prepared and used to transform BKL403 cells. Four transformants were chosen for further analysis. The plasmids prepared from them were found to transform BKL403 cells with high efficiency, even though no deletions were detectable in the plasmids. We assume that they all have point mutations or short deletions which allow them to transform *lit*$^-$ recipients. To determine whether the mutations could confer the *gol* phenotype on T4, the plasmids were first transformed into *E. coli* B834. Then plasmid-containing cells were tested to see if they yielded *gol* mutant recombinants after infection by wild-type T4, as in the marker rescue tests. Only one of the four yielded *gol* mutant recombinants. This mutant, *gol* NTG-4, was one of those sequenced and has the same base change as two *gol* mutants induced in the phage with hydroxylamine (Fig. 2). We have repeated this experiment starting with the plasmids pLA1 and pLA3 with essentially the same results; about one in four point mutations which permit transformation of *lit*$^-$ cells confer the *gol* phenotype on T4 bacteriophage.

Those mutations induced in the plasmid which do not confer the *gol* phenotype may merely inactivate gene 23 and thus make T4 with the mutation inviable, or they may alter some other function associated with the *gol* phenotype. As a preliminary step, we investigated the ability of two such mutant plasmids, derived from pLA3, to induce an active gene 23 protein. The experiment is based on the observation (6, 9) that gene 23 in a plasmid will be induced after infection by T4 and will complement gene 23 mutants of the superinfecting virus. If the gene 23 on the plasmid is inactivated by a mutation, no complementation should occur (Table 3). Neither of the two mutant plasmids, referred to as mutant 1 and 2 in Table 3, complemented the amber mutation in gene 23 of the superinfecting virus. Thus a sufficient (but not necessarily exclusive) reason they do not confer the *gol* phenotype is that they inactivate the product of gene 23.

DISCUSSION

On the basis of genetic and physiological evidence, we had proposed that *gol* mutations of T4 bacteriophage defined a *cis*-acting site which could prevent all T4 gene expression late in infection. Somehow, one site on the DNA can prevent the expression of the entire T4 genome, including genes transcribed with both polarities and genes which lie considerable distances from the site itself. Our evidence was that *gol* mutations, which overcome the effect of the site, are *cis*-acting for gene expression and are closely clustered at one site in gene 23 of the virus, although they do not act by altering the product of gene 23. In this paper, we have presented DNA sequencing data which identify the mutational changes in each of five independent *gol* mutations. We have also shown that the *gol* site is at least partially active in plasmids, in the absence of the T4 genome, which should greatly facilitate studies of the function of the site.

The DNA sequencing data have confirmed the genetic mapping data; all five *gol* mutations which were sequenced had single-base-pair transitions within 40 bp of DNA, very close to 23 *amB*17 in the part of the DNA coding for the N terminus of gene 23. Some clustering of *gol* mutations is evident. Three mutations induced with two different mutagens had changed the same base pair. Two of these were induced in the phage with hydroxylamine, and one was induced in a plasmid with NTG.

The sequence results also confirm that the *gol* phenotype is not due to an altered protein. The DNA sequence reveals the existence of two open reading frames in the *gol* region: one the gp23 coding sequence and the other the frame in register with gp23 on the other strand (Fig. 2) (A. Christensen, personal communication). The gene 23 protein is not required because nonsense mutations in gene 23 on either side of, and very close to, *gol* mutations do not prevent or alter in any way the Gol phenotype (3). We dismiss as very unlikely the possibility that residual gp23, made as a result of ribosome ambiguity, is responsible. Furthermore, the gene 23 protein will be expressed only at low levels, if at all, from plasmids (7), and some plasmids, such as pA83, which have *gol* site activity, have only about one-third of gene 23 in the T4 insert. As for the other reading frame, there is no indication that it encodes a protein, and, even if it does, some *gol* mutations are silent in this frame. Thus, we think there is almost no possibility that the *gol* phenotype is due to an altered protein. This, of course, does not mean that a protein does not bind to the *gol* site, or is involved in some other way, only that *gol* mutations do not exert their phenotype by altering a protein.

The structural significance of the mutational changes in creating the *gol* phenotype is not obvious from the sequence data thus far. Nevertheless, it is worth noting some of the unusual characteristics of the region where *gol* mutations lie. The DNA has an unusually high guanine-plus-cytosine (G+C) content; the average G+C content of T4 DNA is about 30%, and the region around *gol* mutations has a G+C content of about 70%. Also, this region is recombinationally more active than average. The average recombination frequency for most of gene 23 is constant, but there is a hotspot for recombination in the amino terminus, apparently in the region where the *gol* site is located (2). We do not know if the *gol* site is responsible for the high recombination frequency, but we have not found any effect of *gol* mutations on recombination between amber mutations in this region (data not shown). Another interesting sequence is the G-C-G-C-G-C about 30 bp from the nearest *gol* mutation. This can be extended to eight alternating purines and pyrimidines if one counts from the T preceding the sequence, to give T-G-C-G-C-G-C-A. If one then proceeds beyond the C which precedes and the G which follows the 8 bp, a total of 18 out of 20 base pairs are alternating purine and pyrimidine. Again there is no evidence linking this sequence to the *gol* phenotype. A final observation concerns the extensive symmetries in the *gol* region. If one draws some of the possible secondary structures of a single-stranded polynucleotide with 100 bp encompassing the *gol* region, all three transitions known to give the *gol* phenotype lie in unpaired regions (G. Stormo, personal communication). The significance of this, as with the other observations, is unclear.

The clustering of *gol* mutations at position 167 and probably at position 207 suggests that these sites may define different functions or different domains of the *gol* site. However, we have found no differences between *gol* mutations at different sites. The *gol* mutations at both sites 167 and 207 are *cis*-acting, and phage with each of the mutations do not interfere with the gene expression of each other in mixed infections of *lit*$^-$ hosts (data not shown).

The experiments with plasmids containing T4 DNA inserts show that the T4 *gol* site also affects transformation; i.e., in general, the presence of the wild-type *gol* site in a plasmid prevents transformation of a *lit*$^-$ recipient by that plasmid. The evidence is very good that the site on T4 DNA which is preventing plasmid transformation of *lit*$^-$ recipients is also the site which is preventing T4 gene expression late in infection of *lit*$^-$ hosts. Probably the strongest evidence is that *gol* mutations, which overcome the effect on T4 gene expression, also permit transformation of *lit*$^-$ recipients, and, conversely, that about one-fourth of the point mutations induced in a plasmid which permit transformation of *lit*$^-$ recipients also confer the *gol* phenotype when crossed into T4 bacteriophage. The other three-fourths are easily explained. At least some of them inactivate the product of gene 23 and so are lethal when crossed into the bacteriophage genome. These other mutations should reveal a broader class of changes affecting the activity of the *gol* site, and some may be very revealing of its molecular structure and properties.

Although mutations which confer the *gol* phenotype are clustered within 40 bp of each other in the *gol* site, it seems clear that sequences outside this site can influence the activity of the site on plasmids. From the behavior of pLA3Δ2, which can transform *lit*$^-$ recipients because of a deletion which ends almost 1,000 bp from the nearest *gol* mutation, we anticipate that the orientation and site of insertion in plasmids will determine whether the *gol* site can prevent plasmid transformation. We can imagine mechanisms by which sequences outside the *gol* site exert their influence. For example, the *gol* site could require transcriptional activation. The behavior of pLA3Δ2 is consistent with the transcriptional activation hypothesis. The *Eco*RI site in pBR322 is transcribed in both directions (24), so both strands of the T4 DNA insert will be transcribed in both pLA1 and pLA3. However, the leftward promoter is probably deleted in pLA3Δ2, so only the R (late) strand of pLA3Δ2 may be transcribed. Thus, if it is transcription of the L (early) strand which is required, the *gol* site in pLA3Δ2 would not be activated. In this regard, it is intriguing that gene 23 is transcribed at a low level early in infection (29), presumably on the L strand. We also have observed transcription of the L strand of the *gol* region by dot blots (R. Green, unpublished data), but do not yet know if this transcription is required for the activity of the *gol* site.

Because the *gol* site is active on plasmids, in the absence of the T4 genome, it is apparent that *E. coli* gene products interact directly with the site. However, plasmid transformation is inefficient, so it would be difficult to determine how the *gol* site blocks plasmid transformation. We recently cloned the *gol* site into λ bacteriophage, and it prevents λ multiplication in *lit*$^-$ hosts (K. Bergsland, unpublished data). Thus, we are now in a better position to ask what happens when the *gol* site of T4 bacteriophage enters a *lit*$^-$ bacterium.

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