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THE GOL SITE: A BACTERIOPHAGE T4 REGULATORY REGION THAT CAN AFFECT EXPRESSION OF ALL THE T4 LATE GENES

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THE *GOL* SITE: A BACTERIOPHAGE T4 REGULATORY REGION THAT CAN AFFECT EXPRESSION OF ALL THE T4 LATE GENES

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ABSTRACT

THE *gol* SITE: A BACTERIOPHAGE T4 REGULATORY REGION THAT CAN AFFECT EXPRESSION OF ALL THE T4 LATE GENES

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Escherichia coli mutants have been isolated which prevent T4 late (replication coupled) gene expression at temperatures below 34°C., primarily by acting at the level of transcription. The *E. coli* mutants have been mapped to 25 min., where they define a new gene, *lit*.

Because of the severe block to late gene expression, wild-type T4 does not form plaques on *lit*$^-$ mutant hosts at low temperatures. T4 mutants which have been named *gol*, for *g*row *o*n *l*it$^-$ hosts, are able to form plaque.

In a mixed infection of a *lit*$^-$ host by *gol*$^+$ and *gol*$^-$ phage, the *gol* mutation is able to restore gene expression only in *cis*. All *gol* mutations map within the coding region for p23, the major T4 capsid protein. However, *gol* mutations do not exert their effect by altering p23. Rather, they seem to affect a site on T4 DNA, which in the wild-type form interferes with gene expression in a *lit*$^-$ host. The nucleotide sequence of several *gol* mutations has been determined. However, from the sequence data accumulated thus far, the structural significance of the *gol* mutations is not clear. I speculate that the T4 *gol* site plays a role in the template processing that has been proposed to be required for T4 late gene expression.

Besides affecting T4 late gene expression in a *lit*$^-$ host, the T4 *gol* site has an effect when cloned into plasmid DNA. If a plasmid contains a fragment of T4 DNA which includes the wild-type *gol* site, it cannot be used to transform a *lit*$^-$ host, but can be used to transform a *lit*$^+$ host. However, if the plasmid contains a T4 fragment which is *gol*$^-$ or is deleted for the *gol* region, it can be used to transform a *lit*$^-$ host. Therefore, the presence of the wild-type *gol* site in a plasmid prevents the stable transformation of *lit*$^-$ *E. coli*.

The ability of the T4 *gol* site to affect both T4 and plasmid DNA may reflect that a common structure is shared by the two DNAs; further study of the *gol* site may reveal the *in vivo* function of such a DNA structure.

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INTRODUCTION

Despite the wealth of current knowledge about transcriptional regulation in prokaryotes, the control of the expression of the T4 late genes remains an intriguing mystery. It has been known for some time that T4 late gene expression is coupled to concurrent replication and it is thought that a structural alteration of the template occurs, during replication, which activates the late promoters. The nature of the template alteration is not known and it has not been possible to reproduce, *in vitro*, the *in vivo* state of the "competent" T4 late transcriptional template.

It is becoming increasingly evident that "promoter-operator" models for transcriptional regulation do not completely describe all regulation, even in prokaryotes. Mechanisms operating in T4 phage may well also operate in *E. coli* and higher organisms, as well. Therefore, elucidation of T4 late transcriptional regulation may add to our understanding of regulatory mechanisms in general.

Another aspect of the problem of late gene expression in T4 phage is that of coordinating transcription with other intracellular events. The phage life cycle requires that one to two hundred phage equivalents of DNA be replicated, an amount of DNA which is greater than ten times that of the bacterial nucleoid. The intracellular DNA is in a highly complex, condensed structure, in which recombination is very active. The late transcriptional units are highly active in order to produce the structural proteins for at least one hundred phage. Furthermore, encapsidation of DNA into phage heads begins at a

time when all these events are actively occuring; therefore replication, recombination, transcription and packaging all occur simultaneously. It seems likely that mechanisms exist which regulate the coordination of these events; analogous mechanisms might operate in the even more complex coordination problems of higher cells.

One approach to the study of T4 late transcriptional regulation, taken in our laboratory, has been to study T4 genes which encode functions involved in late transcription. One such gene, pseT, encodes the T4 5' polynucleotide kinase, 3' phosphatase. The pseT gene product is required for T4 late gene expression on the E. coli strain CTr5x (K. Sirotkin, W. Cooley, J. Runnels and L. Snyder, 1978). The in vitro activities of the enzyme suggest that it might have the capability, and in vivo role, of altering T4 DNA structure to make it competent for late transcription. Also, it seemed that E. coli encodes at least one function, which is altered in CTr5x, which is involved in T4 late gene expression. If so, mechanisms for transcriptional regulation, similar to those operating in T4 might exist in E. coli; this possibility inspired further study of CTr5x restriction of pseT$^-$ mutants.

In the hope of simplifying genetics and biochemical analysis of the pseT$^-$-restrictive locus in CTr5x, attempts were made to isolate host mutants, in a K-12 background, that would mimic the pseT$^-$-restrictive phenotype of CTr5x.

The E. coli mutants which resulted from a search for "CTr5x-like" mutants have been called lit and are described in the publication in Appendix A (Cooley et al., J. Bacteriol. 140:83-91, 1978). Lit mutants restrict pseT$^-$ deletions but not pseT$^-$ point mutations at 37^{o}C. At temperatures below 34^{o}C, even wild-type T4 is restricted. T4 fails to

multiply on *lit*$^-$ hosts because of a severe block to T4 late gene expression at the restrictive temperature.

Spontaneous T4 mutants arise which can form plaques on *lit*$^-$ host mutants at the restrictive temperature. These T4 mutants have been called *gol*. In Article I, which is published in J. Mol. Biol. 155:395 (1982), evidence is presented that *gol* mutations alter a site on T4 DNA which affects the expression of all the late T4 genes. The *gol* site may be involved in forming or maintaining the DNA template structure which is thought to be required for T4 late gene expression. Such a site may be a prototype for a new class of prokaryotic regulatory sequence.

Besides affecting T4 gene expression, the *gol* site affects the transforming capability of a plasmid which contains it, i.e. a plasmid which contains a *gol*$^+$ sequence will not transform a *lit*$^-$ host. In contrast, if the plasmid contains a *gol* mutant sequence or is deleted for the *gol* region, it can transform a *lit*$^-$ host. Therefore, the *gol* sequence must affect the replication, expression or segregation of plasmids which contain it. This work, as well as DNA sequence analysis of *gol* mutations, is described in Article II.

The literature survey discusses work that has been done on T4 late transcription, both *in vivo* and *in vitro*. Because some aspect of T4 DNA structure is required for late transcription, studies on intracellular T4 DNA are reviewed. Finally, regulatory mechanisms which involve DNA structure and which may be relevant to T4 late transcriptional regulation are discussed.

In the final section, the results presented in Articles I and II and the appendicies are discussed, and recommendations for further study are made.

LITERATURE SURVEY

The T4 Genome

The T4 genome is 1.66 kilobase pairs, enough to code for approximately 160 genes. The linear molecules from phage particles are slightly longer because they are terminally redundant. They are also circularly permuted, giving use to a circular genetic map. About 140 of the T4 genes have been characterized. (Wood and Revel, 1976)

The T4 transcripts can be classified into three major groupings: 1) those which are expressed prior to DNA replication; 2) those which are expressed after DNA replication and 3) those which are expressed throughout infection. The first category, the "early genes," includes the genes whose products function in phage DNA metabolism. Genes in the second category, the "late genes," encode phage assembly functions. The late gene products begin to appear 2.5 to 3.5 minutes after DNA replication begins, which usually occurs at 5 to 6 minutes after infection at $30^{o}C$. Most early protein synthesis starts to be shut off at about the time late synthesis begins, but there is a class of genes, the quasi-lates, which are expressed both early and late. The only quasi-lates functions identified are the T4 tRNAs. (Young et al., 1980).

The T4 genes are arranged on the genome such that the early genes are clustered into two major groups and the late genes are clustered into three major groups, separated by the early clusters. (Wood and Revel, 1976). Within each group are multiple transcription units; many genes are expressed as both mono and multicistronic transcripts (Young and Menard, 1981). The early genes are all transcribed with the same polarity, off

the "l" strand (Notani, 1973). The late genes are transcribed off the "r" strand (Guha et al., 1971).

The rate of chain elongation in T4 is 1100 nucleotides per minute at 30°C, about one half the rate in uninfected E. coli. In general, but with some exceptions, the mRNAs are immediately translated.

Prereplicative (Early) Transcription

Immediate early and delayed early

Within the prereplicative period, the first 5-6 minutes after T4 infection, many species of early proteins appear in a defined temporal sequence (O'Farrell and Gold, 1973). More than one regulatory class of prereplicative messages exists, based on the following evidence (reviewed in Rabussay and Geiduschek, 1977): 1) the mRNA synthesis of a small number of early proteins cannot be initiated within the first one to two minutes after infection (O'Farrell and Gold, 1973), and the synthesis of these proteins is sensitive to the addition of rifamycin added immediately after infection; 2) the transcription of some genes does not occur in the absence of protein synthesis; and 3) the relative abundance of mRNAs changes between five and twenty minutes (Salser et al., 1970). The different regulatory classes have generally been termed immediate early (IE), delayed early (DE), and quasi-late.

Immediate early genes are proximal to promoters; delayed early genes are distal to promoters and appear after a time lag of 1.5 minutes at 30°C (Salser et al., 1970). Synthesis of the delayed early genes is sensitive to chloramphenicol addition (Brody et al., 1970). The chloramphenicol block is probably due to induced polarity (Young, 1975) mediated by rho (Caruso et al., 1979; cf Young and Menard, 1981).

Recently, Young et al (1980) measured the levels of early mRNAs which hybridized to selected early gene clones. They found that messages for genes 30, 39, 52, 41, 42, βgt and the tRNAs appeared first, were made at their highest rates between zero and four minutes and were insensitive to the addition of chloramphenicol. These genes would conform to the designation "immediate early genes." Messages for genes 43, rIIA and rIIB appeared after those for the IE genes, peaked during four to eight minutes, and were not made in the presence of chloramphenicol (CAM); these genes would be the "delayed early genes."

The CAM sensitivity of the switch-on of the delayed-early genes suggests that a phage-coded factor plays a role in the switch from immediate-early to delayed-early synthesis. Mutants have been isolated which may be in such a factor (Mattson et al., 1974). These mutants, called mot, reduce the expression of tRNA, 32, rIIB, 43, 45 and 46, but delay the shut-off of 44, rIIA and 52. An in vitro RNA synthesizing system has been developed (Thermes et al., 1976) in which the mot phenotype and the chloramphenicol effect of restricting transcription to promoter-proximal regions can be reproduced.

The proposal has been made (cf Pulitzer et al., 1979) that mot acts as an antiterminator. However, this proposal remains unproven and the assignments of genes to the DE versus IE categories by mot effect do not entirely correspond to the kinetics of mRNA production, as measured by Young et al (1980)

Sizing of early mRNAs has shown that some of the messages are synthesized in multiple transcripts, lending support to the model that DE mRNAs are synthesized by readthrough from IE genes (Young and Menard, 1981). However, attempts to size the CAM RNA of early genes

in order to show that CMA RNA is shorter than normal transcripts, were unsuccessful (Young and Menard, 1981) because extensive degradation of RNA, both in the presence and absence of CAM, makes sizing RNAs difficult. Thus, the early regulatory switch between IE and DE synthesis is not yet well understood.

Quasi-Lates

The prereplicative transcripts which are present at five minutes but increase at twenty minutes have been called the "quasi-lates." Based on the presence of the gene product both before and after replication, several genes have been identified as quasi-lates, including 32, 41, 43, 44, 63, 57 and the internal head proteins (cf Wood and Revel, 1976). However, by following the kinetics of hybridization of pulse-labeled mRNA to cloned early genes, Young et al. (1980) did not find that the mRNA of genes 41 and 43 increased at twenty minutes; they did not study the mRNA of other putative quasi-lates. They did find that the synthesis of the tRNAs showed quasi-late behavior.

Shutoff of early genes

The synthesis of most early gene products, with the exception of the quasi-lates, ceases after 12 to 14 minutes of infection at $30^{o}C$ (Wiberg et al., 1962; Hosoda and Levinthal, 1968). The synthesis of many early genes is not shut off synchronously (O'Farrell and Gold, 1973) and some of the genes are autogenously regulated (reviewed by Rabussay and Geiduschek, 1977). Thus, a variety of regulatory mechanisms are important in early gene shutoff.

To a large extent, early gene shut off occurs as the result of transcriptional regulation (Bolle et al., 1968a; Salser et al., 1970).

However, post transcriptional mechanisms also operate because, for many early proteins, the stability of early mRNAs decreases at the time early shutoff occurs (Sauerbier and Hercules, 1973). Furthermore a gene has been identified, reg A, which can affect the stability of early messages. In reg A mutant infections, many early messages have greater stability, an effect which is enhanced if replication does not occur.

In general, when replication does not occur, the early gene products continue to be synthesized (Wiberg et al., 1962; Bolund, 1973). Also, in infections in which gene 55 is mutant (and therefore late protein synthesis is not turned on) early gene expression does not cease (Bolund, 1973). Because late genes are also not expressed in replication-defective infections, these observations could be interpreted to mean that a late gene product plays a role in early shutoff. But recently, several situations have been described in which early gene shutoff occurs even though no late gene expression occurs. These situations are 1) in an infection in which cytosine DNA rather than hydroxymethyl cytosine, DNA is produced (Snyder et al., 1976); 2) RNA ligase or pseT mutant infections of the host CTr5x (Runnels et al., 1982); and 3) wild-type T4 infections of lit$^-$ hosts at 30^{o}C (Champness and Snyder, Article I of this dissertation). It therefore seems that the simple explanation that a late gene product shuts off early gene expression must not be correct.

Postreplicative (Late) Transcription

The post-replicative gene products begin to appear about 2.5 to 3 minutes after the onset of replication; i.e., at about 8.5 to 9.5 minutes after infection at 30^{o}C. (Bolle et al., 1968a; O'Farrell and Gold, 1973). The temporal regulation of late gene expression is at the level of mRNA transcription (Young et al., 1980).

The late genes are distributed into three clusters which are separated by regions encoding early genes (Wood and Revel, 1976). Because most of the late genes are transcribed from the "r" strand, with an opposite polarity to the early genes (Guha et al., 1971; Notani, 1973), they cannot be transcribed by readthrough from early promoters. Rather, many new promoters must be utilized. Information about the location and utilization of late promoters comes from a recent study of transcription of the contiguous late genes 21, 22 and 23 (Young et al., 1981). The late genes 21, 22 and 23 are co-transcribed as two minor polycistronic messages and gene 23 is also transcribed as a major monocistronic message. Because the three messages have the same distribution in both steady-state and pulse-labelling conditions, it seems likely that the multiple messages result from the use of multiple promoters and not from processing of a single large precursor.

Regulation of late gene expression

Transcription of the T4 late genes requires the association of several T4-specified proteins with the host RNA polymerase. Early after infection, the RNA polymerase is modified by ADP-ribosylation of the α subunits (Goff, 1974). Several low molecular weight polypeptides bind to the modified RNA polymerase. These include the products of genes 33, 55 and 45 (Horvitz, 1973; Ratner, 1974), and 15K and a 12K dalton protein (Stevens, 1972). The products of genes 33, 55 and 45 are directly and continuously required for late transcription (Bolle et al., 1968b, Pulitzer, 1970; and Wu et al., 1975). The product of gene 45 is required directly, in transcription, and indirectly, in replication (Wu et al., 1975).

In addition to modification of RNA polymerase, T4 late gene expression is thought to require processing of the DNA template. This idea has resulted from the observation that late gene expression is normally coupled to concurrent DNA replication (Riva et al., 1980), but in the absence of replication, mutations in genes which lead to the formation or stabilization of interruptions in DNA permit late gene expression (Bolle et al., 1968b; Hosoda and Levinthal, 1968; Wu et al., 1975). Therefore, it has been proposed (Riva et al., 1970b) that nicks or gaps in DNA might be required for late transcription.

Because late gene expression is replication-coupled it is appropriate at this point to discuss T4 DNA replication.

T4 DNA Replication

T4 replication proteins

The replication complex consists, at least, of the products of genes 43 (DNA Polymerase), 32 (helix-destabilizing protein), 44 and 62 (a complex with DNA-dependent ATPase activity), 41 (DNA-dependent ATPase activity), 45 (which stimulates the activity of the products of gene 44 and 62) and 61. The above complex can be reconstituted to carry out replication *in vitro* (Hibner and Alberts, 1980). The *in vitro* system mimics *in vivo* replication with respect to rate of chain elongation, RNA priming of Okazaki fragments and the general structure of the replication fork. In addition to the above proteins, other T4 proteins, including the products of gene 1 (deoxynucleotide Kinase) and 42 (cCMP hydroxymethylase) may have structural roles in replication.

Recently, a T4 Type II topoisomerase has been identified as the product of genes 39, 52 and 60. Mutants in these genes had been

characterized as the DNA-delay mutants because DNA replication rates in 39^-, 52^- or 60^- infections are lower and delayed relative to wild-type (Naot and Shalitin, 1973; Mulfi and Bernstein, 1974). The T4 topoisomerase probably acts at replication forks (McCarthy, 1979). Topoisomerase mutants (DNA-delay) are cold-sensitive and vary in the severity of their replication defects on B versus K-12 strains. These phenomena may reflect that an E. coli enzyme (probably gyrase) can substitute for the T4 topoisomerase under some conditions.

The replication process

The first event after T4 injection into the cell is attachment of the DNA to membrane, at what will be the origin of replication (cf Siegel and Schaechter, 1973). DNA-membrane attachment occurs without phage protein (Earhart, 1970) or DNA synthesis (Earhart et al., 1973), but some RNA synthesis is required. A candidate for the required RNA is an RNA-DNA copolymer (Buckley and Kozinski, 1972) which is synthesized early, hybridizes to the "1" strand and is an obligatory intermediate in DNA synthesis.

Attachment of the DNA to membrane is sensitive to energy poisons but this sensitivity is reversible after removal of the poison (Earhart et al., 1973).

Within five minutes after infection, deoxynucleotides are added to the parental DNA, increasing the size of the parental molecules by 6% (Murray and Mathews, 1969a). This addition occurs in DO mutants and is apparently mediated by E. coli enzymes. Surprisingly, this "early DNA" does not hybridize to mature T4 DNA at a detectable level (Murray and Mathews, 1969a), but does hybridize to vegetative DNA. A possible

explanation, which has not been investigated, is that the early DNA is copied from a sequence which becomes amplified in vegetative DNA. In this regard, Kozinski et al., (1980) have recently found that, in the early stages of replication, the replication origins are repeatedly amplified by replication. The amplification extends through much of the major late cluster of genes.

T4 replication initiates at multiple sites, primarily in the regions of genes 50-5 and 25-29 (Halpern et al., 1979), and proceeds bidirectionally until 20 to 25 phage equivalents are produced. These DNA molecules recombine (Tomizawa, 1967), through the action of the gene products 46, 47, and 59, to produce concatemers (cf. Broker and Doermann, 1975). DNA synthesis is not a prerequisite for concatemer formation because concatemers are formed in a gene 44$^-$ (DNA replciation negative) infection (Murray and Mathews, 1969b). Replication continues in a complexly interconnected structure (Huberman, 1968) which is distributed throughout most of the intracellular volume (Hamilton and Pettijohn, 1976).

Intracellular DNA structure

The complex structure of T4 DNA late in infection is poorly understood, despite considerable experimentation designed with the purpose of describing intracellular DNA structure. The main experimental approach has been to gently lyse infected cells and study the sedimentation behavior of the released T4 DNA. Because different workers have used a variety of lysis procedures, the reported results are difficult to compare. There is agreement among workers (cf. Siegel and Schaechter, 1973) that both rapidly sedimenting and slowly sedimenting forms are seen, but the actual S values of the slow and fast forms vary from report to report.

Altman and Lerman (1970), using a low salt, lysozyme, SDS lysis procedure, reported four differently sedimenting species: 1) "slow" or <100S; 2) "fast" or 170-650S; 3) mature phage 650-950S; and 4) "bottom" or >950-3500S. Within the first five minutes after infection the slow form, consisting of parental DNA, appears. Half of the parental DNA is rapidly converted to >950-3500S, the form in which new DNA appears. This "bottom" DNA has membrane associated with it. Later, some of this DNA is converted to the "fast" form, 170-650S. From this pool, mature phage (650-950S) are drawn. Some of the "bottom" DNA goes to a slow form (<100S) and never is encapsidated. Also, 40% of the DNA in the bottom fraction neither moves to the slow form nor is encapsidated into mature phage.

Cox and Conway (1975a, b) attempted to determine the template properties of the differently sedimenting forms of vegatative T4 DNA. They found that a 150S form appeared at 12 minutes after infection (at $30^{o}C$). When DNA from this fraction was recovered from a sucrose gradient, it was an active template for protein synthesis; the protein products were identified as structural (late) proteins. In contrast, the DNA from their fast fraction, 1000 to 2000 S, was an active template for early proteins. They found that the slow form was missing when the gene 55 product is missing (the product of gene 55 is normally required for late protein synthesis). The absence of a slowly sedimenting form in a gene 55 mutant infection has also been observed by Snyder and Geiduschek (1968). The "slow" DNA could be created by the action of a late gene or could be the template from which late genes are expressed; Cox and Conway (1975b) favored the latter possibility. But the proteins synthesized in their gene 55 mutant infections closely resembled those

produced from their slow DNA template; because it is known that gene 55 mutant infections do not produce late proteins, this discrepancy raises doubt about their identification of "late" proteins.

Mutants which are defective in recombination do not make fast sedimenting DNA (Cox and Conway, 1975b; Shah, 1976) because they are defective in concatemer formation. Nevertheless, they do make significant amounts of late gene products. Concatemers are also not formed in infections by gene 52 mutant (a component of the T4 topoisomerase) infections, which also produce late gene products (Naot and Shalitin, 1973). Therefore, the fast sedimenting form *per se* must not be required for late synthesis.

In summary, the studies that have been done do not lead to conclusions about which DNA fraction is the *in vivo* template for late gene expression.

Many phage mutants which are defective in head-filling accumulate a 200S form of DNA. Phage which are mutant in gene 49 also do not package DNA into capsids; they accumulate DNA in a "very fast sedimenting" form of 1-2000 S (Kemper and Janz, 1976). The DNA, when visualized by electron microscopy, consists of tangles, loops and free ends (Kemper and Brown, 1976). The DNA can be activated for head-filling if an infection with a ts mutant in gene 49 is returned to the permissive temperature.

Curtis and Alberts (1976) further studied the role of the gene 49 product and found that it is involved in creating single-stranded gaps in the DNA at an average length of one to two genome distances. The gaps are probably protected by the gene 32 product. Curtis and Alberts also studied the structure of the fast-sedimenting complexes and found that they were unaffected by Pronase, Proteinase K or pancreatic RNase treatment.

Also, the sedimentation rate was higher than could be accounted for by the amount of DNA, They concluded that the T4 DNA must be condensed into a compact formation, a conclusion that is in agreement with the electron micrographs of intracellular DNA (Huberman, 1968; Kemper and Brown, 1976; Hamilton and Pettijohn, 1976). Curtis and Alberts suggested that the DNA was cross-linked or branched, at least partially because of recombination forks. In light of current knowledge about the ability of T4 topoisomerase to concatenate and knot DNA (Liu et al., 1980) it is likely that some of the compactness of the intracellular DNA may be due to condensation by topoisomerase. This hypothesis is consistent with the finding of Hamilton and Pettijohn (1976) that gp 39 and gp 52 (now identified as topoisomerase components) are associated with compact DNA.

Hamilton and Pettijohn (1976) also attempted to quantitate the amount of supercoiling in T4 DNA, They measured the sedimentation, after ethidium bromide intercalation, of condensed complexes isolated ten minutes after infection. The bulk of the DNA was not supercoiled but one eighth of the DNA appeared to be supercoiled with a superhelical density similar to that of other naturally occurring supercoiled DNAs. The supercoiled DNA seemed to be segregated into domains which were somehow held physically separate from the bulk of unsupercoiled DNA.

Replication-Uncoupled Late Gene Expression

Although late gene expression normally requries concurrent replication, some late gene expression can occur in the absence of replication, for example, in an infection by DO mutant phage (phage which do not synthesize DNA). Such "replication-uncoupled"

transcription is delayed with respect to that which occurs in a wild-type infection, is temperature-sensitive, and depends on high multiplicity of infection (Wu et al., 1975). However, the normal replication dependence of late transcription is not due simply to the need to accumulate DNA or to achieve a high rate of replication because mutations in several genes, including 30 (DNA Ligase) or 46, 47 (required for recombination and concatemer formation), restrict DNA replication but allow significant late transcription (Wu et al., 1975).

Wu and Geiduschek (1977) attempted to clarify the relationship between replication-coupled and replication-uncoupled late transcription by studying the protein requirements for the two modes of late transcription. They found that proteins required for replication-uncoupled transcription accumulate later than the proteins required for normal late transcription. It is known that, in the absence of replication, breaks accumulate in the parental DNA. As discussed earlier, interruptions in the primary DNA structure may be important in late transcription. If so, a protein which is synthesized relatively late may create some of the breaks which are normally created during replication. Nuclease activities which appear late have been described (Altman and Meselson, 1970; Kemper and Hurwitz, 1973); and one of these nucleases could activate the template. There may be an analogy to the T5 bacteriophage system. There is a T5 exonuclease, the product of the D15 gene, which is essential for T5 late transcription (Chinnadurai and McCorquodale, 1973).

Wu and Geiduschek (1977) also found that the product of gene 55 (which is an RNA polymerase-binding protein and required for both replication-coupled and -uncoupled late transcription) interacts differently

with replication-coupled and replication-uncoupled transcription units, possibly through interaction with the DNA polymerase.

In vitro systems

Because of the complexity of the requirements for T4 late transcription, in vitro late transcription systems have been difficult to develop. The first systems were crude preparations from T4-infected cells which could synthesize late RNA in vitro only from an endogenous template (Snyder and Geiduschek, 1968). More recently, Rabussay and Geiduschek (1977), have developed an in vitro system in which a T4-infected cell lysate is gently lyzed and suspended on a cellophane disc. This system initiates late RNA transcripts, yields a high proportion of late transcripts and is active for at least one hour after lysis.

The development of such an in vitro transcription system has allowed preliminary analysis of the in vitro relationship between replication and late transcription. In an attempt to distinguish between actual involvement of the DNA replication proteins in late transcription and a requirement for replication-coupled processing of the late transcription template, Rabussay and Geiduschek (1979) compared the effects on late transcription of metabolically blocking DNA replication by adding FUdR (which blocks DNA synthesis at the level of thymidylate production) and by thermally inactivating the products of the replication genes 42 and 43. These two different ways of interfering with the normal replication process have both quantitatively and qualitatively distinguishable effects on late transcription which mimic their effects in vivo.

In vivo, temperature-sensitive mutations in the two DNA replication genes, gene 43 (DNA polymerase) and gene 42 (dCMP hydroxymethylase),

reduce late transcription at the non-permissive temperature (Rabussay and Geiduschek, 1979). However, although both mutations reduce DNA replication equally, they have different quantitative effects on late transcription, i.e., at high temperature the temperature-sensitive gene 43 mutation reduces late transcription more than the temperature-sensitive gene 42 mutation. The in vitro effects of ts gene 42 and 43 mutations are similar. An in vitro system prepared from a temperature-sensitive gene 43 lysate synthesizes less late RNA than a temperature-sensitive gene 42 system. However, if DNA replication is limited in vitro by the absence of dNTPs, although the temperature-sensitive gene 43 infection produces negligible late transcription, the temperature-sensitive gene 42 system yields as much late transcription as it does at the permissive temperature. This result suggests that the temperature sensitivity of the gene 43 system may be due more to the absence of functional gene 43 product than to the absence of replication.

The addition of FUdR blocks DNA replication severely, but does allow a residual amount of synthesis. In vivo, FUdR addition as early as six to eight minutes after infection at 37^{o}C blocks most DNA replication but allows appreciable late transcription. The in vitro effect of FUdR is similar to the in vivo effect.

The RNA synthesis which occurs in the presence of FUdR may be due to the residual DNA synthesis. Alternatively, the reduced levels of late RNA synthesis in temperature-sensitive gene 43 infections may reflect a requirement for DNA polymerase involvement in late transcription, either by processing DNA to create a "late-transcription competent" template, or by physical interaction with the transcription proteins.

In summary, considerable evidence has accumulated for the interaction of the late transcription complex with DNA replication

proteins including at least the DNA polymerase and the gene 45 product. In addition, it has been recently shown that one of the previously unidentified small molecular weight polypeptides which binds to RNA polymerase is the product of gene 60 (C. Goff, Cold Spring Harbor Phage Meeting, 1981). The product of gene 60 is also a subunit of the T4 topoisomerase (liu et al., 1979).

Effects of Nucleotide Modification on Late Gene Expression

T4 DNA normally contains hydorxymethylcytosine (HMC) instead of cytosine. In addition, the HMC residues are α or β glucosylated. Appropriate phage and host strains can be used to force T4 to incorporate C instead of HMC into its DNA (Revel and Georgopolous, 1969; Runnels and Snyder, 1978), but the substitution of C for HMC prevents late gene expression. Some r-strand RNA is transcribed but has an unknown defect which prevents its translation (Wu and Geiduschek, 1975). The gene responsible for prevention of late transcription from C-DNA (cytosine-containing DNA) has been identified; mutations in this gene, alc, allow late transcription on C-DNA (Snyder et al., 1976).

The role of the alc gene product in late transcription

Besides preventing late transcription from C-DNA, the T4 alc gene product is responsible for unfolding the host nucleoid shortly after T4 infection (Snustad et al., 1976; Sirotkin et al., 1977). Alc-T4 are also defective in the shutoff of some host RNA synthesis. (Sirotkin et al., 1977; Tigges et al., 1977). Because the supercoiled domains of the E. coli nucleoid (Worcel and Burgi, 1972) are partially maintained by RNA (Stonington and Pettijohn, 1971; Pettijohn and Hecht, 1973), the alc

gene product could act to shut off host RNA synthesis and thereby unfold the nucleoid, or could unfold the nucleoid directly and thereby shut off host RNA synthesis which depends on the nucleoid structure.

The latter hypothesis is supported by experiments reported by Pearson and Snyder (1980). They studied the effect of T4 superinfection on λ- producing cells and found that the alc gene product shuts off the late transcription of λ, including that which is already underway. They proposed that the alc function does not act simply as an RNA polymerase-binding protein which is effective on cytosine-containing promoters, but rather, acts on specific DNA structures and thereby prevents the utilization of promotors and causes premature termination of transcription. By this model, T4 late transcription, λ late transcription and some E. coli transcription share a common DNA template structural requirement; the T4 alc function normally unfolds the host nucleoid but cannot distinguish T4 C-DNA from E. coli C-DNA and therefore destroys the T4 DNA structures which are required for T4 late gene expression.

Expression of Late Genes from Clones

As an approach to simplify the study of the expression of T4 late genes, attempts have been made to study the expression of cloned late genes after superinfection by T4 phage. A cloned gene will efficiently complement an infecting amber mutant phage (for example, amber g23), providing that the phage contains mutations to prevent breakdown of the plasmid DNA (Jacbos et al., 1981). The effects of infecting with three genotypes of phage have been examined (Jacobs and Geiduschek, 1981): 1) the infecting phage are alc^+ and HMC-containing; 2) the phage are alc^+ and C-containing, and 3) the phage are alc^- and C-containing.

In cells uninfected by T4, some plasmid-mediated expression of gene 23 occurs, presumably from a plasmid promoter. This expression is shut off after infection by HMC-containing alc$^+$ phage but not after infection by C-containing alc$^-$ phage.

After T4 infection, plasmid-mediated expression of gene 23 requires the gene 55 product, as do the chromosomal late genes. However, the three cases have different requirements for DNA replication and DNA replication proteins. In case 1 (infecting phage have HMC-DNA and are alc$^+$) expression of plasmid gene 23 requires the functioning replication proteins, DNA replication, topoisomerase activity (gene 52 product), and functioning gene 46 activity (46 is normally required for recombination). These requirements are similar to the requirements for the expression of the chromosomal late genes, with the exception that late chromosomal replication occurs in the absence of the gene 46 product. It is not known whether the required replication must take place on the chromosome or on the plasmid, for example, to incorporate HMC into one strand of the plasmid which then undergoes a gene-46 mediated reaction. In case 3, (phage have C-DNA and are alc$^-$) replication, replication proteins and gene 46 product are not required and significant plasmid-mediated synthesis occurs in their absence. Finally, in case 2 (phage produce C-DNA and are alc$^+$), no plasmid gene expression occurs; no chromosomal late expression occurs under these conditions either.

The T4 PseT Gene

More clues to the requirements for T4 late gene expression have come from the study of two T4-induced enzymes, the products of the pseT gene and gene 63. Both of these enzymes are required, under some conditions, for T4 late gene expression.

The T4 pseT gene encodes an enzyme which has two distinct in vitro activities, a 5' polynucleotide kinase activity and a 3' phosphatase activity (Cameron and Uhlenbeck, 1977; Sirotkin et al., 1978). The 3' phosphatase removes terminal 3' phosphates from DNA or deoxyribonucleotides or, less preferentially, from RNA or ribonucleotides (Becker and Hurwitz, 1967). The 5' polynucleotide kinase phosphorylates 5'-OH terminated DNAs or RNAs by transferring the gamma phosphate from ATP. It will phosphorylate single-stranded molecules, molecules with protruding 5' ends, and, less efficiently, nicks or gaps in double stranded DNA (cf. Kleppe and Lillehaug, 1979).

Clues to the biological role of the pseT gene product come from the phenotype of pseT$^-$ mutants on the E. coli strain CTr5x,on which they are nonviable (Depew and Cozzarelli, 1974; Sirotkin et al., 1978). In a pseT mutant infection of CTr5x, no phage are produced because DNA replication (Depew and Cozarelli, 1974) and late gene expression are defective (Sirotkin et al., 1978). The reduced rate of replication caused by a pseT$^-$ mutation occurs before late gene expression begins, suggesting that the pseT gene product may act directly in DNA replication.

Mutants in the pseT gene usually lack both of the in vitro activities. However, one mutant, pseT 1 lacks the phosphatase activity but retains the kinase activity and another mutant, pseT 47, lacks the kinase activity but has a low level (about 3%) of the phosphatase activity (Soltis and Uhlenbeck, personal communication). PseT 47 and pset 1 have the same phenotypes as other pseT mutants and do not complement each other, suggesting that either it is the lack of phosphatase that is critical to the mutant's loss of viability or that, in vivo, the kinase and phosphatase act in concert.

Gene 63

Gene 63 maps very near to the pseT gene and codes for a protein product which acts both in tail fiber attachment during phage morphogenesis (Wood and Bishop, 1973) and as an RNA ligase (Snopek et a., 1977). The RNA ligase activity and the tail fiber attachment activity of gene 63 are probably unrelated (Snopek et al., 1977, Runnels et al., 1982).

The RNA ligase activity of gene 63 catalyzes the joining of single-stranded polynucleotides in vitro. It will cyclize an RNA polymer by joining the 3' hydroxyl-terminated "acceptor" end to a 5' phosphate "donor" end and it will join 3' hydroxyl and 5' phosphate termini of separate polynucleotides. The enzyme shows a strong preference for RNA as the acceptor molecule, but almost any ribo or deoxyribo oligonucleotide which has been activated by the addition of AMP in a 5'-5' linkage can serve as the donor molecule (England and Uhlenbeck, 1978). Therefore, the enzyme can join not only RNA to RNA but also DNA to RNA and also stimulates "blunt-end joining" by T4 DNA ligase in vitro (Sugino et al., 1977).

Mutants in the RNA ligase activity are viable on most E. coli laboratory strains but, on E. coli CTr5x, RNA ligase mutants have phenotypes that are indistinguishable from pseT$^-$ mutant phenotypes (Runnels et al., 1982). The similarity in phenotype observed in RNA ligase$^-$ and pseT$^-$ mutants, and the observation that mutants in both genes are suppressed on CTr5x by the same T4 extragenic suppressor has led to the hypothesis that the two enzymes interact in vivo in some reaction which is required for normal replication and which activates the DNA template for T4 late gene expression (Runnels et al., 1982).

Not enough information is currently available to propose precisely at what step in DNA metabolism and transcription the pseT and RNA ligase functions are involved. Possibilities include removal of nicks in DNA formed during the removal of RNA primers, formation of DNA structures required for replication and late transcription, or processing of an RNA which is involved in the formation of T4 "nucleoids."

E. coli CTr5x

CTr5x is a strain derived from CT196, a clinical isolate of E. coli. The original CT196 isolate restricted the growth of pseT mutants but also restricted wild-type T4. CT196 was crossed with various Hfr donor strains until a recombinant was obtained which restricted pseT mutants but not wild-type T4 (Depew and Cozzarelli, 1974).

CTr5x restricts T4 mutants in pseT and RNA ligase as described above. A derivative of CTr5x which contains an efficient amber suppressor no longer restricts any pseT mutants, even those which are deletions. It seemed likely that CTr5x might contain an amber mutation in a gene which is required for the growth of pseT$^-$ and RNA ligase$^-$ phage and which is suppressed in the amber-suppressing CTr5x (cf. Sirotkin et al., 1978). This hypothesis was the inspiration for the search for E. coli mutants in a K12 background which would emulate the restrictive behavior of CTr5x. Lit mutants, which resulted from this search, are described in Appendix A (Cooley et al., 1979).

Recently, it has proven possible to map the locus in CTr5x which is responsible for the restriction of pseT$^-$ and RNA ligase mutants (A. Jabbar and L. Snyder, personal communication). The locus, named prr, lies between his and trp on the E. coli map at about 29 min. Interestingly, when transferred to a heterologous genetic background, the prr

recombinant strain no longer restricts pseT$^-$ or RNA ligase$^-$ mutants at 37^{o}C, only at lower temperatures. However, when the prr locus is transferred to a lit$^-$ host, the restriction of pseT$^-$ and RNA ligase$^-$ mutations is complete at 37^{o}C, suggesting an interaction between the prr locus and the lit gene. This observation is more fully discussed in the Summary section.

DNA Structure and Transcription

Evidence is accumulating that gene expression can be differentially affected by DNA structure. The best documented structural influence on transcription is supercoiling, i.e., the degree of superhelical density can differentially activate promoters.

In Escherichia coli, the omega protein, Topoisomerase I, relaxes negatively supercoiled DNA. The structural gene for Topoisomerase I has recently been identified (Trucksis and Depew, 1981) as topA in E. coli and sup X in Salmonella typhimurium. The sup X locus was discovered some time ago as a suppressor of promoter mutations in the leu operon of Salmonella (Mukai and Margolin, 1963). Sup X mutations also restored expression of mutant lac operons which had inactive CAP-binding sites, and affected many other promoters as well (cf. Smith, 1981). In view of the in vitro activity of omega, a likely explanation for the sup X phenotype is that, in the absence of Topoisomerase I, intracellular DNA is more negatively supercoiled (by DNA gyrase) and the increased supercoiling allows some mutant leu and lac promoters to be utilized.

Further evidence that supercoiling can differentially affect E. coli promoters comes from experiments on the in vivo effects of naladixic acid and coumermycin. These antibiotics are inhibitors of DNA gyrase

and when they are added to cells they inhibit transcription from some operons but not others (Sanzey, 1979).

In vitro, supercoiled DNA is often transcribed more readily than relaxed DNA. For example, purified RNA polymerase transcribes λ RNA five times more efficiently if the DNA is supercoiled (Botchan, 1976).

The condensed E. coli genome is separated into supercoiled domains. DNA gyrase, presumably the topoisomerase which supercoils E. coli DNA, introduces about forty-five breaks per chromosome in the presence of oxolinic acid (Snyder and Drlica, 1979). If breaksites correspond to DNA-gyrase binding sites, there may be one binding site per loop. Perhaps differential supercoiling within domains functions to modulate the transcriptional activity of certain genes clustered in a domain.

A variety of topoisomerases have been isolated from different cell types (Baldi et al., 1980; Hsieh and Brutlag, 1980). However, no enzyme other than E. coli gyrase has been reported to introduce negative supercoils into DNA. For example, the T4 topoisomerase does not exhibit any gyrase activity in vitro (Liu et al., 1980). Nevertheless, domains of supercoiling apparently do exist in intracellular T4 DNA (Hamilton an Pettijohn, 1976). E. coli gyrase can substitute for T4 topoismerase in replication (McCarthy, 1979); it may be able to introduce the supercoils observed in T4 DNA. Alternatively, an unidentified T4 or E. coli subunit may interact with the T4 topoisomerase in vivo, giving it gyrase activity.

Some in vivo activity of T4 topoisomerase plays an important role in T4 late gene expression. In the absence of T4 topoisomerase, the E. coli gyrase can provide a substitute activity, but in the absence of both enzymes, T4 late gene expression is abnormal (Gisela Mosig, personal communication).

ARTICLE I

THE *GOL* SITE: A *CIS*-ACTING BACTERIOPHAGE T4 REGULATORY REGION THAT CAN AFFECT EXPRESSION OF ALL THE T4 LATE GENES

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The *gol* Site: A *Cis*-acting Bacteriophage T4 Regulatory Region that can affect Expression of all the T4 Late Genes

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We have shown that mutations in the *Escherichia coli lit* gene can prevent the expression of the late genes of bacteriophage T4 at temperatures below 34°C. The defect in late gene expression occurs, at least partially, at the level of transcription, and neither DNA replication nor DNA encapsidation into phage heads is significantly affected. Rare T4 "*gol*" mutations overcome the defect in late transcription. Refined mapping experiments place *gol* mutations within gene *23*, but an altered gene *23* protein is not responsible for the phenotype. Rather, *gol* mutations seem to alter a *cis*-acting site in T4 DNA, the wild-type form of which interferes with late transcription in *lit*$^-$ hosts.

1. Introduction

The intracellular structure of the DNA of even simple procaryotes and viruses has proved relatively intractable to biochemical analysis. The DNAs are highly folded and often supercoiled, but beyond this little is known. It seems reasonable to suppose that there exist periodic sites on DNA that are involved in the formation and/or maintenance of DNA structures. However, it is difficult to predict how one might identify such sites, since it is not clear what effect mutations in the sites would have on phenomena such as replication, recombination and transcription.

It has been proposed that a DNA structure created during replication is required for the transcription of the true-late genes of bacteriophage T4 (Riva *et al.*, 1970). The genes are called the "true-late" genes to distinguish them from those early genes that are transcribed early as well as late after infection, but whose transcription, at least at early times, is not coupled to the replication of the DNA. The DNA structure is probably fragile because infected cells must be lysed and treated in particular ways to achieve true-late transcription *in vitro* (Rabussay & Geiduschek, 1977). There may be alternative ways of creating the DNA structures other than replication, since some transcription of these genes occurs in the absence of replication, but it is delayed and dependent on the multiplicity of infection (Wu & Geiduschek, 1975).

While most studies of T4 true-late gene expression have focused on the role of T4

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gene products, it seems likely that host genes are also involved. Such host genes would be of particular interest because they could be involved in transcriptional regulation in uninfected cells. We have identified a candidate for such an *Escherichia coli* gene, the *lit* gene, at 25 minutes on the genetic map (Cooley *et al.*, 1979). The wild-type (*lit*$^+$) allele of this gene is required for normal T4 late gene expression, and cells with the mutant allele (*lit*$^-$) fail to support late gene expression at temperatures below 34°C. Only late gene expression and not T4 DNA replication are affected, so the effect is specific to gene expression.

Rare T4 mutants exist that can multiply in a *lit*$^-$ host (Cooley *et al.*, 1979). These "*gro-lit*" or "*gol*" mutants exhibit normal rates of late gene expression in *lit*$^-$ hosts. We showed that *gol* mutations are co-dominant for late gene expression with wild-type T4 and that they are closely linked to an amber mutation in gene *23* (Cooley *et al.*, 1979). In this paper, we present evidence that *gol* mutations define a site on T4 DNA rather than a diffusible gene product. In a *lit*$^-$ host something happens (or doesn't happen) at this site that prevents the late transcription of T4 bacteriophage.

2. Materials and Methods

(a) *Bacterial and phage strains*

(i) *T4 strains*

The amber mutants were obtained from R. Vanderslice and W. B. Wood or were from our stock collection. The mutations will be referred to by gene and by name. For example, amber mutation H11 in gene *23* is *23am*H11. T4 *gol6B* was isolated as a spontaneous plaque-forming mutant on *E. coli* MPH6, a *lit*$^-$ host (Cooley *et al.*, 1979).

(ii) E. coli *strains*

CR63 (supD) was used for crosses and B834 (su$^-$) (Wood, 1966) was used to detect am$^+$ recombinants and as the *lit*$^+$ host for physiological experiments. In the original experiments, the effect of host *lit*$^-$ mutations on T4 development was studied using the K-12 strains from which *lit*$^-$ mutants were first isolated (Cooley *et al.*, 1979). It is difficult to achieve synchronous infections of K-12 strains, so we transferred a *lit*$^-$ mutation into the B strain, *E. coli* B834, as follows. An Hfr strain, RH041, which is *purB*$^-$ and transfers clockwise from 97 min, was obtained from B. Bachmann and the Yale Stock Collection. A *lit*$^-$ mutation, *lit-6*, was transduced with bacteriophage P1 into this strain from MPH6 using *pur*$^+$ as the selected marker. An Hfr *lit*$^-$ recombinant was then mated with *E. coli* B834 made *his*$^-$ by mutagenesis with nitrosoguanidine (Adelberg *et al.*, 1965). Two out of 80 *his*$^+$ recombinants of the mating were *lit*$^-$. One of these, *E. coli* B834-*lit*403, was the *lit*$^-$ host used for all the experiments to be described. Since only one *lit*$^-$ host was used it will be referred to simply as "the *lit*$^-$ host".

(b) *Crosses and infections for labeling*

Crosses and infections were performed in M9 medium as described previously (Snyder *et al.*, 1976). To measure phage yields the cells were grown in tryptone broth at 37°C and infected at a total multiplicity of infection (m.o.i.) of 10. After 5 min, T4 antiserum (Cappel) was added and at 13 min the cells were diluted 10^3 times. At 90 min chloroform was added, and the phage were diluted further and plated. In some experiments it was necessary to determine accurately the ratio of *gol*$^-$ and *gol*$^+$ phages in mixed infections. To accomplish this, the mixture of phage to be used or the phage released were plated on *lit*$^+$ *E. coli*. A sector of the plate that contained about 100 plaques was chosen and the percentage of the plaques due to *gol*$^-$ mutants was determined by toothpicking all of the plaques in this sector onto a plate with *lit*$^-$ indicator.

(c) *Analysis of T4 protein and RNA synthesis*

To label proteins, [^{35}S]methionine (1000 Ci/mmol, 10 μCi/ml) was added to cells infected at 30°C in M9 medium without Casamino acids. The procedures and buffers of Studier (1973) were used for the processing of the samples and slab gel electrophoresis. The gels were 11% (w/v) acrylamide, with the exception of the gel shown in Fig. 3, which was 9·5% acrylamide. Gels were stained (to ensure that total protein per column was uniform), dried, and used to expose XRP-1 Kodak X-ray film for varying lengths of time. To label RNA, [5-^{3}H]uridine (26 Ci/mmol, 10 μCi/ml) was added to cells infected at 30°C as above. The RNA was extracted with phenol as described by Bolle *et al.* (1968), except that the RNA was precipitated with alcohol after 4 extractions and treated with 10 μg RNAase-free DNAase/ml (Worthington) for 200 min at 37°C before the final 2 extractions with phenol. The purified "*l*" and "*r*" separated complementary strands of T4 RNA were a very generous gift from Dietmar Rabussay, who also helped with the hybridization assays. Hybridization was by the liquid method in 2 × SSC (SSC is 0·15 M-NaCl, 0·015 M-sodium citrate) for 8 h at 60°C. The hybrids were collected on nitrocellulose filters and washed with 0·5 M-KCl, 0·01 M-Tris·HCl (pH 7·9).

3. Results

(a) *T4 gene expression in the* lit$^-$ *host*

We reported previously that host *lit*$^-$ mutations prevent T4 late gene expression (Cooley *et al.*, 1979). Figure 1 (columns A and a) shows the synthesis of T4 proteins late in infection of the *lit*$^+$ and *lit*$^-$ host, respectively. At this labeling time, some T4 late protein synthesis occurs in the *lit*$^-$ host, but it is reduced in rate compared to the *lit*$^+$ control. At later labeling times, T4 protein synthesis has almost totally ceased in a *lit*$^-$ host (Cooley *et al.*, 1979).

Host *lit*$^-$ mutations only prevent T4 late gene expression at temperatures below 34°C (Cooley *et al.*, 1979). Furthermore, we have found that the temperature at which the cells were grown *prior* to infection is important. More late protein synthesis occurs in *lit*$^-$ cells grown at 37°C and infected at 30°C than in cells grown and infected at 30°C (data not shown). This indicates that the temperature dependence of late gene expression in a *lit*$^-$ host is not due to the temperature dependence of a particular reaction, but rather to differences in cells grown at different temperatures.

It is interesting to contrast the block imposed on T4 late gene expression by host *lit* mutations with that imposed by most T4 mutations, which prevent the synthesis of true-late proteins; for example, DNA-negative or gene *55* mutants. In the latter cases, early protein synthesis continues and the product of T4 gene *32* is sometimes grossly overproduced (cf. Gold *et al.*, 1976). After infection of the *lit*$^-$ host by wild-type T4, true-late proteins are made in reduced amounts, but early protein synthesis also ceases, probably somewhat earlier than usual, and the gene *32* product is not overproduced. This is demonstrated in Figure 1, where we show the results of infecting with T4 having amber mutations in genes *44* and *42*, the products of which are required for DNA replication. Now, as mentioned above, the synthesis of the products of many early genes, including *42* and *43*, continued late into infection (Fig. 1, lanes b and c). In fact, the program of gene expression in the *lit*$^-$ mutant was not very different from that in the *lit*$^+$ host (compare with Fig. 1, lanes B and C). A similar result was obtained with a T4 gene *55* mutant, except that

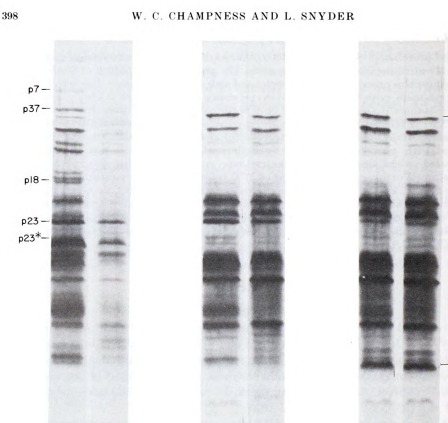


FIG. 1. T4 protein synthesis after infection of the *lit*$^-$ host. Proteins (identified as gene products) were labeled from 18 to 21 min. and electrophoresed and autoradiographed as described in Materials and Methods. Lanes A, B and C, *lit*$^+$ *E. coli*; a, b and c, *lit*$^-$ *E. coli*. A and a, T4; B and b, *42am*N122; C and c, *44am*N82.

in this instance the gene *32* product was overproduced in both hosts (data not shown). Therefore, the phage mutations that prevent true-late gene expression are epistatic over the host *lit* mutation in that they prevent the shutoff of early gene expression even in the *lit*$^-$ host.

In an earlier paper (Cooley *et al.*, 1979), we proposed that host *lit* mutations block T4 late gene expression at the level of transcription. To prove this we performed the experiment shown in Table 1. We radioactively labeled RNA late in infection of the *lit*$^+$ and *lit*$^-$ hosts and hybridized the RNA to the complementary *r* and *l* strands of T4 DNA. If the defect occurs at the level of transcription, we would not expect

TABLE 1

Hybridization of RNA labeled after infection of the lit$^-$ *host to the separated complementary strands of T4 DNA*

RNA	Cts/min input	Cts/min hybridized *r* strand	Cts/min hybridized *l* strand	% Of input RNA hybridized to *r* strand
E. coli lit$^+$ *gol*$^+$ T4	637	334	60	52
E. coli lit$^-$ *gol*$^+$ T4	941	99	86	10
E. coli lit$^-$ T4 *gol6B*	1064	759	63	71

RNA was labeled from 28 to 31 min after infection. The specific activities of the RNAs were, from top to bottom: 2·6, 3·4 and 2·4 cts/min per ng.

much hybridization to the *r* strand with the RNA made in the *lit*$^-$ host since T4 late RNA hybridizes mostly to the *r* strand of T4 DNA. With RNA prepared after infection of the *lit*$^+$ host, 52% of the input RNA hybridized to the *r* strand of T4 DNA, while in the *lit*$^-$ case only 10% hybridized to the *r* strand. Not only did the percentage of RNA hybridizing to the *r* strand drop, but the total hybridization to the separated strands also dropped. The latter result is reproducible and suggests that the distribution of the RNA may be grossly altered, or that the RNAs may be much smaller, or that some host RNA may be synthesized. In any case, it seems clear that the host *lit*$^-$ mutation affects T4 late transcription and this is probably sufficient to explain the defect in late gene expression and phage production.

(b) *Effect of T4* gol *mutations on T4 late gene expression*

T4 *gol* mutations are single point mutations that overcome the defect in late protein synthesis in *lit*$^-$ hosts (Cooley *et al.*, 1979). If the defect in late transcription is causing the defect in protein synthesis, we would also expect *gol* mutations to overcome the effect on late transcription. In Table 1 we have included hybridization results from experiments with RNA labeled in the *lit*$^-$ host after infection with the *gol* mutant. This late RNA hybridized with an efficiency of 77% to the separated strands of T4 DNA and 71% of the input RNA hybridized to the *r* strand. Thus the RNA labeled after infection by the *gol* mutant was more like normal T4 late RNA in that most of it hybridized to the *r* strand of T4 DNA. We conclude that T4 *gol* mutations overcome the effect on late RNA synthesis caused by host *lit* mutations.

(c) *Genetic analysis of T4* gol *mutations*

In an earlier paper, we reported that *gol* mutations are closely linked to T4 gene *23* (Cooley *et al.*, 1979). The 200 *gol* mutants we have tested so far all had mutations in this region. If mutations to the Gol phenotype can occur elsewhere, they must be less frequent by at least two orders of magnitude.

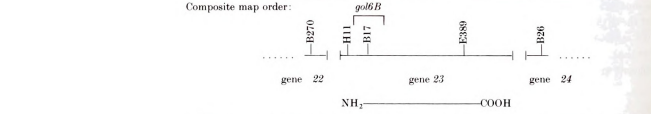
To localize further a particular *gol* mutation, we performed three-factor crosses. The results are shown in Table 2. This particular *gol* mutation, *gol6B*, apparently lies within gene *23*, clockwise (or carboxy-terminal) to the very amino-terminal

TABLE 2

Mapping of a gol *mutation by three-factor crosses*

Cross	Frequency of *gol* mutants among am^+ recombinants	% *gol* mutants	Order deduced
1. *am*H11, *gol6B* × *am*B270	13/96	14	B270-H11-*gol*
2. *am*H11, *gol6B* × *am*B17	117/126	93	H11 [*gol* B17]
3. *am*B17, *gol6B* × *am*H11	6/80	8	H11 [*gol* B17]
4. *am*B17, *gol6B* × *am*B26	17/148	12	[*gol* B17] — B26
5. *am*B17, *gol6B* × *am*E389	13/93	14	[*gol* B17] — E389
6. *am*E389, *gol6B* × *am*B17	138/146	94	[*gol* B17] — E389
7. *am*E389, *gol6B* × *am*B270	105/143	73	B270 — *gol* — E389

Composite map order:



Double mutants consisting of the *gol6B* mutation and amber mutations in genes *22*, *23* and *24* were crossed against other single *am* mutants and am^+ recombinants were tested for growth on the *lit*$^-$ host. The crosses unambiguously place the *gol* mutation between the gene *23* mutations *am*H11 and *am*E389, very close to *am*B17. The crosses do not clearly show on which side of *am*B17 the *gol* mutation lies. We have indicated this by brackets.

*23am*H11 and close to the *23am*B17 mutation. A. H. Doermann (personal communication) has confirmed this map position using "marker rescue" mapping techniques and has further concluded that this *gol* mutation lies clockwise (or carboxy-terminal) to *23am*B17. An expanded map of this region is shown in Figure 2. The product of gene *23* is the major capsid protein. During head formation, the N-terminal portion of the polypeptide is removed by proteolytic cleavage (Laemmli, 1970) and the site of the cleavage lies between *23am*H11 and *23am*B17.

(d) *Evidence that* gol *mutations define a* cis*-acting site on T4 DNA*

In spite of the map position of *gol* mutations, the Gol phenotype is not due to an altered gene *23* product. In the experiment shown in Figure 3(b) (lane 3) a double mutant, having both the *gol* mutation and *23am*E389, was used to infect the *lit*$^-$ host. The *gol* mutation stimulated the synthesis of all the T4 late proteins, including the amber fragment of gene *23*. Thus the Gol phenotype is unchanged

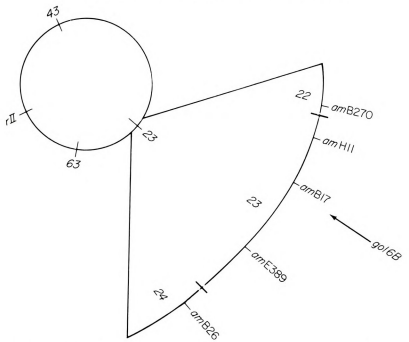


FIG. 2. An expanded map of the gene *23* region of T4 showing the position of a *gol* mutation.

even in the absence of a functional gene *23* polypeptide. We repeated this experiment with the mutation *23am*H11, which, as mentioned above, is N-terminal to the *gol* mutation and in the part of gene *23* coding for the fragment removed during head formation. Now the amber fragment was too small to be detected, but the *gol*, *23am*H11 double mutant synthesized all the late proteins except the gene *23* product (data not shown). We conclude that, since the *gol* mutation could stimulate the expression of the other late genes in the absence of the gene *23* product or even the N-terminal fragment, the Gol phenotype is not due to an alteration of either the gene *23* protein or the N-terminal fragment of the gene *23* protein.

The possibilities remain that the Gol phenotype is due to altering the gene *23* mRNA rather than protein; or to altering a gene whose sequence overlaps that of gene *23* or is on the opposite DNA strand. Alternatively, *gol* mutations may define a site. The evidence presented immediately below suggests that *gol* mutations define a site on the DNA rather than a diffusible gene product.

If *gol* mutations define a site on the DNA, they may be more closely linked than if their target is a diffusible gene product. Of the five spontaneous *gol* mutations we have tested, four seemed to be in the same base-pair, since they gave recombination frequencies of less than 0·01%, the frequency of recombination between mutations in adjacent base-pairs. The other mutation gave a frequency of about 0·04% with the other *gol* mutations, which is consistent with its being about four base-pairs away. Therefore, the "target size" for *gol* mutations seems to be very small.

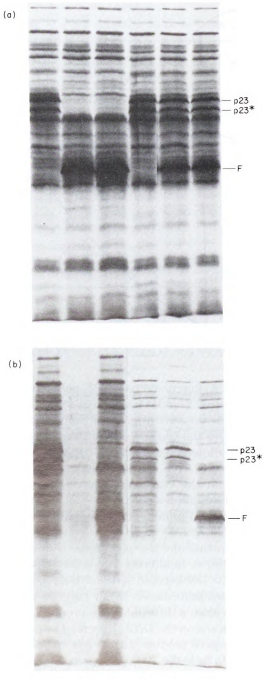


FIG. 3. Synthesis of T4 gene 23 product (p23) in mixed infections with a gene 23 amber mutation and a *gol* mutation on *lit*$^-$ *E. coli*. An electropherogram of T4 proteins labeled from 28 to 31 min after infection is shown. (a) *lit*$^+$ *E. coli*; (b) *lit*$^-$ *E. coli*. Lanes 1, T4 *gol6B*; 2, *23amE389*; 3, the double mutant

If *gol* mutations define a site on T4 DNA they should be *cis*-acting for the expression of T4 late genes in mixed infections. To test this, we performed the experiment shown in Figure 3, lanes 5 and 6. We coinfected cells with the T4 *gol* mutant and gol^+ T4 with the *23am*E389 mutation (lane 5) and, conversely, with gol^+ T4 and a double mutant having the *gol* mutation and *23am*E389 (lane 6). In lit^+ *E. coli*, gene *23* was expressed from both the *gol* mutant and gol^+ DNA since the *am* fragment and complete polypeptide were synthesized at approximately equal rates (Fig. 3(a), lanes 5 and 6). In contrast, in the lit^- *E. coli* gene *23* was only expressed from the DNA with the *gol* mutation. In other words, if the gol^+ DNA had the amber mutant allele, only the complete gene *23* polypeptide was synthesized (Fig. 3(b), lane 5), but if the *gol* mutant DNA had the amber allele, only the amber fragment was synthesized (Fig. 3(b), lane 6). Thus, the *gol* mutation stimulated the synthesis of the gene *23* product only in *cis*.

We attempted a semi-quantitative determination of the stimulation of the complete polypeptide and the *am* fragment in each experiment by determining the area under each band, using a scanning and integrating densitometer. A background was subtracted that was taken to be the area in the same region of the gel when the *am* mutant or wild-type T4, respectively, had infected the lit^- host without the coinfecting *gol* mutant. Subject to the limitations of this method, it appears that the presence of a *gol* mutation on the same DNA stimulated the synthesis of either the complete polypeptide or the amber fragment at least 300-fold without stimulating gene expression from the gol^+ DNA in the same cell. The synthesis of the *am* fragment appears to the eye to be stimulated more by the *gol* mutation than the complete polypeptide, but the densitometer reveals that this is an illusion based on the width of the bands in different parts of the gel.

To determine if the *cis* effect of *gol* mutations extends to late genes other than *23*, we repeated the experiment shown in Figure 3, but with an amber mutation in gene *18*. The results are shown in Figure 4. Like the gene *23* product, the gene *18* product was only synthesized from the DNA with the *gol* mutation. We tried the same experiment with gene *34*. The results were less clear but there did seem to be some *cis* effect on this gene as well (data not shown). Apparently, the *cis* effect of *gol* mutations extends some distance, and in both directions, from the site of the *gol* mutation.

One could argue, from the experiments shown in Figures 3 and 4, that *gol* mutations are totally recessive, but that a subset of the cells received *gol* mutant phage exclusively. Since these were the only cells in which late proteins were synthesized, only one allele of gene *23* or gene *18* was expressed, because this was the only allele in those cells that were making any late proteins. This seems unlikely. The rate of late protein synthesis in the mixed infections was about 30% that of the lit^+ control, so 30% of the cells could not have been infected by gol^+ T4. But to eliminate this trivial explanation for the *cis* effect, we designed the experiment shown in Table 3. The experiment is based on the observation that host

*23am*E389, *gol6B*: 4, wild-type T4 plus T4 *gol6B*: 5, *23am*E389 plus T4 *gol6B*; 6, the double mutant *23am*E389, *gol6B* plus wild-type T4. In the single infections (lanes 1 to 3) the m.o.i. was 10. In the mixed infections (lanes 4 to 6) the m.o.i. was 10 of each. The gene *23* product and the amber fragment left by *23am*E389 (F) are identified.

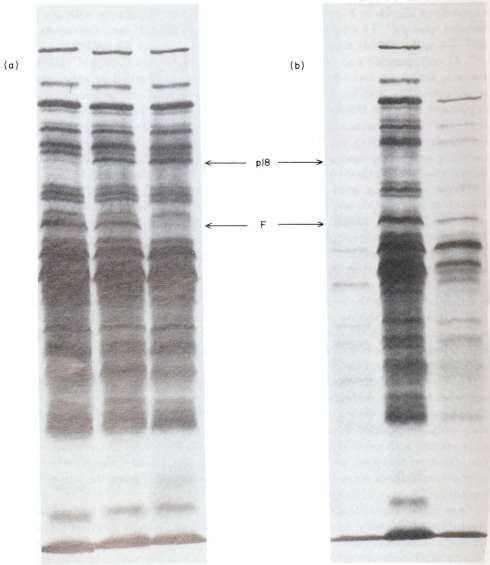


FIG. 4. Synthesis of T4 gene *18* product in mixed infections with a gene *18* amber mutation and a *gol* mutation in *lit*$^-$ *E. coli*, shown in an electropherogram of the T4 proteins labeled from 28 to 31 min after infection. (a) *lit*$^+$ *E. coli*; (b) *lit*$^-$ *E. coli*. Lane 1, *18am*E18; 2, the double mutant *18am*E18, *gol6B* plus wild-type T4; 3, T4 wild-type; 4, *18am*E18; 5, the double mutant *18am*E18, *gol6B*; 6, the double mutant *18am*E18, *gol6B* plus wild-type T4. For single infections (lanes 1, 3, 4 and 5) the m.o.i. was 10. For the mixed infections (lanes 2 and 6), the m.o.i. was 10 of each. The T4 gene *18* product (p18) and the amber fragment left by *18amE18* (F) are identified.

TABLE 3

Production of gol$^+$ *and* gol *mutant phage in mixed infections*

Phage	Bacterium	Phage per infected cell	% *gol6B* input	% *gol6B* output
T4+	*E. coli lit*$^+$	55	0	0
T4 *gol6B*	*E. coli lit*$^+$	43·5	100	100
T4 *gol6B+23am*H11	*E. coli lit*$^+$	15	51	33
*am*H11	*E. coli lit*$^+$	4×10^{-4}	0	0
T4+	*E. coli lit*$^-$	0·75	0	0
T4 *gol6B*	*E. coli lit*$^-$	135	100	100
T4 *gol6B+23am*H11	*E. coli lit*$^-$	27	51	67
*23am*H11	*E. coli lit*$^-$	$4{\cdot}5 \times 10^{-4}$	0	0

At least 100 plaques were tested for the Gol phenotype to determine the percentages of *gol* mutants produced. In this experiment, the yield of T4 *gol6B* was higher in the *lit*$^-$ mutant than in *lit*$^+$ *E. coli*. This is not generally the case.

lit mutations do not substantially affect T4 DNA replication; so, in a *lit*$^-$ host, both *gol*$^+$ and *gol* mutant DNA is replicated. Thus, even if *gol* mutations are *cis*-acting for gene expression, those late proteins synthesized from the *gol* mutant DNA could package *gol*$^+$ DNA in the same cell. Therefore, in a mixed infection of the *lit*$^-$ host, such as that shown in Figures 3 and 4, both *gol*$^+$ and *gol* mutant phage should be produced if *gol* mutations are *cis*-acting. In contrast, if the explanation for the apparent *cis* effect is that *gol* mutations are recessive and the only cells synthesizing late proteins are those infected exclusively with *gol* mutants, then only *gol* mutant phage will be produced in mixed infections. Even if *gol* mutations are *cis*-acting we expect some bias toward the *gol* mutant phage because those cells that are infected mostly with *gol* mutants will produce more phage, since they will synthesize more late proteins. In the experiment shown in Table 3, the *gol*$^+$ phage had an amber mutation, *23am*H11, so any *gol*$^+$ phage produced must obtain their major coat protein from the *gol* mutant genomes in the same cell. The results were that in the mixed infection about nine phage per cell or 33% of the total phage released were of the *gol*$^+$ genotype. This represents a stimulation in the production of *gol*$^+$ phage of about 20,000 times more than that produced after infection by the *gol*$^+$ phage alone. It is also many more phage than are produced after *gol*$^+$ phage without an amber mutation infect the *lit*$^-$ host (see Table 3). We conclude that late proteins are synthesized in cells infected by both *gol*$^+$ and *gol* mutant phage, and that *gol* mutations are not simply recessive. However, there is some interference from *gol*$^+$ genomes on the expression of *gol* mutant genomes in the same cell. In mixed infections of *gol*$^+$ and *gol* mutant phage, such as those shown in Figures 3 and 4, the rate of late protein synthesis was less than half that of normal even if half of the infecting phage had the *gol* mutation.

4. Discussion

Host *lit*$^-$ mutations severely alter the transcription of the T4 genome late in infection and thereby prevent the expression of the late genes. One site, the *gol* site on T4 DNA, is responsible for the defect in late gene expression, and T4 with

mutations in this site multiply normally on *lit*$^-$ hosts. Our evidence that *gol* mutations define a site rather than a diffusible gene product is twofold. First, they are very closely linked. Second, they are *cis*-acting for the expression of the late genes, e.g. when *lit*$^-$ cells are mixedly infected with *gol*$^+$ and *gol* mutant phage having different alleles of a late gene, only the allele on the *gol* mutant DNA is expressed. There is no *cis* effect on DNA packaging, however, and both *gol*$^+$ and *gol* mutant DNA are packaged in late proteins made from the *gol* mutant DNA.

We have demonstrated a *cis* effect on the expression of genes *23* and *18*, both of which are closely linked to the *gol* mutation. Somewhat unexpectedly, we also observed some *cis* effect on the expression of gene *34*, which is about 100° away on the circular map (or about 10 μm on DNA) from the site of the *gol* mutation, although the results were less dramatic because of the lower rate of expression of gene *34* than that of either gene *23* or *18*. Because T4 recombination is so active, one might not expect to be able to demonstrate a *cis* effect on the expression of a gene so far away, since the *gol* mutation will often be separated from the gene *34* amber mutation by recombination. Nevertheless, this result need not vitiate our interpretation. Most recombination occurs late in T4 development (Levinthal & Visconti, 1953), perhaps later than the times at which we labeled the proteins.

Models of the function of the T4 *gol* site must await information on the nature of *gol* mutations and of the function of the host *lit* gene product. In a *lit*$^-$ host, T4 late gene expression is temperature-dependent. This temperature dependence is at least partially due to differences in *E. coli* cells that have been grown at different temperatures and not to the thermal sensitivity of a protein or reaction. Also, the effect of host *lit* mutations on T4 gene expression is qualitatively different from that of most types of phage mutations that block late gene expression. When true-late gene expression is prevented by mutations in replication genes or gene *55* or *33*, the synthesis of many early proteins continues late into infection. However, late into infection of a *lit*$^-$ host, we see the cessation of synthesis of even those early gene products that are normally synthesized throughout infection. Other situations are known in which true-late gene expression is prevented and the synthesis of the early gene products also ceases. These occur when T4 DNA replicates to contain cytosine and the T4 are *alc*$^+$ (Snyder *et al.*, 1976), and when polynucleotide kinase or RNA ligase-deficient mutants of T4 infect *E. coli* CTr5x (Sirotkin *et al.*, 1978; Runnels *et al.*, 1982). It is interesting that the *alc* gene product also unfolds the bacterial nucleoid after infection (Sirotkin *et al.*, 1977; Tigges *et al.*, 1977) and that some of the original *lit*$^-$ mutants restricted polynucleotide kinase-deficient mutants of T4 (Cooley *et al.*, 1979). Perhaps all these gene products are involved in a common pathway.

We can only speculate on how the wild-type *gol*$^+$ site can interfere with late gene expression in a *lit*$^-$ host. Host *lit* mutations might prevent the utilization of the *gol*$^+$ site, and *gol* mutations alter the site so it can function in a *lit*$^-$ host. Alternatively, in a *lit*$^-$ host, something may happen to the *gol*$^+$ site that prevents the expression of the genome harboring the site. For example, host *lit*$^-$ mutations may activate a restriction-like nuclease, which makes a break specifically at the *gol* site. If this is true, then late gene expression must be particularly sensitive to cleavage at this site, since after infection by gene *46* or *47* mutants, for example, double-

stranded breaks accumulate in T4 DNA (Hosoda *et al.*, 1971) but late gene expression is not affected. Furthermore, the observation that *gol*$^+$ genomes can be packaged in mixed infections of *lit*$^-$ hosts argues against the "restriction-site" hypothesis, since packaging requires concatameric genomes.

We have recently observed that plasmids with T4 DNA "inserts" containing the wild-type (*gol*$^+$) site can be used to transform *lit*$^+$ but not *lit*$^-$ *E. coli*. In contrast, if the T4 DNA insert contains the *gol* mutant site or deletions of this site, then either *lit*$^+$ or *lit*$^-$ *E. coli* can be transformed. Apparently, something happens at this site even in the absence of the rest of the T4 genome. Note that these observations support the latter of the two possibilities mentioned above: that something happens at the *gol*$^+$ site in a *lit*$^-$ host that is deleterious to the expression of genomes harboring the site. This raises the possibility that the *gol* site is not normally required for late gene expression *in vivo* but can act as a "spoiler" under some conditions. It may not be required for late transcription because other similar sites can substitute for it or because its real role is to co-ordinate some other event, such as DNA packaging, with late transcription. In any case, even if the *gol* site is not normally involved in transcriptional activation, an understanding of how an event at one place in the DNA can affect the expression of genes so far away should enhance our understanding of the intracellular structure of T4 DNA and how this structure activates the transcription of the late genes.

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ARTICLE II

THE BACTERIOPHAGE T4 GOL SITE: SEQUENCE ANALYSIS AND EFFECTS OF THE SITE ON PLASMID TRANSFORMATION

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ABSTRACT

T4 *gol*$^-$ mutations overcome, in *cis*, the block to T4 late gene expression imposed by *Escherichia coli lit* mutants. We have proposed (Champness and Snyder, *J. Mol. Biol.*, 1982) that *gol*$^-$ mutations alter a site on T4 DNA that has a regulatory function in T4 late gene expression. This work reports nucleotide sequence analysis of *gol*$^-$ mutants; the sequence data supports the proposal that *gol* mutations do not alter a protein product, but rather, alter a regulatory site on T4 DNA. Besides affecting T4 gene expression, when cloned into a plasmid the T4 *gol* site has an observable effect on plasmid behavior. If a plasmid contains the *gol*$^+$ T4 sequence, it cannot transform a *lit*$^-$ host although it can transform a *lit*$^+$ host. But if the plasmid contains a *gol*$^-$ sequence, or is deleted for the *gol* region, it can transform a *lit*$^-$ host. Therefore, the presence of the *gol*$^+$ DNA interferes with the ability of a plasmid to stably transform *lit*$^-$ *E. coli*.

INTRODUCTION

Escherichia coli lit$^-$ mutants prevent T4 late gene expression at temperatures below 34^{o}C. T4 *gol* mutations were discovered because they overcome the block to T4 development imposed by *E. coli lit* mutants (Cooley et al., 1979). Evidence has been presented that *gol* mutations affect gene expression only in *cis* in a mixed infection of a *lit*$^-$ host by *gol*$^+$ and *gol*$^-$ phage and therefore do not alter a diffusible gene product. Rather, it has been proposed that gol mutations alter a site, the wild-type form of which interferes with normal gene expression in a *lit*$^-$ host.

Expression of the late genes of T4 seems to require template "processing," (Rabussay and Geiduschek, 1977) which is normally coupled to DNA replication. The nature of the processing events remains undefined but evidence has been accumulated for the involvement of single-stranded nicks or gaps (Riva et al., 1970b; Wu and Geiduschek, 1974), concurrent DNA replication (Riva et al., 1970a), and replication protein-transcription complex interactions (Wu et al., 1975; Wu and Geiduschek, 1977); Rabussay and Geiduschek, 1979).

There is also a requirement for some activity of the T4 topoisomerase type II (Liu et al., 1980) in T4 late gene expression (Gisela Mosig, personal communication). Because the *E. coli* gyrase can substitute for the T4 topoisomerase, the topoisomerase could be required for supercoiling. Although there is some evidence that domains of supercoiling exist in T4 DNA (Hamilton and Pettijohn, 1976), it is not known if the T4 topoisomerase is capable of catalyzing supercoiling; *in vitro*, the enzyme can not introduce supercoils. Therefore, the

topoisomerase may create some other kind of tertiary conformation in intracellular T4 DNA.

It is tempting to speculate that the gol site plays a role in the template processing required for late gene expression. But, because of the complexity of T4 DNA organization at times when gol mutations exert their effect, it is difficult to study the effects of gol mutations on intracellular T4 DNA.

However, we have found that gol DNA has an effect when cloned into plasmids; this paper describes the effects of the presence of gol^+ and gol mutant DNA on plasmid transformation.

It also reports the sequence of some gol^- mutations, both spontaneous and mutagen-induced, 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 their source or reference are listed in Table 1.

Hydroxylamine mutagenesis

Mutagenesis was performed as described by Tessman (1968).

Growth of Cytosine-Containing T4 Phage

Because T4 DNA contains glucosylated 5-hydroxymethyl cytosine (HMC) in place of cytosine, the phage DNA is insensitive to most restriction endonucleases. However, by using appropriate phage and host mutants, T4 can be grown with mostly cytosine in its DNA. T4 which is mutant in the genes 56 (deoxycytidine triphosphatase); den A (endo II, a double-stranded-DNA-specific nuclease which degrades the host chromosome); den B (endo IV, a single-stranded DNA-specific nuclease which destroys T4 progeny DNA molecules which contain cytosine); and alc (a gene whose function prevents true-late transcription from a template containing cytosine) will have up to 95% cytosine in its DNA. (Snyder, et al., 1976). Growth of these phage on the E. coli strain B834 galU56 will ensure that the residual HMC residues are not glucosylated and that HMC-containing revertants will not accumulate (Runnels and Snyder, 1978). This host strain is partially deficient in uridine diphosphoglucose, which is the source of glucose found in phage DNA; in this host mutant, an intact rgl restriction system recognizes unglucosylated HMC residues in phage DNA, but unmodified C-containing DNA is not degraded because the strain lacks a functioning r_B restriction system.

Table I

A. E. coli Strains

Strain	Relevant Characteristics	Source or Reference
K803	Su II^{++} $r_k^- m_k^-$	
B834	$r_B^- m_B^-$ rgl^+,su^-	Snyder et al., 1976
B834 galU56	$r_B^- m_B^-$, rgl^+,su^-,UDPG$^-$	Runnels and Snyder, 1978
B834 lit 403	lit^-, su^-	Champness and Snyder, 1982
MPH 7	lit^-, suI^+	Cooley et al., 1979
JM103	Δlac pro, $r_k^- m_k^+$; F' traD35,proAB,$lacI^q$, ZΔM15	Bethesda Research Laboratories

B. Bacteriophage T4 Strains

Strain	Relevant Characteristics	Source or Reference
Dec 8	56^-(amE51), den A^-, den B^-, alc^-	our laboratory
Gol 6B	$\underline{gol}^-$, spontaneous	Champness and Snyder, 1982
Dec 8, H1	$\underline{gol}^-$, hydroxylamine mutagenesis	our laboratory
Dec 8, H2	$\underline{gol}^-$, hydroxylamine mutagenesis	our laboratory

In order to digest gol mutant DNA with restriction enzymes, it is necessary that the mutant be in a genetic background which allows the phage to multiply with cytosine rather than hydroxymethyl cytosine in its DNA. Therefore, gal 6B, was crossed into a 56$^-$, den A$^-$, den B$^-$, alc$^-$ background selecting on B834 golU56 for cytosine, and on a lit$^-$ host for the gol mutation. Other gol mutants were obtained by mutagenizing a 56$^-$, den A$^-$, den B$^-$, alc$^-$ strain with hydroxylamine.

Cytosine-containing phage were purified on a CsCl step gradient as described previously (Sirotkin et al., 1978) then phenol extracted and the DNA was dialized against IM NaCl overnight, then H_2O overnight.

Enzymes

Eco Rl, Hind III, 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.

DNA Manipulations

Restriction enzymes were used according to the supplier's recommendations. DNA restriction fragments were separated on a 0.7% (w/v) agarose gel in 0.09 M Tris-borate, pH 8.3 and the desired fragment was recovered by electrophoresis onto filter paper, followed by centrifugation to elute the DNA and finally, two ethanol precipitations. Ligation of T4 restriction fragments to plasmid DNA was done with an equimolar ratio of plasmid to restriction fragment, at a total DNA concentration of 40 µg/ml and incubated overnight at 18^oC. For ligation of 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 µl and incubated for 24 hours at 9^oC.

Cloning in M13mp7

The M13 lac vector which was used (Messing et al., 1981) carries an insert coding for the first 145 amino acids of the β-galactosidase gene. The phage is therefore a donor for alpha complementation. The lac DNA contains a 42 base pair Eco R1 fragment which does not inhibit α complementation and which contains cloning sites for Eco R1, Bam H1, Pst 1, Sal 1, Acc 1 and Hinc II. The intact phage forms blue plaques when plated under conditions of IPTG induction with X-gal as a substrate. DNA insertion into any of the cloning sites disrupts β-galactosidase production and causes the appearance of white plaques.

Transfection

Exponentially growing JM103 was pelleted by centrifigation at 7000 g for 5 min, resuspended in one half the growth volume in 50 mM $CaCl_2$, then incubated on ice for 20 minutes. The cells were centrifuged again, then resuspended in one tenth the growth volume in 50 mM $CaCl_2$. 0.3 ml of cells were then added to 12 ng of a ligated mixture of M13mp7 and T4 DNA, incubated on ice for 40 minutes, then heat shocked at 42°C for two minutes. Immediately, 0.2 ml of exponentially growing JM103, 3 ml of YT soft agar, IPTG (final concentration, 0.3 mM) and x-gal (final concentration, 0.03%) were added and then plated on YT agar plates. (YT agar contains, per liter, 8 gm tryptone, 5 gm NaCl, 5 gm yeast extract and 18 gm agar). After overnight incubation at 37°C, intact phage gave blue plaques and recombinant phage gave white plaques. White plaques (putative recombinants) were plaque-purified on JM103, then an individual plaque was suspended in 0.5 ml 0.85% saline for marker rescue (see below).

Identification of Clones by Marker Rescue

If T4 infects a cell containing a clone of T4 DNA in a plasmid or M13, recombination can occur between the cloned DNA and the infecting DNA. If the cloned DNA contains a gol mutation some of the progeny virus will be gol mutants and will multiply on a lit host. This allows one to determine if the cloned DNA contains the gol mutation. The actual method used is somewhat different for plasmid and M13 cloning vectors.

1. Plasmids. First, exponentially-growing MPH 7 su^+1 (lit^-) was plated with top agar. Then, approximately 10^6 cells of a culture of B834 containing the putative recombinant plasmid were spotted onto the plate. Finally, about 10^7 wild-type T4 were spotted directly onto the first spot and plates were incubated overnight at 28^oC. Under these conditions, wild-type T4, when spotted alone or spotted onto B834 containing pACYC184, gave no plaques within the spot. A clone which contained a T4 Eco R1 gol^- fragment produced discrete plaques within the spot, as a result of marker rescue of wild-type T4 by the gol mutation on the clone.

2. M13 clones. Exponentially growing MPH 7 su^+1 was plated as above. Spotting was done in the following order: first JM103 ($\sim 10^6$ cells) was spotted onto the MPH 7 su^+1 lawn, then 1 μl of the recombinant M13 phage suspension, then 10^7 wild-type T4. After overnight incubation at 28^o the spots were examined for plaque formation. Under these conditions M13 spotted onto JM103 gave no plaques; wild-type T4 spotted onto non-recombinant M13 plus JM103 gave a background of no more than 1-5 plaques; and test 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 done by the method of Selzer et al. (1978). Cells were spread on tryptone plates (10 gm tryptone, 20 gm agar, 5 gm NaCl, 10 gm Casamino acids per liter) with 25 μg/ml ampicillin or 10 μg/ml tetracycline.

Preparation of M13 DNA for Sequencing

Recombinant plaques which tested positively for the presence of gol mutant DNA were plaque purified as above, then a single white plaque was transferred with a sterile toothpick into 5 ml of YT medium. 50 μl of an exponentially growing culture of JM103 was added and the culture was incubated at 37°C with shaking for 7 hours. One ml was microfuged for 10 minutes, the supernatant was poured off into another tube and the virus were precipitated by incubation in 4% PEG 6000 and 0.5 M NaCl at room temperature for 30 minutes. After microfuging for 5 minutes, the supernatant was discarded, the inside walls of the tubes were wiped clean and the virus were resuspended in TES buffer (20 mM Tris pH 7.5, 10 mM NaCl, 0.1 mM Na_2 EDTA). The virus were then extracted with phenol saturated with 10 mM Tris HCl pH 8.0, 1 mM Na_2 EDTA, for 5 minutes. After microfuging for 5 minutes, the aqueous layer was removed and the DNA was precipitated by the addition of 0.1 M Na Acetate and two volumes of ethanol and overnight incubation at -20°C. The precipitated viral DNA was collected by microfuging for 10 minutes, washing with cold ethanol and then microfuging again. After drying, the DNA was resuspended in 50 μl TES buffer. This procedure routinely gave enough DNA for 10 sets of sequencing reactions.

Selection for Loss of Tetracycline Resistance

Cultures were grown in the absence of tetracycline for a number of generations. Cells which had lost the tetracycline resistance carrying plasmid were selected using fusaric acid (Maloy and Nunn, 1981).

Sequencing Reactions

Sequencing reactions were adapted from the procedures of Sanger et al., (1977) as adapted for M13 (Schrier and Cortese, 1979). 1 µg of M13 template DNA and 5 ng primer (a 26 base pair fragment which is complementary to the lac DNA immediately adjacent to the Eco R1 site) were annealed at 90°C for 5 minutes in 70 mM Tris HCl pH 7.5, 70 mM $MgCl_2$, 500 mM NaCl in a volume of 0.015 ml, then slowly cooled to room temperature. Deoxynucleoside triphosphates, dideoxynucleoside triphosphates were added in the concentrations given by Sanger et al., (1977). α-^{32}P-dATP, 1 unit DNA Pol I large fragment and 5 mM dithrothreitol were added and incubated for 15 minutes at 30°C. 0.025 mM dATP was added and incubation continued for another 15 minutes. Reactions were stopped by addition of 10 µl of formamide dye mix 0.1% (w/v) xylene cyanol FF, 0.1% (w/v) bromophenol blue, 10 mM Na_2 EDTA, 95% (v/v)deionized formamide. 6% sequencing gels were prepared as described by Sanger and Coulson (1978).

Materials

α-^{32}P-dATP was from New England Nuclear (400 Ci/mmol) or Amersham (800 Ci/mmol). Deoxynucleoside triphosphates and dideoxynucleoside triphosphates were from Bethesda Research Laboratories. M13mp7 RF DNA and 26 bp primer were from Bethesda Research Laboratories. Fusaric acid, ampicillin and tetracycline were from Sigma.

RESULTS

Cloning gol Mutants Into pACYC184

The gol 6B mutation maps between 23amB17 and 23amB272 (Champness and Snyder, 1982; Figure 1) in the coding region of the gene 23 product. Other gol mutations have not been so precisely mapped, but all are very close to 23amB17 (W. Champness, unpublished data).

Much of the T4 genome has been cloned as Eco R1 restriction fragments and gene 23 is completely included within a 3.5 Kb Eco R1 fragment (Figure 1). Therefore, cytosine-containing DNA from 56$^-$, den A$^-$, den B$^-$, alc$^-$, gol$^-$ mutants constructed as in Methods were digested to completion with Eco R1 and run on an agarose gel. Fragments of the size 3.5 Kb were recovered and ligated to pACYC184 (Figure 2). Recombinant clones were screened genetically, by marker rescue, for the presence of a gol mutation. Clone pG6B was derived from gol 6B and clones pGH1 and pGH2 were derived from the hydroxylamine-induced mutants, gol H1 and gol H2, respectively.

Cloning gol mutants Into M13mp7

Figure 1 shows the location and size of Hind III restriction sites within the large Eco R1 fragment including gene 23. The gol site is undoubtably included within the 1.1 Kb fragment.

The DNA sequence of this fragment has been determined (A. Christensen, personal communication). The sequence predicts that four Hpa II sites occur within the 1.1 Kb Hind III fragment (Figure 1), generating fragments of 460, 180, 75, 270, and 155 bases. It is also possible to assign the probable positions of many of the gene 23 amber mutations. The relative positions of the Hpa II sites and 23amB17 and

Fig. 1. Correlation of genetic and physical maps in the T4 gene 23 region

A. Gentic map. The locations of gene 22 and 23 amber mutations with respect to Hind III restriction sites are taken from Young et al. (1980). The distances between amber mutations are determined by recombination frequency (Celis et al., 1973). The position of gol with respect to gene 23 ambers was determined previously (Champness and Snyder, 1982). Hpa II sites were predicted from the sequence of gene 23 (A. Christensen, personal communication).

B. Physical map of clones derived from pBR322 (pLA1, pLA4, pLA5, pLA3Δ2) is taken from Jacobs et al. (1981).

C. Structure of gol mutant DNA-containing clones derived from pACYC184, pG6B, pGH1 and pGH2; and the gol^+-DNA-containing clone pA67.

Abbreviations: H3, Hind III; R1, Eco R1; H2, Hpa II; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; ⌒ indicates deleted region.

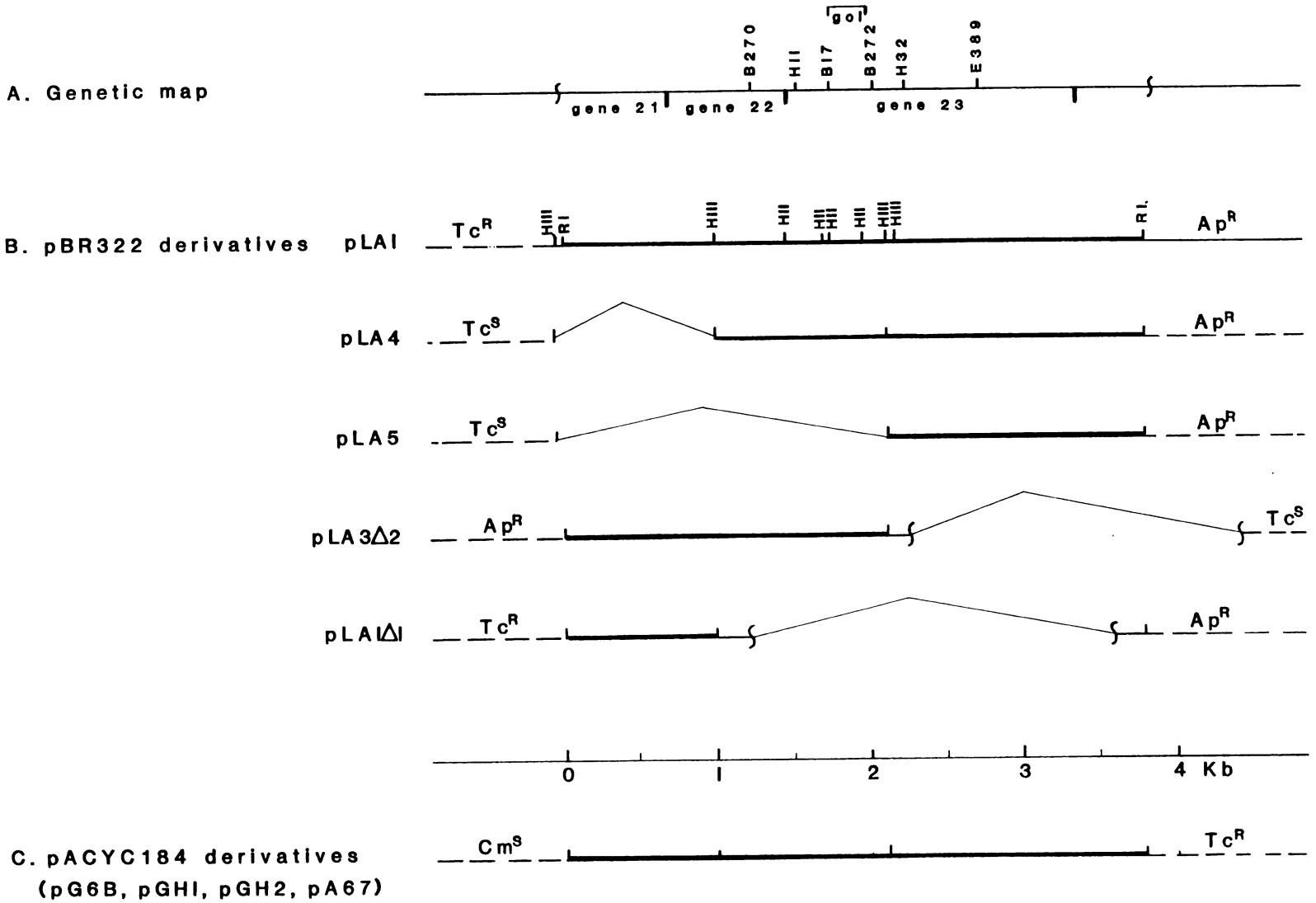
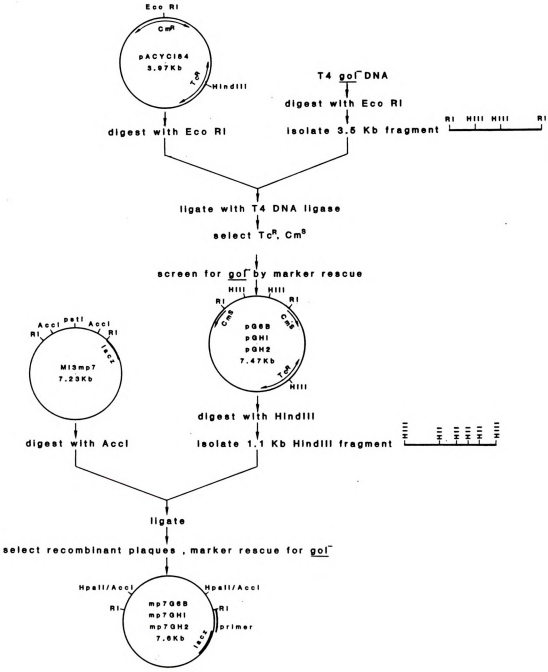

A. Genetic map
gol
B270
H11
B17
B272
H32
E389
gene 21
gene 22
gene 23
B. pBR322 derivatives
pLA1
TcR
ApR
pLA4
TcS
ApR
pLA5
TcS
ApR
pLA3Δ2
ApR
TcS
pLA1Δ1
TcR
ApR
0
1
2
3
4 Kb
C. pACYC184 derivatives
(pG6B, pGHI, pGH2, pA67)
CmS
TcR

Fig. 2. Cloning strategy. T4 cytosine-containing *gol*$^-$ DNA was first digested with Eco R1, then the 3.5 Kb fragment which contains the *gol* region was cloned into the plasmid pACYC184 (Chang and Cohen, 1978). The 270 base pair Hpa II fragment which contained *gol* (see Figure 1) was then cloned into the Acc I site in M13mp7.

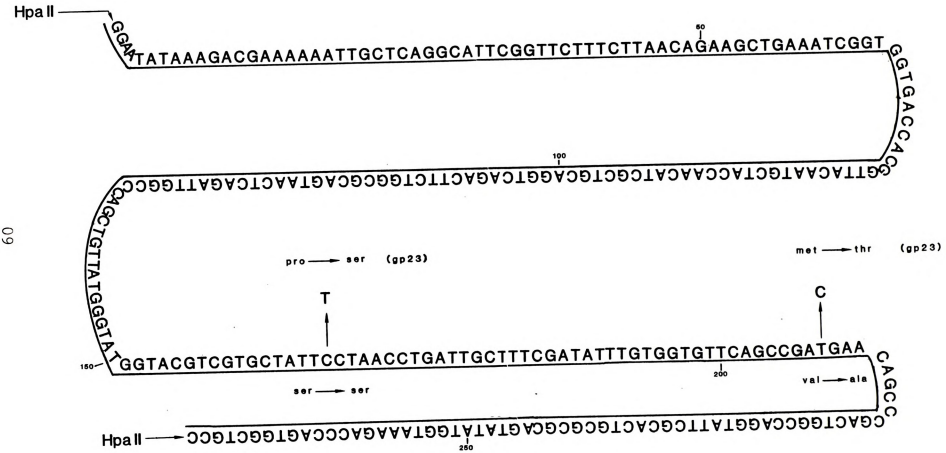

Eco RI
Cm^R
pACYC184
3.97Kb
Tc^R
HindIII
digest with Eco RI
T4 gol⁻ DNA
digest with Eco RI
isolate 3.6 Kb fragment
R1 HIII HIII R1
ligate with T4 DNA ligase
select Tc^R, Cm^S
screen for gol⁻ by marker rescue
HIII HIII
R1 R1
Cm^R Cm^R
pQ88
pQH1
pQH2
7.47Kb
Tc^R
HIII
digest with HindIII
isolate 1.1 Kb HindIII fragment
AccI PstI AccI
R1 R1
lacZ'
M13mp7
7.23Kb
digest with AccI
ligate
select recombinant plaques , marker rescue for gol⁻
HpaII/AccI HpaII/AccI
R1 R1
mp7Q88
mp7QH1
mp7QH2
7.6Kb
lacZ'
primer

23amB272 predict that the 270 base pair fragment might include the gol 6B mutation. Therefore, plasmids pG6B and pGH1 and pGH2 were digested with Hind III and the 1.1 Kb Hind III fragment was then isolated and digested with Hpa II. Hpa II ends are compatible with Acc I ends so the fragments could be cloned in the Acc I site of M13. Following ligation, recombinant M13 plaques (Figures 2) were selected and tested by marker rescue for the presence of a gol mutant DNA insert. By this method, M13mp7 clones mp7G6B, mp7GH1, and mp7GH2 (containing gol mutations 6B, H1 and H2 respectively) were obtained. As shown below, all three of the gol mutations were located within the 270 bp Hpa II fragment.

Sequence Analysis

M13 clones mp7G6B, mp7GH1 and mp7GH2 were sequenced by the dideoxy chain termination method. The sequence changes due to mutations are indicated in Figure 3. In each case, there was only one base change within the insert sequence. Because each insert was shown genetically to contain a gol mutation, these changes found must be the biologically relevant changes. The spontaneous mutant, gol 6B, changes T/A to C/G at position 207. The two hydroxylamine-induced mutants H1 and H2 are both changes of C/G to T/A at position 167. We have observed similar clustering of gol mutations when mapped genetically; most spontaneous gol mutations gave little or no recombination as if they were in the same base or within a few base pairs of each other, but one mutation (hydroxylamine-induced) gave a recombination frequency with gol 6B consistent with its being close but at a distinct site from the gol 6B mutation (L. Snyder, unpublished data).

Fig. 3. Sequence of the 270 base pair Hpa II fragment and changes due to gol$^-$ mutations. The mutant gol 6B changes T/A to C/G at position 207. The mutants gol H1 and gol H2 change C/G to T/A at position 167.


Hpa II
GGAA
TATAAAGACGAAAAAATTGCTCAGGCATTCGGTTCTTTCTTAACAGAAGCTGAAATCGGT
GGTGACCAG
GGTTACAATGCTACCAACATCGCTGGCAGGTCAGACTTCTGGCGCAGTAACTCAGATTGGCC
CAGCTGTTATGGGTAT
GGTACGTCGTGCTATTCCTAACCTGATTGCTTTCGATATTTGTGGTGTTCAGCCGATGAA
CAGCC
CGACTGGCCAGGTATTCGCACTGCGGCGCAGTATATGGTAAAGACCCAGTGGCTGCC
Hpa II
60
100
150
200
250
pro → ser (gp23)
met → thr (gp23)
T
C
ser → ser
val → ala

Transformation Studies

To determine if the gol site exerts an effect when cloned in plasmids we tested the ability of the clines to transform lit$^-$ E. coli, selecting for antibiotic resistance on the plasmid. Both pACYC184 and pBR322 can be used to transform lit$^-$ E. coli. However, as shown in Table II, a plasmid which contains the 3.5 Kb T4 DNA insert which is gol$^+$ cannot be used to transform a lit$^-$ host. Such a plasmid will transform a lit$^+$ host. The failure to transform occurs whether the insert is cloned into pACYC184 (plasmid pA67) or pBR322 (plasmid PLA1) and regardless of orientation. In contrast, plasmids which contain an insert with a gol mutation, pG6B, pGH1, or pGH2, can transform the lit$^-$ host indicating that the failure to transform is due to the gol site.

Occasionally, rare antibiotic resistant colonies arose following attempted transformation of the lit$^-$ strain with a gol$^+$ plasmid. These transformants could be grouped into two classes. The transformants in one class were phenotypically lit$^+$. To test whether these colonies were revertants of the lit$^-$ mutation or if the presence of the plasmid was affecting the lit phenotype, loss of the plasmid was selected for by growing cells in the absence of antibiotic and then selecting for fusaric acid resistance (see Methods). Lit$^+$ cells remained lit$^+$ after loss of tetracycline resistance and presumed loss of the plasmid. Therefore, these transformants were true lit$^+$ revertants and the lit$^+$ phenotype was not an effect due to the presence of the plasmid.

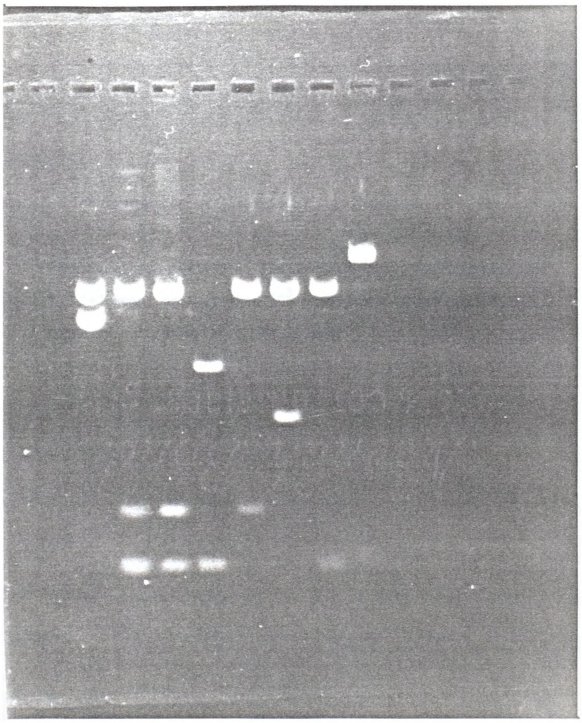
The second class of antibiotic resistant transformants were lit$^-$ and resulted from the acquisition of a plasmid which had undergone a deletion of the region which contains gol. One such deleted plasmid, pLA1 1, is shown in Figure 4. Digestion of the plasmid by

Fig. 4. Restriction enzyme digests of plasmids used in transformation studies.

Shown is an ethidium bromide stained 0.7% agarose gel on which were run restriction enzyme digests of the plasmids shown in Figure 1B.

Lane 1 and 6, DNA digested with Eco R1; lanes 2, 3, 4, 5, 7, DNA digested with Eco R1 and Hind III; lane 8, DNA digested with Hind III.

Lane 1 and 2, pLA1; lane 3, pLA4; lane 4, pLA3Δ2; lane 5, pLA5; lanes 6, 7, 8, pLA1Δ1.



1 2 3 4 5 6 7 8

Table II. Transformation of *lit*$^-$ *E. coli* by plasmids.

Transformation ability of the plasmids shown in Figures 1 and 4. "+" means greater than 10^3 transformants per μg DNA; "-" means zero transformants per μg DNA.

plasmid	T4 insert	transforms **E. coli** lit$^+$	lit$^-$
pBR322	−	+	+
pLA1	gol$^+$	+	−
pLA1Δ1	Δgol	+	+
pLA4	gol$^+$	+	−
pLA5	Δgol	+	+
pLA3Δ2	gol$^+$	+	+
pACYC184	−	+	+
pA67	gol$^+$	+	−
pG6B	gol$^-$	+	+
pGH1	gol$^-$	+	+
pGH2	gol$^-$	+	+

by both Eco R1 and Hind III shows that the plasmid contains the intact 1.0 Kb Eco R1-Hind III Fragment, but the 1.1 and 1.4 Kb bands are missing. Because no new band appears, the major portion of these two fragments has been deleted.

Taken together, these data indicate that the presence of the *gol*$^+$ T4 DNA sequence in a plasmid prevents the stable transformation of *lit*$^-$ *E. coli*.

To further define the exact region on the 3.5 Kb Eco R1 fragment which prevents transformation, transformation studies were done with subclones of the 3.5 Kb Eco R1 fragment. A series of plasmids which are derived from pLA1 (*gol*$^+$) have been constructed and described (Jacobs et al., 1981). These were tested to see if they could transform *lit*$^-$ *E. coli*. The structure of these plasmids is shown by restriction digestion in Figure 4 and drawn schematically in Figure 1. pLA4, which contains the 1.1 Hind III and 1.4 Kb Hind III-Eco R1 fragments and therefore includes *gol*$^+$ DNA, does not transform the *lit*$^-$ strain. pLA5, which contain only the 1.4 Kb Hind III-Eco R1 fragment and therefore does not include *gol*$^+$, is capable of transforming the *lit*$^-$ strain. pLA3Δ2 has a large portion of the 1.4 Kb Hind III-Eco R1 fragment deleted as the result of exonuclease III digestion. The precise endpoint of the deletion within the 1.4 Kb Hind III-Eco R1 fragment is not known but it ends short of the Hind III restriction site at 2.1 Kb. Therefore, this plasmid contains the *gol*$^+$ sequence. Nevertheless, this plasmid is capable of transforming the *lit*$^-$ strain. We do not know if this plasmid contains a small deletion or some other change within the *gol* region. If it does not, it would appear that another region on the plasmid besides the *gol*$^+$ site is involved in the failure of transformation by pLA1, pLA4 and PA67.

In general, however, there is a strong correlation between the presence of *gol*$^{+}$ T4 DNA in a plasmid and the failure of such a plasmid to transform a *lit*$^{-}$ host strain.

DISCUSSION

Nucleotide sequencing of the mutational lesions in gol mutants has confirmed the genetic mapping; all the mutations sequenced so far lie close to 23amB17 and all are single base changes. The results also confirm that the gol phenotype is not due to altering a protein.

From the mucleotide sequence (this work and work communicated by A. Christensen), it is evident that the only open reading frame on the "r" strand is that which encodes gp23. Two of the three reading frames on the "l" strand are blocked by nonsense codons, but the third is open for some distance in the region of 23amB17-23amB272. But mutations H1 and H2 would not change the amino acid which could be encoded in this frame. We have previously presented evidence that although gol mutations are located within the coding region for the gene 23 product, the gol phenotype does not occur as the result of altering p23 (Champness and Snyder, 1982). Therefore, the sequence data confirms the conclusion that gol mutations do not exert their effect by altering a protein product.

The structural significance of the mutational changes in creating a gol$^-$ phenotype is not obvious from the accumulated sequence data. Clearly, it would be necessary to sequence more mutants, both spontaneous and mutagen-induced, in order to define the limits of the region which can be mutated and the variety of changes within that region which can create a gol$^-$ phenotype. The necessity of maintaining a functional p23 places constraints on the kinds of gol$^-$ mutations that will be obtained using the selection for phage growth on a lit$^-$ host. But it may be possible to obtain a wider range of gol$^-$ mutants by selecting plasmid mutants which can transform lit$^-$ hosts. (see below)

It is interesting to note some of the unusual characteristics of the DNA sequence in the region where gol mutations lie. The DNA has an unusually high G-C content; although the average G-C content of T4 DNA is about 30%, 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 0.011% per nucleotide, but there is a "hotspot" for recombination in the amino terminus, apparently in the region where gol is located. (Celis et al., 1973). 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).

The studies on the transformation ability of gol-DNA-containing plasmids show that, besides affecting T4 late gene expression, the T4 gol site also affects the transforming ability of plasmids, i.e., in general the presence of the wild-type gol site in a plasmid prevents transformation of a lit^- host by that plasmid. We do not yet know if lit^- restriction of T4 gol^+ phage and the failure of lit^- cells to be transformed by gol^+ plasmids result from the same mechanism, but gol^- mutations selected in phage overcome both restrictions. In this regard, it is important to note the anomolous behavior of pLA3Δ2, which transforms lit^- cells although its deleted region ends short of where gol point mutations are located. Several explanations can be proposed which could account for the anomolous behavior of pLA3Δ2: the gol site is much larger than the point mutations studied thus far would indicate; another element close to gol interacts with gol; or the transformation block is not strictly analogous to the T4 development block and another close-by

element is involved in the transformation block. Selection for mutations that allow a *gol*$^+$ plasmid to stably transform would allow further study of the relationship between these two phenomena.

We do not yet know at what level *gol*$^+$ plasmids are blocked in transformation, i.e., breakage of the plasmid DNA; failure of the plasmid to be expressed, replicated or segregated; or lethality of the plasmid to the recipient cell. Further knowledge about the *gol* effect on plasmid DNA may give us our best insights into the *in vivo* function of the T4 *gol* site.

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SUMMARY

Probably the most interesting aspect of this work is the discovery of gol mutations, which affect T4 late gene expression. Evidence has been presented that gol mutations define a site on T4 DNA which affects, in cis, the expression of the T4 late genes from the entire genome.

Not enough information is currently available about T4 intracellular structure to define the role the gol site plays in late gene expression and T4 development. However, the observation that gol affects both T4 gene expression and some aspect of plasmid growth suggests that both plasmids and T4 require some common structure. If the relationships between the gol effect on T4 and the gol effect on plasmid DNA can be clarified, then knowledge about the defect in plasmid growth will be important in understanding the defect in T4 development.

Based on our current knowledge, speculative models for the effects of gol on T4 and plasmid DNA structures can be proposed. One model invokes the supercoiling of DNA. Increased superhelical density could be required for activation of the T4 late promoters. Supercoiling could also be required for plasmid transcription or replication. Plasmids are known to undergo a transient decrease in superhelical density during the growth cycle (Timmis et al., 1976; Crosa et al., 1976). Gol^+ DNA might interfere with the relaxation, maintenance, or introduction of supercoils at the proper time in the plasmid growth cycle.

Besides, or instead of, activation of promoters, supercoiling might be required because it forces the DNA into a conformation, for

example, a hairpin loop (Lilley, 1980), which is required in some reaction. Panayotatoes and Wells (1981) have observed that supercoiled plasmid DNA is cut by S1 nuclease at a preferred site because supercoiling forces the formation of a hairpin structure at that site; the *in vivo* significance of that particular hairpin is not presently known. In contrast, if an artificial, large hairpin is introduced into a plasmid, it is lethal to the cell (Lilley, 1981). It is not known what aspect of plasmid growth is affected or why the hairpin is lethal. The large hairpin could reduce or eliminate supercoiling or could have a direct negative effect on some process. Perhaps the *gol* sequence, when introduced into a plasmid, alters the tertiary conformation of the DNA.

Another possibility is that *gol* is a site at which a topoisomerase acts. As defined by the sequenced mutations, the *gol* site seems large for a protein-nucleic acid interaction site (at least 40 base pairs). But precedents for such large interaction sites do exist; for example, RNA polymerase recognizes at least 50 base pairs in the *lac* promoter (Reznikoff and Abelson, 1978).

An important observation about the *gol* site is that the wild-type *gol* site "poisons" at high multiplicity, as if it strongly competes with the mutant site (Appendix B, Figure 1B). This "competitive" effect suggests that when wild-type T4 infects a *lit*$^-$ mutant, the *gol*$^+$ sequence is not simply ignored but participates in a deleterious reaction.

Considering that *gol* has an effect on both plasmid and T4 DNA, it may be possible to learn something about what happens at the *gol* site by learning what happens to *gol*$^+$ plasmid DNA when it enters a *lit*$^-$ cell. Because plasmid transformation is such a low frequency event, it would be difficult to follow the fate of the transforming plasmid DNA or

of the recipient cells. One approach to this problem would be to clone gol DNA into bacteriophage lambda and after infection of lit$^-$ cells at a high multiplicity, follow the fate of the lambda DNA.

A possible interpretation of the data that have been accumulated on gol mutants, which has not been previously mentioned, is that the gol phenotypes results from changes of an RNA which is encoded in the gol region. In light of the data on the cis effect of gol mutations, such an RNA would have to be cis-acting. Recently, Lacatena and Cesareni (1981) have shown that a cis RNA interaction is important in determining plasmid Col El incompatability. A possible function of a "gol" RNA might be to base pair with the complementary DNA and form T4 "nucleoid-like" structures.

Konarska et al. (1981) have recently described a wheat germ RNA ligase which forms a 2' phosphomenoester, 3'5'-phosphodiester linkage, which may be either an intermediate or end product of the ligation reaction. If T4 RNA ligase can also generate such a structure, the 2' phosphate might provide a branch through which an RNA can anchor DNA. The chain of evidence that connects gol with the pseT and RNA ligase gene products makes it tempting to speculate that an RNA encoded in the gol region is processed by the pseT and RNA ligase functions to create a mature RNA which forms and/or maintains T4 DNA structure.

We do not yet have very much information about the function of the lit gene product in E. coli. Lit$^-$ mutants do not have any phenotype that we have observed except in the lit double mutants discussed in Appendix A. However, an interesting observation has been made which may be relevant to the regulation of the lit function.

When wild-type T4 infects in lit⁻ host at 30°C, less than one percent of a normal burst of phage is produced (Article I, Table 3). When infective centers are measured, it is seen that 10 percent of the lit⁻ cells produce an infective center; this subpopulation of cells produces a significant burst, about five to ten phage per cell (W. Champness, data not shown). In a gol mutant infection, the number of infective centers produced is normal.) The percentage of semi-permissive cells which produce a small burst is independent of the multiplicity of infection. The permissivity of lit⁻ cells for T4 may depend on what stage of the cell cycle the cells are in (Cooper and Helmstetter, 1968). Thus, the synthesis or activity of the lit function may be coordinated with the cell cycle.

It is interesting to speculate that the lit function plays a role in regulating some E. coli transcription which has structural requirements similar to those for T4 late transcription, although we do not now have evidence for such a type of transcriptional regulation in E. coli. However, an unusual E. coli locus, rdg, has recently been described (Froelich and Epstein, 1981) which has an interesting effect on transcription. In temperature sensitive rdg mutants, the rec A gene product is required for cell viability at high temperature; the simultaneous loss of rec A and rdg prevents most RNA transcription. Although lit and rdg are different genes and we have no reason to suspect that the rdg locus is even related to lit, this observation by Froelich and Epstein points out that there are many aspects of transcriptional regulation which we do not yet understand, even in prokaryotes. Further study of the E. coli lit gene and the T4 gol site should add to our

understanding of transcriptional regulation, especially, how intracellular DNA structure can effect regulation.

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APPENDICES

APPENDIX A

A NEW GENE OF *ESCHERICHIA COLI* WHOSE PRODUCT PARTICIPATES IN T4 BACTERIOPHAGE LATE GENE EXPRESSION: INTERACTION OF *LIT* WITH THE T4-INDUCED POLYNUCLEOTIDE 5'-KINASE 3'-PHOSPHATASE

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A New Gene of *Escherichia coli* K-12 Whose Product Participates in T4 Bacteriophage Late Gene Expression: Interaction of *lit* with the T4-Induced Polynucleotide 5′-Kinase 3′-Phosphatase†

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We isolated five *Escherichia coli* mutants deficient in their ability to support the late (replication-coupled) gene expression of T4 bacteriophage at 30°C. These mutants, which we call Lit mutants, define at least one novel gene at 25 min on the *E. coli* map. They were selected in an attempt to obtain mutants which restrict the growth of T4 mutants deficient in polynucleotide 5′-kinase 3′-phosphatase but not that of wild-type T4 at 37°C. Some of the mutants do have these phenotypes under some conditions. Studies of the block in T4 development in some of the *E. coli* mutants suggest that Lit mutants are affected in a gene product involved in the metabolism of deoxyribonucleic acid nicks or single-strand gaps. None of the Lit mutants is deficient in the major, bacterial, 3′-phosphatase activity in crude extracts.

Some types of bacteriophage transcription may require covalent alterations of the DNA template. For example, T5 induces a DNA 5′-exonuclease which enhances its late transcription (5, 9). Also, late transcription of T4 is greatly enhanced by phage DNA replication (12), and DNA ligase mutations relieve the requirement for DNA replication (13) presumably by preventing the repair of nicks in DNA.

It would be of interest to know whether the types of bacteriophage transcription which require DNA alterations have common features, and whether an analogous type of transcription exists in uninfected bacteria. A first step in addressing this question is to isolate and study host mutants which cannot support a type of bacteriophage transcription that requires DNA alterations. Presumably, some of these host mutants would be deficient in a function which participates in the phage transcription and, hopefully, in an analogous type of host transcription. For the selection, it may be best to start with phage mutant in a known function thought to be involved in transcription, since using the wild-type bacteriophage for the selection is technically difficult and would reveal little about the actual functions involved. The host mutants of interest are those that can propagate wild-type bacteriophage but not the phage mutants.

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We have proposed that the T4 polynucleotide 5′-kinase (10, 11) is required, under some conditions, for T4 late transcription (14). This enzyme is also a 3′-phosphatase (3, 14), so it will henceforth be referred to as the T4 polynucleotide 5′-kinase 3′-phosphatase. This enzyme is normally not required for phage development on standard laboratory strains of *Escherichia coli*, since T4 *pseT*$^-$ mutants, which do not induce it (7, 14), multiply almost normally (7, 4, 14). However, T4 *pseT*$^-$ mutants are restricted on *E. coli* CTr5x, a hybrid of *E. coli* K-12 and *E. coli* CT196 (a clinical isolate) (7), because subnormal amounts of late gene products are made (14) and also, possibly, because of a DNA packaging defect (7). Presumably, *E. coli* CTr5x has an amber mutation in a gene whose product is required to support the multiplication of *pseT*$^-$ T4 because, with the acquisition of an efficient amber suppressor, it becomes permissive for all *pseT*$^-$ mutants, even deletions (14). It is tempting to speculate that the host gene with the amber mutation codes for an enzyme which is analogous to the phage polynucleotide 5′-kinase 3′-phosphatase and can normally substitute for it during T4 development. However, other explanations are possible.

Rather than try to map the putative amber mutation in *E. coli* CTr5x which makes it restrictive for *pseT*$^-$ T4 (which could be difficult because the strain is distantly related to *E. coli* K-12), we isolated similar restricting mutants of a convenient laboratory strain of *E. coli* K-12.

These mutants, which we call Lit$^-$ mutants, for late inhibitors of T4, are similar in some, but not all, respects to *E. coli* CTr5x. They define at least one, and possibly two, new genes of *E. coli*.

MATERIALS AND METHODS

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

Isolation of Lit mutants. Lit mutants were isolated as mutants which plated wild-type T4 but not a T4 *pseT* deletion mutant, Δ*pseT*1, at 37°C. Potential mutants were subsequently screened to determine whether the restriction originates from the absence of the *pseT* gene or from the absence of another T4 gene included in the deletion.

The mutants we were seeking would not propagate the T4 *pseT* deletion mutant but would be killed by it. The latter presents problems for the selection, which can be dealt with in a number of ways. The first Lit$^-$ mutant, *E. coli* MPH5, was isolated using a variation of the "tab" procedure of Takahashi et al. (16). In this procedure, a carefully predetermined number of phage and mutagenized bacteria are plated together, and *E. coli* mutants that survive are tested for the desired phenotype. This method is very sensitive to the amount of phage and bacteria as well as the condition of the plates, and often MPH5 did not survive a reconstruction of the selection conditions. Therefore, we designed a type of sibling selection procedure by which we isolated the remaining four Lit$^-$ mutants.

This procedure succeeds because, on an undisturbed plate, the descendants of a mutant bacterium will be clustered and will protect each other by failing to produce the T4 *pseT* deletion mutant progeny. *E. coli* AB2495 were mutagenized with nitrosoguanidine by the procedure of Adelberg et al. (1), and about 10^8 were spread on a tryptone plate with 10^7 T4 Δ*pseT*1. The plate was incubated overnight at 37°C. This plate was then replicated onto another plate on which about 10^9 T4 Δ*pseT*1 had been spread, and this plate was also incubated overnight. About 500 to 1,000 colonies appeared, mostly due to resistant mutants. The MPH5 prototype survived a reconstruction of this procedure even when mixed with 10^8 of the unmutagenized parental cells beforehand. MPH5 mutant colonies grew with a characteristic "lumpy" appearance, like a bunch of grapes, which helped distinguish them from resistant colonies, which tended to be round and often slimy. The lumpy colonies from the mutagenized plates were picked under a dissecting microscope and streaked across dried streaks of both T4 Δ*pseT*1 and T4 wild type, in that order, both at 10^9/ml, and the plates were incubated at 37°C. The mutants that seemed to be cleared by wild-type T4 but not by Δ*pseT*1 were purified by two cycles of "streaking out" and used as indicator bacteria to plate T4 Δ*pseT*1 and wild-type T4 at 37°C. We found about one Lit$^-$ mutant for every 200 lumpy colonies tested, and MPH6, -7, -21, and -24 were isolated by this procedure. All five of the Lit$^-$ mutants were probably due to independent mutational events because they either originated from different mutagenized stocks or had different phenotypes.

TABLE 1. *Bacteriophage and bacterial strains*

	Strain	Relevant characteristics	Source or reference
T4	Δ*pseT*1	Deletion including *pseT*	14
	*pseT*2	Point mutant in *pseT*	7
	BL292	Gene 55 amber	8
	N82	Gene 44 amber	8
	N81	Gene 41 amber	8
	NG576	Gene 52 amber	8
	N134	Gene 33 amber	8
	M69	Gene 63 amber	8
	B17	Gene 23 amber	8
	E727	Gene 49 amber	8
	N54	Gene 31 amber	8
E. coli	CT196	Clinical isolate	7
	CTr5x	Hybrid K-12: CT196	7
	AB2495	F$^-$ multiple auxotroph *trp-35 his-4 supE44*	CGSC[a]
	Hfr Broda 8	Transfers clockwise from 8 min	CGSC
	Hfr KL99	Transfers clockwise from 22 min	CGSC
	Hfr KL96	Transfers counterclockwise from 49 min	CGSC
	MA1008	*pyrC46*	CGSC
	W3110	*trp*$^+$	CGSC
	PCO254	*purB51*	CGSC

[a] CGSC, *E. coli* Genetic Stock Center, Department of Human Genetics, Yale University School of Medicine, New Haven, Conn.

Mapping Lit mutants. The procedure of Curtiss et al. (6) was used for Hfr crosses. P1 transduction was with P1*vir* in tryptone broth supplemented with 2.5 mM $CaCl_2$. The presence or absence of the Lit phenotype among recombinants was determined by streaking across T4 Δ*pseT*1 and T4 wild type as in the isolation procedure.

Assays of *E. coli* 3′-phosphatases. The assay conditions were essentially those of Depew and Cozzarelli (7). The preparation of 3′-[^{32}P]dTMP was from 500 ml of *E. coli* cells labeled for about six generations in Tris medium with 10^{-3} M PO_4 and 15 mCi of $^{32}PO_4$. The cells were centrifuged and incubated overnight in 1% sodium dodecyl sulfate and 0.5 M NaOH at 37°C to degrade the RNA. The extract was neutralized, blended in a Vortex mixer, extracted twice with phenol, and dialyzed against 1 M NaCl and then water. The dialysate was precipitated with 5% (wt/vol) trichloroacetic acid and washed with 80% ethanol and then ether before drying. The pellet was suspended in 2 ml of water and was digested with micrococcal nuclease (Worthington) to 40 μg/ml in 0.01 M Tris (pH 8.7)–2.5 mM $CaCl_2$ for 1 h at 37°C, neutralized, and further digested by adding spleen phosphodiesterase (Worthington) to 50 μg/ml three times, 1 h apart. The 3′-dTMP from the digest was purified by paper electrophoresis in 0.05 M ammonium acetate (pH 3.5), followed by paper chromatography with 1.8% NH_4OH, 62% isobutyric acid, and 10^{-3} M EDTA and by a repeat of the paper electrophoresis (14). Very high backgrounds were observed if the 3′-dTMP was contaminated with either 5′-dTMP or 3′-UMP. Any 3′-UMP

left over from RNA after dialysis will be separated from 3′-dTMP by the above procedure. However, 5′-dTMP is not separable, so the micrococcal nuclease and spleen phosphodiesterase must be free of contaminating DNase activities.

To prepare extracts for the assays, cells were grown to 4×10^8/ml in tryptone broth (10 g of tryptone and 5 g of NaCl per liter of water), and, if infected, phage was added at a multiplicity of infection of 10 for 15 min at 37°C. The cells were chilled, centrifuged, resuspended at one-tenth the volume in 0.01 M Tris (pH 7.5) with 1 mM mercaptoethanol, and lysed by sonication. *E. coli* B extracts (0.1 ml) could be assayed directly, but *E. coli* K-12 extracts first needed to be diluted about 1:100 with resuspension buffer. For further purification, the extracts were cleared by centrifugation for 20 min at 75,000 × *g* and precipitated with an equal volume of 0.5% protamine sulfate. The supernatant fluid was assayed then or purified further by $(NH_4)_2SO_4$ precipitation and DEAE chromatography by the procedure of Becker and Hurwitz (2). Approximately 30 to 40% of the activity in crude extracts from *E. coli* B or K-12 strains was recovered in the DEAE fractions.

Labeling of proteins and DNA. Because of the multiple auxotrophies of the *E. coli* AB2495 parent, Lit⁻ mutants were difficult to infect synchronously with T4, necessitating the following procedure. First *thy*⁺ revertants were used. To label proteins, the cells were grown at 37°C to 4×10^8/ml in M9 (5.5 g of Na_2HPO_4, 3 g of KH_2PO_4, 0.5 g of NaCl, 1.0 g of NH_4Cl, 0.5% glucose, 10^{-3} M $MgSO_4$) supplemented with thiamine at 50 μg/ml and all 19 amino acids at 100 μg/ml except methionine. The cells were concentrated 10 times by centrifugation and infected at a multiplicity of 10 with T4 which had been purified on CsCl step gradients to remove ghosts and prevent ghost exclusion. After 4 min for absorption, the infected cells were diluted 1:10 into fresh medium at 30°C and labeled with 10 μCi of [^{35}S]methionine per ml (865 Ci/mmol) at the times and for the periods indicated.

To label DNA, M9 medium supplemented with 1% casein hydrolysate, tryptophan (50 μg/ml), and thiamine (50 μg/ml) was used, and the cells were labeled with [*methyl*-^{3}H]thymidine at 10 μCi/μg (1 μg/ml) to measure the rate of incorporation and 100 μCi/μg (1 μg/ml) to measure the size of DNA. For the measurements of the rate of incorporation, cells were precipitated with 5% trichloroacetic acid, resuspended with 2% KOH, and reprecipitated with 5% trichloroacetic acid before being collected on glass fiber filters.

Alkaline sucrose gradients. The procedures of Depew and Cozzarelli were followed (7), layering 0.1 ml of an unconcentrated lysate on the gradient.

Slab gel electrophoresis and autoradiography. The apparatus and procedure of Studier (15) were used. Gels were stained with Coomassie blue to check the total amount of protein applied to each well.

RESULTS

Isolation of Lit mutants. We isolated five *E. coli* mutants which restrict the growth of the T4 Δ*pseT*1 mutant but not that of wild-type T4 at 37°C. Three of the five mutants (MPH5, -7, and -21) were indistinguishable in some respects. They multiplied with the same generation time as their parent and absorbed T4 normally. Wild-type T4 produced plaques on them with an efficiency of about 0.2 at 37°C, whereas T4 *pseT* deletions such as Δ*pseT*1 plated with an efficiency of less than 10^{-4}. It was perhaps fortunate that we carried out the selection at 37°C because there was no difference between the plating efficiencies of the *pseT* deletions and wild-type T4 at either higher or lower temperatures. At 30°C both wild-type T4 and T4 Δ*pseT*1 were almost totally restricted, plating with efficiencies of less than 10^{-4}. At 42°C, even the T4 *pseT* deletions plated normally. The effect of varying incubation temperature on wild-type T4 plating efficiency of Lit⁻ mutants was remarkably abrupt, the plating efficiency going from 10^{-8} to 0.2 in the temperature range of 34 to 37°C. None of these three Lit⁻ mutants was particularly restrictive for the growth of T4 *pseT* point mutants even at 37°C.

The other two *E. coli* mutants (MPH6 and -24) were different in some respects. MPH6 was noticeably restrictive for T4 *pseT* point mutants at 37°C, giving very small plaques at a frequency of about 10^{-2}. It absorbed T4 more slowly than the parental strain, made slimy, opaque colonies, especially on minimal media, and did not plate bacteriophage P1. It was cold sensitive for T4 multiplication, as were the other Lit⁻ mutants.

MPH24 grew poorly, particularly at 42°C. It was more permissive for wild-type T4 at 30°C than the other Lit mutants. It absorbed T4 slowly and did not plate P1. It was not particularly restrictive for the growth of T4 *pseT* point mutants.

Mapping mutations responsible for the Lit phenotypes. MPH5, -7, and -21 behaved similarly to each other in Hfr crosses and P1 transduction, as though they have mutations in the same gene. With Hfr Broda 8 as donor, approximately 80% of the Trp⁺ recombinants were Lit⁺, whereas only 34% of the His⁺ recombinants were Lit⁺. With Hfr KL208, only 30% of the Trp⁺ recombinants were Lit⁺. Thus, the conjugation data with these donors suggested that the Lit mutation lay counterclockwise and within about 10 min of *trp*. This conclusion was also supported by the mapping data with Hfr KL99, which transferred the region of the Lit mutation very early, placing it clockwise of 22 min. P1 transduction was used to further localize the Lit mutation (Table 2). We found no cotransduction with *pyrC* or with *trp*. There was, however, about 70% cotransduction with *purB* and the Lit⁻ marker in all three Lit mutants, indicating that all three had mutations at about 25

TABLE 2. *Mapping of lit mutations with P1 transduction*

Donor	Recipient	Selected marker	Unselected marker	No. tested	Percent co-transduction
MPH5	MA 1008	*pyrC*$^+$	*lit*	110	0
W3110	MPH5	*trp*$^+$	*lit*$^+$	148	0
MPH5	PC0254	*purB*$^+$	*lit*	63	78
MPH7	PC0254	*purB*$^+$	*lit*	76	66
MPH21	PC0254	*purB*$^+$	*lit*	39	51

min on the *E. coli* map. Since the mutations were closely linked and had similar phenotypes, we think they arose in the same gene, although we have not established this point with complementation tests. We have tentatively assigned the name *lit* to the gene and given the mutations in MPH5, -7, and -21 the names *lit-5*, *lit-7*, and *lit-21*, respectively. The one Lit$^-$ transductant of PC0254 we tested was similar to the original MPH5 mutant in all of its phenotypes. We take this as additional evidence that a mutation in one gene was causing the multiple phenotypes of MPH5, -7, and -21.

In contrast, the two other mutants, MPH6 and MPH24, were probably double mutants, having *lit* mutations and at least one other unlinked mutation in a cistron whose product interacts with the *lit* function. The evidence for this is as follows. When MPH6 and -24 were crossed with Hfr Broda 8 and Trp$^+$ was the selected marker, most of the recombinants made very poor lawns on plates. If, as suggested by its map position, the mutation crossed out of MPH6 and MPH24 was a *lit* mutation, we would expect the recombinants to accumulate *lit* mutations upon culturing. This prediction was fulfilled, at least for the MPH6 mutant. Furthermore, we were able to isolate a *lit* single mutant as a recombinant from the original MPH6 mutant. This mutant was similar to our other Lit$^-$ mutants, and we call its mutation *lit-6*. It was used for some of the experiments discussed below.

Because of their similarities, we assume that both MPH6 and MPH24 carried *lit* mutations as well as second-site mutations in the same other gene. We think that it is the mutation in this other gene that caused MPH6 and -24 to be restrictive for T4 *pseT* point mutants, because the recombinants, when first isolated (at least in the case of MPH24), restricted T4 *pseT* point mutants but were completely permissive for wild-type T4 at any temperature.

Effect of *lit* mutations on T4 development. Since at least some of our *lit* mutations did not affect bacterial growth or T4 absorption, we could study their effect(s) on T4 development at 30°C to determine why T4 does not multiply on them. The rate of T4 DNA synthesis was not significantly affected (Fig. 1A). Also, alkaline sucrose gradient centrifugation revealed little difference in the single-strand length of the T4 DNA that was made (Fig. 2A). The remaining parts of Fig. 1 and 2 will be referred to below.

Slab gel electrophoresis of proteins and autoradiography can be used to analyze T4 gene expression during infection because only T4 proteins will be labeled after infection, since T4 shuts off host protein synthesis. It can be seen that T4 early gene expression is not significantly affected by the *lit* mutation (Fig. 3A and E). However, there is a dramatic effect on T4 late protein synthesis, especially very late in infection (Fig. 3B, C, and D and F, G, and H). It can be seen that T4 late protein synthesis began normally on the mutant host but then decreased

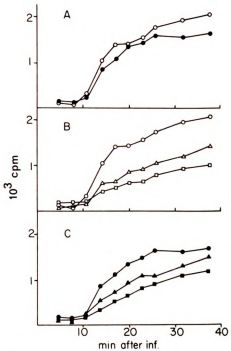


FIG. 1. *The rate of T4 DNA synthesis after infection of the Lit$^+$ parent, E. coli AB2495, and a Lit$^-$ recombinant of MPH6 with T4 wild-type and a T4 Gol mutant. E. coli AB2495, open figures; MPH6 recombinant, closed figures.* (●, ○) *Wild-type T4;* (■, □) *T4 Gol mutant;* (▲, △) *mixed, T4 Gol plus wild-type T4 at a multiplicity of infection of 5 of each. The wild-type T4 experiments shown in (B) and (C) are replots of the data in (A) for comparison. Surviving bacteria were less than 0.1% at 2 min after infection in all cases.*

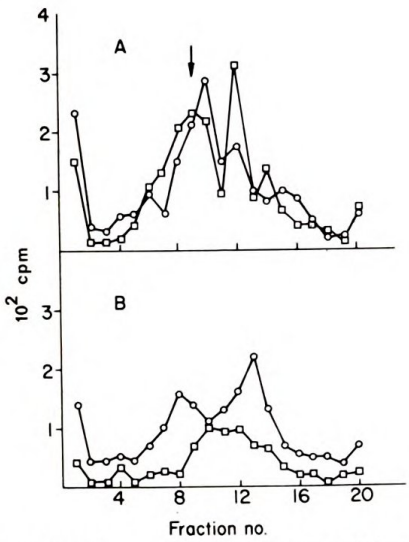


FIG. 2. *Alkaline sucrose gradient centrifugation of T4 DNA pulse-labeled from 14 to 16 min after infection of E. coli by wild-type T4 and a T4 Gol mutant. (A) ○, wild-type T4 on E. coli AB2495; and □, wild-type T4 on the E. coli MPH6 recombinant. (B) ○, T4 Gol on E. coli AB2495; and □, T4 Gol on the E. coli MPH6 recombinant. The arrow shows the approximate position of a viral T4 DNA marker on a similar gradient. Shown are the trichloroacetic acid-precipitable counts.*

in rate relative to the Lit$^+$ control. We assume that the effect on late gene expression is sufficient to explain the inability of T4 to plate on a Lit$^-$ mutant host at 30°C. T4 *pseT* deletion mutants, such as Δ*pseT*1, were no more deficient in late gene expression than wild-type T4 at 30°C (data not shown). We have not yet analyzed protein or DNA synthesis at 37°C.

Physiological and genetic characterization of T4 mutants that multiply on *E. coli* Lit mutants. Plaques formed at a low frequency when wild-type T4 was plated on Lit$^-$ mutants at 30°C or when T4 *pseT* deletions were plated at 30°C or 37°C. These plaques were almost normal size and were due to the growth of T4 mutants because, when the phage from them were isolated, they gave plating efficiencies of 100% on Lit$^-$ mutants. They also gave 100% plating efficiencies on the Lit$^+$ parent. We call the mutants T4 Gol mutants, for grow on Lit. A T4 Gol mutant isolated from any Lit$^-$ mutant plated on any other Lit$^-$ mutant. This was one of the first indications that the *E. coli* Lit$^-$ mutants all had a common molecular basis for their phenotypes.

The frequency of Gol mutants in lysates of some T4 *pseT* deletion mutants was three orders of magnitude higher than in wild-type T4 lysates. There are three possible explanations: the *pseT* deletions may be mutagenic; the Gol phenotype may require two mutations, one of which is in the region included in the *pseT* deletion; or Gol mutants may be selected when the *pseT* deletion mutants multiply, even on a Lit$^+$ host. We think the third explanation, that the Gol mutants were selected, is the correct one because the *pseT* deletion mutants did not accumulate mutations to other phenotypes and because the Gol phenotype did not depend on the *pseT* deletion and could be separated from it by recombination.

In an attempt to map the T4 mutation responsible for the Gol phenotype, we plated the T4 amber mutants shown in Table 1 on *E. coli* MPH5 (*supE*). The phage that made plaques were amber, Gol double mutants; they were crossed with wild-type T4, and the progeny were plated on a *supE*$^+$ but Lit$^-$ derivative of MPH6. Only the Gol, amber$^+$ recombinants should plate, and the recombination frequency between the Gol mutation and the amber mutation can thus be determined. We found no linkage with T4 genes 55, 44, 41, 52, 33, and 63. We had difficulty selecting Gol mutants with amber mutants in genes 23, 49, and 31, so we crossed a Gol mutant with amber mutants in these genes and tested the progeny for wild-type recombinants, since we knew these would be viable. We found very close linkage with gene 23, which is in the late region of the T4 map. Four other independent Gol mutants we tested showed the same linkage to gene 23. We are presently undertaking more precise mapping studies.

Since Gol mutations can almost completely alleviate the affect of the host *lit* mutations on T4 development, studies of T4 Gol mutant development should give us insights into the function of the host *lit* gene product. In particular, if the effect of *lit* mutations on T4 late gene expression is responsible for the inability of T4 to grow on a Lit$^-$ host, then Gol mutations should overcome this effect. Figure 4 shows the results of slab gel electrophoresis and autoradiography of the proteins made late in infection by a T4 Gol mutant. The Gol mutation had no effect on late gene expression in the parental host strain (Fig. 4A and B). However, the Gol mutation did affect late gene expression on the mutant host, completely overcoming the effect of the host *lit* mutation (Fig. 4D and E). It should be noted that some of the T4 late proteins affected (e.g., p37) are coded by genes which are some distance from the site of the Gol mutation and which are separated from it by early genes.

Also shown are the results of a complementation test in which cells were infected by wild-

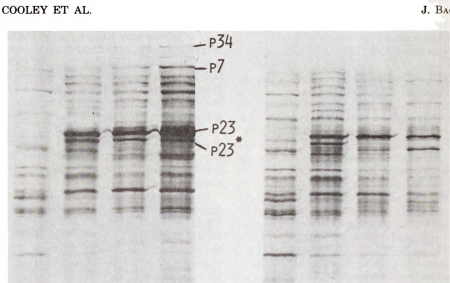


FIG. 3. *Rate of synthesis of T4 proteins after infection of the recombinant of E. coli MPH6. Slab sodium dodecyl sulfate-12% polyacrylamide gels and autoradiograms of proteins pulse-labeled with [^{35}S]methionine after T4 infection at 30°C, multiplicity of infection of 10. Labeling period: (A) and (E), 6 to 9 min; (B) and (F), 18 to 21 min; (C) and (G), 28 to 31 min; (D) and (H), 38 to 41 min. (A to D) E. coli AB2495 Lit$^+$; (E to H) E. coli Lit$^-$. The products of some late genes are identified. P23* is the processed form of the product of T4 gene 23.*

type T4 and the Gol mutant simultaneously and the effect on T4 late gene expression was determined. The Gol mutant was codominant with wild-type T4 for late gene expression on the Lit$^-$ host (Fig. 4F). This is not a special property of this Gol mutation, because two other mutants we have tested were also codominant. It is surprising that codominant mutations could enhance T4 late gene expression since T4 late transcription undoubtedly occurs from many promoters simultaneously and there are many T4 genomes present late in infection. We shall return to possible explanations in the Discussion.

The only T4 Gol mutant we tested also showed a defect in T4 DNA replication, and this occurred on any host. Figure 1B and C show that the rate of T4 DNA synthesis was sharply reduced when a T4 Gol mutant infected the parent *E. coli* AB2495 (Fig. 1B) or a Lit$^-$ mutant (Fig. 1C). Again, the effect of the Gol mutation on the rate of T4 DNA replication seemed to be codominant, because in mixed infections the rate of T4 DNA synthesis was intermediate between the T4 Gol and wild-type T4 rates.

The T4 Gol mutation affected not only the rate of DNA synthesis but also the size of the T4 DNA that was made. In Fig. 2B, alkaline sucrose gradient centrifugation revealed that the single strands of the T4 Gol DNA labeled in a 2-min pulse of [^{3}H]thymidine were significantly shorter in either a Lit$^-$ mutant or its parent. All of the experiments above were done with a T4 Gol mutant which we had shown to have a mutation close to gene 23 in the late region. We have not absolutely ruled out the possibility of two mutations in the Gol mutant, of which one is responsible for the effect on late protein synthesis and the other for the effect on replication. However, this seems very unlikely, since the Gol mutant was a spontaneous mutant and both the effects on late gene expression and replication were codominant.

Effect of *lit* mutations on host 3′-phosphatase activities. We began the isolation of *E. coli* Lit$^-$ mutants with the thought that we might find a host gene that codes for a product analogous to the T4 phage-induced polynucleotide 5′-kinase 3′-phosphatase. If so, Lit$^-$ mutants may be deficient in a similar enzyme. No one has been able to detect a polynucleotide 5′-kinase activity in uninfected bacteria (10, 11). In 1967, Becker and Hurwitz (2) reported a 3′-phosphatase from *E. coli* which was nonspecific for

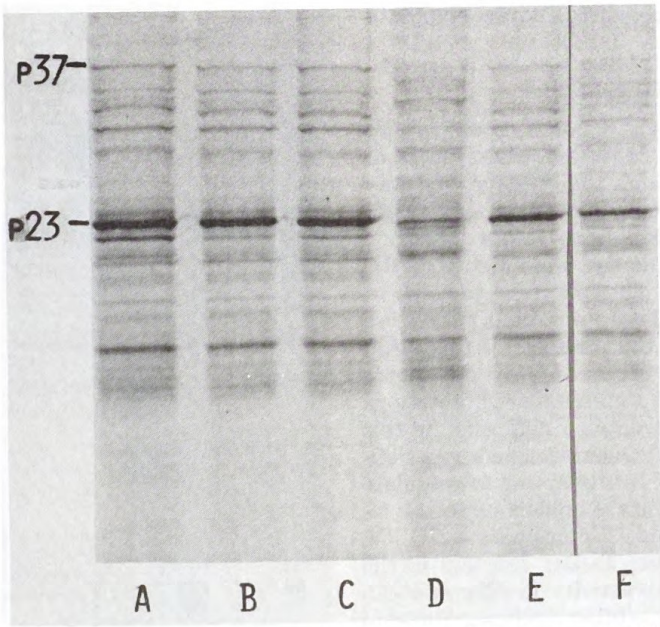


FIG. 4. *T4 late protein synthesis by wild type and a T4 Gol mutant on E. coli AB2495 (Lit⁺) and E. coli MPH7 (Lit⁻). Shown are sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiograms as in Fig. 3. Time of labeling, 35 to 38 min after infection. (A) and (D) Wild-type T4, multiplicity of infection of 10; (B) and (E) T4 Gol, multiplicity of 10; (C) and (F) T4 Gol plus wild-type T4, multiplicity of 5 for each. (A to C) E. coli AB2495 (Lit⁺); (D to F) MPH7.*

3′- or 5′-phosphates on mononucleotides, but which, on polynucleotides, would only remove 3′-terminal phosphates. This enzyme was reported to be much more active in extracts of *E. coli* C than *E. coli* B. We found that not only *E. coli* C, but also *E. coli* K-12 and *E. coli* CTr5x, displayed almost 100 times as much 3′-phosphatase activity in crude extracts as *E. coli* B. The major activity in *E. coli* K-12 was not deficient in any of our Lit⁻ mutants or in *E. coli* CTr5x (see Table 3). We think the differences in activities shown in Table 3 are not significant and are due to the difficulty of assaying the enzyme in crude extracts, because they were not reproducible and disappeared upon further purification of the enzyme.

We do not know if the major 3′-phosphatase detected at much lower levels in B strains was due to the same or a different enzyme. It purified much the same through the DEAE chromatography step of Becker and Hurwitz and could use Co^{2+} instead of Mg^{2+} as a divalent cation, as could the K-12 enzyme.

We consistently observed an inhibition of the *E. coli* B enzyme after T4 infection, as indicated by the lower level of 3′-phosphatase activity in T4 *pseT*2-infected cells (i.e., in the absence of phage-induced enzyme) compared to uninfected cells (see Table 3). We think this inhibition is real because it has appeared dozens of times in our assays. The inhibition of the host enzyme was more apparent after partial purification of the enzyme with protamine sulfate (see Materials and Methods), when almost no activity could be detected (data not shown). Thus, the activity which persisted in T4 *pseT*2-infected cells in Table 3 was probably due to other competing activities in crude extracts. In contrast, the major 3′-phosphatase activity in *E. coli* K-12 did

TABLE 3. *3′-Phosphatase activities in Lit mutants and extracts of T4-infected E. coli B*

Expt no.	Source of crude extract	3′-Phosphatase activity[a]
I	*E. coli* AB2495	4.8
	MPH5	5.4
	MPH6	3.3
	MPH7	7.7
	MPH21	5.1
	MPH24	3.2
	CTr5x	8.7
II	*E. coli* B	0.11
	T4⁺-infected *E. coli* B	0.17
	T4 *pseT*2-infected *E. coli* B	0.04

[a] Nanomoles of PO_4 released from 3′-dTMP in 30 min at 37°C per 4×10^8 cell equivalents.

not seem to be inhibited after T4 infection (data not shown).

DISCUSSION

Some Lit⁻ mutants of *E. coli* K-12 restrict T4 *pseT*⁻ point mutants more than wild-type T4 at 37°C. The Lit⁻ mutants that behave this way seem to have mutations in another, as yet uncharacterized, gene. It appears that a deficiency in this other gene was the direct cause of the restriction of *pseT*⁻ mutants of T4 in some of the Lit⁻ mutants. There must be a very close interaction between the products of this other gene and the *lit* gene because Lit⁻ recombinants made poor lawns and accumulated *lit* mutations as suppressors. Apparently, a deficiency in this other function must be compensated by a deficiency in *lit* function. It is tempting to speculate that this other function is analogous to the 5′-polynucleotide kinase 3′-phosphatase of T4. None of the Lit⁻ mutants was deficient in the major 3′-phosphatase activity in *E. coli* K-12. However, there may be other 3′-phosphatases in *E. coli*, as suggested by our observation that an activity in *E. coli* B, which is present at much lower levels, is inhibited after T4 infection, which is not true of the major K-12 activity.

A comparison of Lit⁻ mutants and *E. coli* CTr5x is interesting. In both *E. coli* CTr5x and Lit⁻ mutants, T4 late gene expression is defective. Furthermore, the effect on late gene expression in both is temperature dependent, since *E. coli* CTr5x allows normal synthesis of T4 late proteins after infection by a T4 *pseT*⁻ mutant at 42°C (see Fig. 5). However, T4 *pseT* Gol mutants do not plate on *E. coli* CTr5x. In spite of this, it seems likely that the restriction of T4 *pseT* mutants by *E. coli* CTr5x and by *E. coli* Lit mutants is related.

We think that *lit* mutations define a new cistron of *E. coli* K-12. The only other known mutations that map in this region and restrict T4 are *galU* mutations. However, *galU* mutations cotransduce with *trp* and not *pur*B and do not exert an effect on T4 in the first generation, and the progeny T4 grown on a *galU* host will plate on *rgl* but not *rgl*⁺ *E. coli*. Those T4 progeny that escaped the *lit* restriction plated equally well on *rgl*⁺ and *rgl* cells (data not shown).

The *E. coli lit* mutations prevented late gene expression and the development of bacteriophage T4 at temperatures below 34°C, suggesting that T4 needs the product of the host *lit* gene for its late gene expression, at least at lower temperatures. The plating efficiency of T4 on host *lit* mutants increased abruptly from 34 to 37°C. This abrupt change occurred in all of our

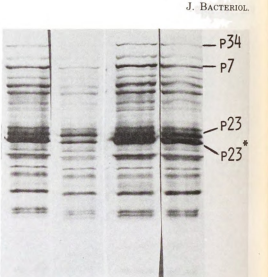
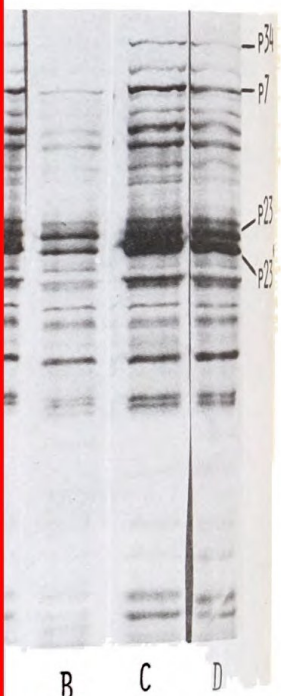


FIG. 5. *T4 late protein synthesis on E. coli CTr5x at 37 and 42°C. Shown are sodium dodecyl sulfate-polyacrylamide gel electrophoresis autoradiograms as in Fig. 3. (A) and (C) Wild-type T4; (B) and (D) T4 pseT2; (A) and (B) 37°C; (C) and (D) 42°C. Time of labeling: (A) and (B), 38 to 41 min; (C) and (D), 28 to 31 min. Multiplicity of infection of 10 throughout.*

lit mutants, even when the *lit* mutation had been transduced into a different genetic background, and is probably a reflection of the function of the product of the *lit* gene.

The participation of the *lit* function in T4 late gene expression presumably occurs at the level of transcription and is the result of altering T4 DNA. Some of the evidence for DNA alteration by the *lit* function comes from the results on the effect of a T4 Gol mutation on T4 DNA replication, since it caused a reduction in the rate of T4 DNA replication and in the length of the newly synthesized DNA even though early gene expression was not affected.

T4 Gol mutations have unusual characteristics. They map in the late region but can affect T4 DNA replication early. They are also codominant with wild-type T4 both in their defect in T4 DNA replication and in their ability to enhance T4 late gene expression on a Lit⁻ host. Explanations for codominance usually invoke either gene dosage effects or *cis*-acting proteins or sites in DNA. The gene dosage explanation for codominance assumes that the Gol function is present in limiting amounts for the phenotype



T4 late protein synthesis on E. coli CTr
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Multiplicity of infection of 10 throughout.

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so that making one-half as much allows one-half as much late protein synthesis. The gene dosage explanation seems unlikely, but the *cis*-acting site (or protein) explanation is in some ways more radical, since the site of the Gol mutation must influence the expression of all the late genes of T4 even though they are expressed from many promoters, most of which are to the 5′ side of the Gol mutations or separated from them by an early region which is transcribed in the reverse direction. Experiments can be designed which should allow us to distinguish between these two possible explanations for the codominance of T4 Gol mutations.

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APPENDIX B

WILD-TYPE T4 GENOMES INTERFERE WITH THE EXPRESSION OF *GOL* MUTANT GENOMES IN MIXED INFECTIONS

In experiments such as those shown in Article I, figures 3 and 4, the rate of T4 late protein synthesis is less than one-half of normal, even if one-half the infecting phage have the *gol 6B* mutation, suggesting that wild-type T4 DNA can interfere with the expression of *Gol* mutant DNA in the same cell. To investigate this further, we studied the effect on late protein synthesis of varying the ratio of wild type to *gol* mutant T4, keeping the total multiplicity constant. We scanned the autoradiograms and determined the area under the peaks due to some T4 late proteins. We then standardized the results by dividing by the area under the same peak in the same experiment when wild-type T4 infected wild-type *E. coli*. The latter is necessary because the density of bands varies somewhat from experiment to experiment. The results of the above calculation applied to three T4 late proteins are shown in Figure B1. The rate of synthesis of the T4 late proteins p23, p18, and p37 expressed as a percentage of the normal rate is plotted against the percentage of the infecting phage which were wild-type T4. If the rate of synthesis of the T4 late protein is proportional to the percentage of genomes which can synthesize that protein, then the results should fall on the dashed straight line. The results vary somewhat from experiment to experiment, but in all cases, the results of the mixed infection of the *lit*$^-$ host by a *gol* mutant and wild-type T4 deviate significantly from the line. In the experiment shown, the

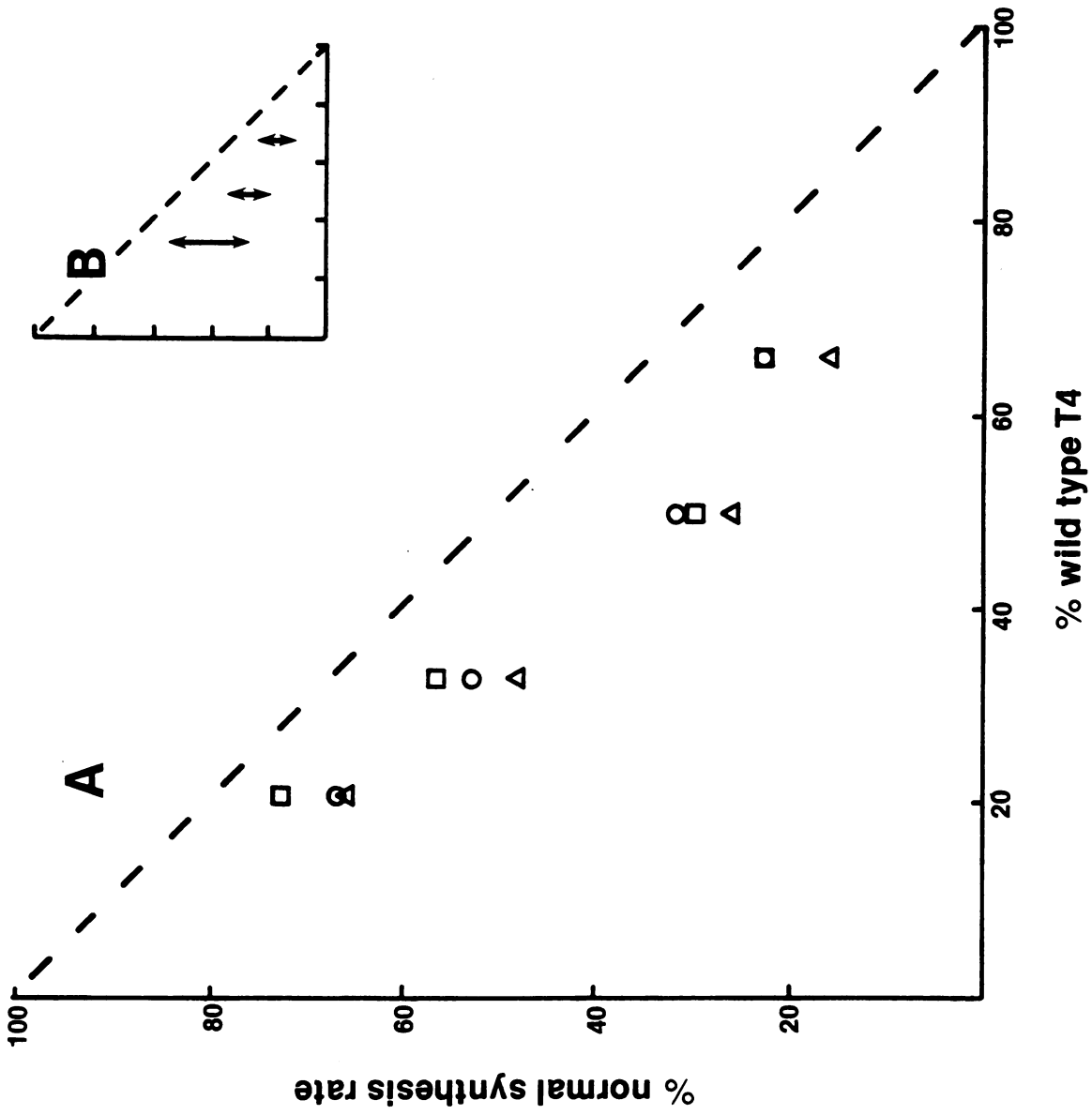
the deviation was less than we normally observe and the insert shows the spread in the highest and lowest values we have obtained from different experiments. Most results fall somewhere in between. We conclude that wild type genomes can "poison" the ability of gol mutant genomes to express their late genes in a lit$^-$ host. It is worth noting that one of the late proteins shown, p37, is coded by a gene almost 10 microns on DNA from the site of the gol mutation.

Fig. 1B. Rate of synthesis of T4 late proteins in mixed infections of wild-type T4 and a gol mutant on lit⁻ E. coli. Infections were done at 30°C with wild-type T4 and gol 6B in varying ratios. Total multiplicity of infection was 24.

(A) The synthesis rates of various T4 late proteins are expressed as a percentage of the normal protein synthesis rate when wild-type T4 infects wild-type E. coli.

□ - gene 18 product, 0 - gene 23 product, Δ - gene 37 product.

(B) ↔ indicates range in values obtained for the gene 23 product in different experiments.


A
B
100
80
60
40
20
% normal synthesis rate
100
80
60
40
20
% wild type T4