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TWO BACTERIOPHAGE T4 GENE PRODUCTS WHICH REGULATE GENE EXPRESSION AFFECT DNA STRUCTURE:

I. The *Alc* Gene Product Unfolds the Host Nucleoid

II. The *PseT* Gene Product Can "Shuttle" DNA Phosphates from 3' to 5' Termini

By

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ABSTRACT

TWO BACTERIOPHAGE T4 GENE PRODUCTS WHICH REGULATE GENE EXPRESSION AFFECT DNA STRUCTURE:

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When T4 polymerizes cytosine (instead of its usual hydroxylmethylcytosine) into its DNA it does not produce any progeny because it does not express its true-late genes normally. However, previous data indicate that T4's alc gene can be altered so that T4 with cytosine containing DNA develops normally. Such alc$^-$ T4 express combinations of the following pleotrophic defects: 1) defectively unfolding the chromosome of its host, E. coli; 2) failing to induce a polypeptide that normally co-purifies with the host RNA polymerase; and 3) defectively preventing host transcription.

The selection that yields T4 that induce altered alc gene products sometimes yields T4 that induce altered pseT gene products as well. (The T4 pseT gene has previously been shown to be necessary for the induction of 3' phosphatase activity and for growth on an E. coli strain, CTr5x). Genetic evidence is presented here that pseT is the structural gene, not only for 3' phosphatase, but also for 5' polynucleotide kinase. In addition, the pseT gene product

is required for normal T4 true-late gene expression during infection of *E. coli* CTr5x. Adding efficient amber suppressing ability to *E. coli* CTr5x alters it so that all *pseT*$^-$ T4, even deletions, can grow on it. Thus, an amber mutation in an *E. coli* CTr5x gene probably prevents the growth of *pseT*$^-$ T4. It follows then that the normal functioning of a similar host gene in commonly used laboratory strains is probably required for growth of *pseT*$^-$ T4 on those strains.

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INTRODUCTION

Studies of bacteriophage T4 transcription may be used to advance our knowledge of gene expression and its relationship to DNA structure because T4 drastically affects both the gene expression and DNA structure of its host, E. coli. After T4 infection, host gene expression ceases, host DNA organization changes, and, eventually, the host DNA itself largely disappears. Another reason to use T4 to study the relationship between DNA structure and function is that T4 induces many enzymes that are essentially duplications of host enzymes. For example, T4 directs the synthesis of many of the gene products necessary for the replication of its DNA (for example, see Alberts, et al., 1975). These duplications can make it easier to study functions as altered by phage mutants, since host mutants can be more difficult to isolate.

In addition, the regulation of T4's own gene expression is complex and interesting (for a review see Rabussay and Geiduschek, 1977). At least four classes of T4 genes have been described: immediate early, delayed early, quasi-late, and true-late. The early genes are transcribed almost exclusively from T4's l-strand. Models for their control based upon either initiation at specific promoter sequences or termination at specific sequences can be used to explain existing data. The true-late genes are transcribed, generally, from T4's r-strand. Less is known about the control of true-late transcription. However, it is known that the expression of T4's true-

late genes seems to depend on specific DNA structures.

The alc and pseT genes are examples of T4 genes that can be exploited to study gene expression and its relationship to DNA structure. The alc gene product normally blocks T4 true-late gene expression when cytosine (instead of the usual hydroxylmethylcytosine) is polymerized into T4 DNA (Snyder et al., 1976). The T4 alc gene product is also required to "unfold" the host's chromosome and to block transcription of host RNA (Sirotkin et al., 1977 - appendix 1). The alc gene may induce an RNA polymerase subunit (Sirotkin et al., 1977 - appendix 1).

The selection that yields T4 alc$^-$ mutants sometimes yields T4 that are pseT$^-$ as well (table 1 of article 1). In addition, when T4 that are only alc$^-$ are propagated, pseT$^-$ mutants are often recovered (table 1 of article 1). Studies presented in article 1 demonstrate several properties of the pseT gene: 1) the pseT gene product can, in vitro, remove 3' phosphates from DNA termini and add gamma phosphates from ATP to 5' hydroxyl termini; thus it can be said to "shuttle" phosphates; 2) a host gene product can substitute for the pseT gene product; 3) the pseT gene product or its substitute, is required for normal T4 true-late gene expression.

Normally T4 true-late gene expression depends upon recently replicated DNA. The putative substitute for the pseT gene product can therefore be expected to support host gene expression that depends upon recently replicated DNA. Further research is currently using pseT$^-$ mutants in a selection intended to yield host mutants in the gene(s) corresponding to the pseT gene (see appendix 3).

It is not clear how either the alc or pseT gene function in vivo, although some models to explain their function can be suggested (see literature review and article 1). For example, the alc gene product might function either as an RNA polymerase subunit that blocks transcription of cytosine containing DNA, or as an activity that removes the supercoiling of cytosine containing DNA. Some models for pseT function describe the end products of phosphate "shuttling" observed in vitro as intermediates appearing only transiently in vivo. For example, these intermediates may appear as DNA supercoils are created or destroyed. Thus to provide an adequate background for the understanding of the alc and pseT gene products, it is necessary to summarize research concerning DNA supercoiling. In addition, research in five areas more obviously related to the alc and pseT gene products will be discussed: 1) isolated bacterial nucleoids; 2) the structure of replicating T4 DNA; 3) T4 induced modifications of the host RNA polymerase; 4) T4's effect on the organization of host DNA and host gene expression; and 5) the regulation of T4 true-late gene expression. Research exploring the 5' polynucleotide kinase and 3' phosphatase activities has been summarized in article 1 rather than in the literature review.

LITERATURE REVIEW

Isolated Bacterial Nucleoids

E. coli's DNA is about one mm long (Cairns, 1963). Because the dimensions of *E. coli* cells average about one thousandth of this, DNA must be highly convoluted to fit into cells. Transcription, segregation, recombination, and replication all force further constraints on the DNA's organization. Understanding these processes probably requires understanding the restrictions they place upon DNA structure. Although it is reasonable to expect that lysing cells would necessarily destroy all of this organization, this is apparently not the case.

Stonington and Pettijohn (1971) reported the first lysis procedure that retained some *E. coli* DNA organization. Usually, unless the cells were quite dilute, most lysis procedures yielded viscous lysates. Their lysis procedure, however, produced lysates that were not viscous, because the DNA from the lysed cells did not spread throughout the solution. Being compact, this DNA sedimented more quickly than disperse DNA would have sedimented. All further work (see below) on these compact DNA structures, called "nucleoids," retained certain elements of Stonington and Pettijohn's (1971) three step lysis procedure. Cells were first resuspended on ice in a solution containing 20% sucrose, NaCl, and NaN_3. The sucrose and salt gave the solution the correct osmolality to prevent spheroplasts from lysing prematurely. The NaN_3 blocked ATP production via

oxidative phosphorylation. Interestingly, ATP alone, when added to these lysates after the third solution, turned them viscous (K. Sirotkin, R. Pearson, L. Snyder, unpublished), implying that the nucleoids were "unfolded." Lysozyme then turned the cells into spheroplasts. The third solution contained NaCl to a final concentration of at least 1 molar (to inhibit nucleases) and detergent to lyse the spheroplasts. These nonviscous crude lysates were often layered on sucrose gradients to purify or analyze the nucleoids.

Nucleoid structure

Stonington and Pettijohn (1971) reported that either SDS or heat "unfolded" the nucleoid structure, as shown by increased viscosity. Nicks introduced by DNAase, also disrupted the nucleoid structure (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972), with a kinetics that implied that five to forty separate supercoiled loops or domains were involved (Worcel and Burgi, 1972). Surprisingly, both groups reported that RNAase unfolded nucleoid structure. Although in early work Worcel and Burgi, (1972) claimed that one nick, introduced by RNAase, was sufficient to unfold nucleoid structure, later workers (Pettijohn and Hecht, 1973; Drlica and Worcel, 1975), found intermediates after RNAase treatment. Thus, more than one nick was necessary to unfold nucleoid structure. The RNA molecules most tightly bound to the nucleoid and hence implicated in maintaining its structure, were studied by Hecht and Pettijohn (1975, 1976). They found that: 1) the RNA molecules were bound at more than one site (1975); 2) DNA that was either about to be replicated or freshly replicated did not bind more RNA

than did other DNA (1975); 3) about 60-90 molecules were bound per nucleoid (1976); 4) about 300 base pairs per RNA molecules were bound, probably as RNA:DNA hybrids (1976); 5) neither their hybridization kinetics nor their rifampicin mediated disappearance kinetics distinguished these preferentially bound RNA molecules from the bulk of E. coli's RNA; and 6) both rRNA and mRNA species were represented. Rifampicin treatment, which is capable of blocking all RNA initiation, unfolded the nucleoid in vivo so that after the lysis procedure outlined above, the lysates were viscous (Dworsky and Schaechter, 1973; Pettijohn and Hecht, 1973). Whether the nucleoid structure is disrupted before or after lysis, the resulting solution is spoken of as containing "unfolded" nucleoids.

All of these data are consistent with the model that the association of RNA with the nucleoid DNA occurs shortly after the RNA is transcribed, possibly by competing with the non-transcribed strand for hybridization, perhaps at regions that are slightly underwound because of supercoiling (supercoiling is discussed in a following section).

Other cellular constituents than RNA were isolated with nucleoids. Proteins and cell membrane pieces, and even part of the cell wall can be attached to the nucleoid. Depending on the temperature during the incubation with high salt and detergent, either membrane-attached (0°-4°C), membrane-released (22°C) or a mixture of types (10°C) were observed (Worcel and Burgi, 1974). Some of the attachment points, as measured by X-ray mediated breakage and later adherence to Mg^{++}-Sarkosyl crystals, disappeared after rifampicin

treatment, as if they arose during RNA transcription (Dworsky and Schaechter, 1973). Korsch et al. (1976) showed that the membrane attached nucleoids also had cell wall parts attached, and that, because of this, careful attention must be paid to the physiological state of the cells because of physiologically linked variations in lysozyme susceptibility. This varying lysozyme susceptibility was used by Korsch et al. (1976) to explain differences in sedimentation velocities for nucleoids from, for example, amino acid starved cells. By equilibrium CsCl centrifugation of fixed membrane released nucleoids, Giorno et al. (1975a) showed that the DNA to RNA to protein ratios were constant in spite of the observed heterogeniety of sedimentation profiles. The proteins included RNA polymerase (Stonington and Pettijohn, 1971).

Both protein and RNA maintain nucleoid structure (Drlica and Worcel, 1975). Different treatments produced partially disrupted membrane released nucleoids that could be distinguished by both their viscosity and sedimentation rate. Treatments that removed all supercoiling, 2ug/ml ethydium bromide or DNAase, yielded nucleoids that made slightly more viscous suspensions or were slower sedimenting than untreated nucleoids. Treatment with RNAase or incubation at 35°C in low (0.1 M NaCl) salt yielded more unfolded nucleoids. Since nucleic acid hybridization stability decreases at low salt (Marmur and Doty, 1962), and lessened ionic strength can be expected to increase the binding of proteins to nucleic acid, both the RNAase and low salt probably released the RNA:DNA hybrids mentioned earlier, rather than releasing structures stabilized by proteins. Treatment of these above slightly unfolded (35°C 0.1 M NaCl) nucleoids with

trypsin, or treatment of fresh nucleoids with 1% SDS completely unfolded them. These last treatments would be expected to destroy all interactions between proteins and nucleic acids. Thus both RNA and protein stabilize nucleoid structure.

It is important to realize that different groups used different gravitational field strengths, and that this alone can lead to different apparent sedimentation rates for nucleoids (Hecht, et al., 1977). The higher the field strength, the lower the apparent sedimentation rate.

Nucleoids: artifact or biologically significant:

One concern is that the procedures for isolating nucleoids might produce artifactual protein-DNA associations. Along this line, Silberstein and Inouye (1974) reported that the concentration of lysozyme commonly used in nucleoid isolation can lead to artificial DNA aggregation. However, Korsch et al. (1976) reported that the high salt of the lysis prevents such artificial aggregations. By flourescence microscopy, the dimensions and gross structure of the isolated nucleoids appeared similar to the appearance of DNA in intact cells (Hecht et al., 1975), corroborating the preceding result.

If nucleoids occur widely in nature, their universality would imply that some essential organization might be leading to their particular structure, even if their isolation gives rise to structures different from those occurring in vivo. Benyajat and Worcel (1976) isolated chromosomes from Drosophila melanogaster that resemble E. coli nucleoids in having separate supercoiled loops.

But because they were too unstable to purify, it was difficult to detail their structure. Piñon and Salts (1977) isolated supercoiled chromosomes from Saccharomyces cerevisiae. The isolated chromosomes contained at least 60 independent supercoiled domains and protein stabilized their structure. The chromosomes also differed in sedimentation rate depending upon the part of the yeast cell cycle from which they were isolated.

Griffith (1976) presented evidence that procaryotic and eucaryotic DNA had a similar beaded structure when viewed by electron microscopy, although because lysozyme was used in the presence of low salt to lyse the procaryotic cells, artificial associations between lysozyme and DNA might have occurred (Silberstein and Inouye, 1974).

These observations together imply that the structure of isolated nucleoids, even if not exactly corresponding to in vivo structures, at least might arise from fairly widespread in vivo organization.

Nucleoids as a probe of replication related events

Since the first claim that nucleoids from E. coli cells were released from their membrane attachments at the end of a round of replication in amino acid starved cells (Worcel and Burgi, 1974), many workers have investigated nucleoid structure and its relationship to the replication cycle. Ryder and Smith (1974) found evidence that membrane attached nucleoids can be found after lysing amino acid starved cells. They attributed the earlier reported absence to the membrane associated nucleoids being pelleted in a prespin used by Worcel and Burgi (1974). In fact, Ryder and Smith (1974) claimed that envelope associated nucleoids isolated from amino acid starved

cells sedimented about 50% faster than controls. These workers also reported (Ryder and Smith, 1975; Ryder et al., 1975) that a change in the sedimentation rate of isolated nucleoids can be observed that correlates with the initiation of DNA synthesis. They report that the sedimentation rate of isolated bacterial nucleoids increased about 14% at high temperature in a ts dnaC mutant and that the sedimentation rate decreased to normal after shift down to the permissive temperature. Disappointingly, no correlation with DNA content, degree of supercoiling, or number of membrane attachment sites was described by these workers.

Korsch et al. (1976) explained at least some of the preceding with the observation that amino acid starved cells were less affected by lysozyme. They also found that the membrane associated nucleoid contained cell wall material. After adjusting for the different susceptibility to lysozyme, amino acid starved cells gave similar results to control cells. By using cells growing with poor carbon sources so that some cells would not be replicating their DNA (Cooper and Helmstetter, 1968), Korsch et al. (1976) showed that membrane associated nucleoids isolated from cells sedimented at about the same rate whether or not they were replicating their DNA. The slight difference between the two rates could be accounted for by the greater DNA content of the replicating nucleoids. This, however, did not explain the results Ryder and Smith (1974) obtained with the ts dnaC mutant.

Thus, in spite of the fact that about 0.5% of the E. coli chromosome will not replicate without protein synthesis (Marunouchi and Messer, 1973) no convincingly different isolated nucleoids have been

obtained from amino acid starved cells.

After a lysis similar to that used to isolate E. coli nucleoids, DNA near the B. subtilis origin was preferentially associated with membrane components after shearing (Yamaguchi and Yoshikawa, 1977; Imada et al., 1975). Since B. subtilis has a mesosome to which DNA seems to be attached, it is hard to compare these results to results obtained using E. coli.

Nucleoids as transcription and replication templates

Another way to test whether or not nucleoids retain components of their in vivo organization as they are isolated is to try to use them as templates for transcription and replication. Kornberg, et al. (1974) isolated membrane associated nucleoids with 10 mM spermidine replacing 1.0 M NaCl and tried to replicate them. About one percent of the genome replicated at about five percent of the in vivo rate. Although some increased viscosity after incubation in the replication mixture was observed, no decrease in sedimentation rate was detected. Interestingly, if both polymerase I and ligase were added, small DNA fragments were changed to large fragments, as occurred in vivo (Okazaki, et al., 1968).

Giorno, et al. (1975b) studied transcription with nucleoid templates. They found that the size of transcripts and their elongation rate were similar on folded and unfolded nucleoids. However, the number of active RNA polymerase molecules and the chain initiation rate were both greater on the folded chromosome. As is discussed below, it is possible that supercoiling alone accounted for this effect although this would not be established by these

experiments alone.

Enzymes Altering DNA Supercoiling

The first report on an enzyme catalytically relazing supercoiled DNA but leaving the DNA covalently closed, was made by Wang (1971). Burrington and Morgan (1976) purified this "omega" protein from E. coli and confirmed Wang's report that it only relaxed negative DNA supercoils. This is significant because most models for replication involve the generation of positive supercoiling (see Cairns, 1963, for example). However, Morgan (1970) proposed a model with linked progeny strands that would generate negative supercoiling.

Gellert et al. reported (1976a) an ATP dependent reaction capable of catalytically introducing negative supercoils into DNA. This supercoiling enzyme has been named DNA "gyrase." They also reported (1976b) that novobiocin and coumermycin inhibited DNA gyrase purified from cou^s, but not cou^r E. coli. Cou, a gene determining sensitivity to coumermycin, near 82 minutes on the standard E. coli map, is probably then the structural gene for a gyrase component. Very recent reports (Gellert et al., 1977; Sugino et al., 1977) identified a second gyrase component sensitive to nalidixic acid. This component, apparently the product of E. coli gene nalA (48 minutes), had a number of interesting characteristics (Sugino et al., 1977). including the ability to relax supercoiled DNA. It is unlike the omega protein immunologically and in its ability to relax positively supercoiled DNA.

Enzymes capable of relaxing DNA supercoils have been purified from many eucaryotic sources (Bauer et al., 1977; Mattoccia et al., 1976; Champoux and McConaughy, 1976; Baase and Wang, 1976; Pullyblank

and Morgan, 1975; Keller, 1975; Champoux and Dulbecco, 1972; Vosberg et al., 1976). These enzymes could relax both positively and negatively supercoiled DNA and are thus similar to the nalidixic acid sensitive gyrase component.

Their biological significance

Supercoiling probably plays a role in replication. The previously mentioned antibiotic resistance studies (Gellert et al., 1976b; Gellert et al., 1977) imply that novobiocin and nalidixic acid which block replication disturb DNA supercoiling. Gyrase also plays a role in bacteriophage ϕx175 replication. Unless RFIV is supercoiled to RFI, the obligatory gene A product cleavage will not occur (Marians et al., 1977). Because novobiocin and coumermycin inhibit bacteriophage T7 replication in cou^s but not as severely in cou^r hosts (Itoh and Tomizawa, 1977), supercoiling also probably plays a role in T7 replication. Interestingly, nucleoid-like structures are also observed in T7 infected cells, but these structures do not have the same RNAase or proteinase sensitivity as do the E. coli nucleoids (Serwer, 1974; Hiebsch and Center, 1977; Paetkan, et al., 1977).

As they replicate, plasmids exist briefly, with fewer supercoils before becoming fully supercoiled (Timmis et al., 1976; Crosa et al., 1976). This implies that supercoiling might be an essential part of plasmid "maturation." It is not known, however, which plasmid functions require supercoiled DNA.

As mentioned in the introduction to this thesis, it is possible that the pseT gene product alters DNA supercoiling. The alc gene

product might also affect DNA supercoiling. It might, for example, unfold its E. coli host's nucleoid (Sirotkin, et al., 1977 - appendix 1) by removing its supercoiling. R. Pearson and L. Snyder (personal communication) in our laboratory have obtained preliminary results indicating that the alc gene product affects the supercoiling of bacteriophage lambda DNA.

In vitro observations

Three complications impede attributing in vivo biological significance to the biochemistry of DNA supercoiling observed in vitro. The first is common to all biochemistry: the assumption that enzymatic actions observed in vitro also occur in vivo. Although this could be argued, it is certainly a reasonable initial assumption. The second complication concerns the meaning of the finding that ex-extracted DNA is supercoiled. As a few moments of experimentation will demonstrate, to coil anything tightly requires rotation. The supercoiling would only stress the coil to the degree that it is extended. However, the DNA would tend to extend as it diffuses away from a coil that concentrates it. Thus, the equilibrium state of a supercoil must balance the energy stored in the coiling and the energy stored in the increased local DNA concentration. "Supercoiling" therefore refers as much to DNA topology as to any internal stress. This point is independent of the number of strands making up the coil. As discussed in Griffith (1975), if a double helix is slightly denatured, in vivo, by proteins or ionic conditions, it will supercoil in the absence of the denaturing proteins or ionic conditions. (However the interpretation that RNA:DNA hybrids are

maintaining the structure of the isolated bacterial nucleoid (see above) supports the model that supercoiling generates some single stranded regions in vivo). The third complication involves the use of supercoiled DNA substrates. Not only are such experiments necessarily performed in vitro, but in addition, the DNA used may be in a form never found in vivo. Nonetheless, many such experiments have been performed and some information can certainly be gleaned from them.

For example, that an endonuclease specific for single stranded DNA makes some nicks in supercoiled DNA (Wang, 1974) implies that such DNA contains, at least transiently, single stranded regions. Perhaps this tendency towards unwinding explains why RNA polymerase binds much more tightly to supercoiled DNA (Warner and Schaller, 1977), and why there are more, and more stable, promoters on supercoiled DNA (Richardson, 1975). More difficult to interpret, Bochtan et al., (1973) found that more initiation occurs on lambda DNA if it is supercoiled, but that this transcription of the supercoiled template contains a lower proportion of lambda early mRNA and is less sensitive to lambda CI repressor. They assumed that these later facts implied that this transcription of the supercoiled template was not biological and so they reported no controls to see if it was only transcribed from one strand or experiments to determine whether such transcripts can be translated into late proteins. Some late lambda proteins can be made in the absence of the Q gene product (Sato and Campbell, 1970; Echols, 1971), which positively regulates lambda late gene expression. It is tempting to speculate that supercoiling mimics or substitutes for lambda Q gene product function.

In vitro lambda integrative recombination requires closed circular DNA (Mizuuchi and Nash, 1976) and this DNA must be supercoiled (H. A. Nash, personal communication). Holloman and Radding (1976) report that supercoiled DNA can accept DNA fragments *in vitro*, that the *recA* gene product can recombine into the supercoiled DNA *in vivo*. Perhaps the transient single strandedness of supercoiled DNA leads to both of these phenomena.

Intermediates and models

The relaxation complexes of some plasmids might represent some type of intermediate in supercoiled DNA processing, although it is not clear what biological significance they might have or even, in fact, if they involve omega proteins or gyrases at all. When the supercoiled plasmid and its associated (*not* covalently bound) protein are treated with SDS or pronase, one DNA strand is nicked, leaving a 3' hydroxyl (Guiney, 1975). This seems similar to the supercoil relaxing protein, the *nalA* gene product, that leaves a double strand break in the DNA when the nalidixic acid inhibited gyrase complex is treated with SDS (Sugino et al., 1977). The reported intermediate with a single strand nick for the rat liver omega protein (Champoux, 1976) may also be similar.

Sugino et al. (1977) proposed a simple model for the two component gyrase. The *cou* gene product, according to this model, melts the double helix forcing the same number of turns of the double helix into a shorter area thus causing the nonmelted regions to be more positively supercoiled. The *nalA* gene product then relaxes these positive supercoils. When the complex leaves the DNA, the DNA is underwound.

All of these observations might be related to the phosphate "shuttling" performed by the pseT gene product in vitro. Although the relaxation complex has the same phosphate orientation as would be left by the pseT gene product (article 1), no information is available on the mechanics of winding or unwinding of supercoiled DNA. So there is not any information, as yet, on the question of whether any of these other proteins "shuttle" phosphates as they function. The fact that they have not been observed to function in vitro similarly to 5' polynucleotide kinase might simply mean that they assay differently.

The Relationship of Nicking or Gapping Enzymes to Gene Expression

Besides the examples involving T4 DNA ligase and bacteriophage T5 exonuclease mentioned in article 1 of this thesis, some examples involving eucaryotic RNA polymerase and single stranded DNA have been reported. Mammalian RNA polymerase beta can initiate at single stranded DNA breaks if they have 3' hydroxyl termini (Dreyer and Hausen, 1976). ssDNA sequences, obtained using hydroxylappatite, appear disproportionately frequently as chick embryo mRNA (Tapiero et al., 1976), implying that DNA gaps play a role in mammalian gene expression.

The phosphate "shuttling" performed by the pseT gene product in vitro, if significant in vivo, could imply that the pseT gene product affects termini processing. The pseT gene product can alter phosphates at DNA termini so that, if those termini are at nicks or gaps, the ability of other enzymes to process those termini could be altered: a 3' phosphate would interfere with gap closing by DNA polymerase and either a 3' phosphate or a 5' hydroxyl would prevent DNA ligase from

sealing a nick.

Requirements for T4 True-late Gene Expression

Because this topic has recently been thoroughly reviewed (Rabussay and Geiduschek, 1977) and because it is discussed as it relates to the pseT gene product (article 1), this section will only provide a brief overview. Normally, T4 true-late gene expression requires concomitant T4 replication (Riva et al., 1970a), but by inactivating T4 DNA ligase (gene 30) and a T4 exonuclease (gene 46), T4 true-late gene expression can be uncoupled from replication (Riva et al., 1970b). The products of T4 genes 45 (Wu and Geiduschek, 1975), 33 and 55 (Bolle et al., 1969) are also required for T4 true-late gene expression. Unless the alc gene is altered, the DNA template must contain hydroxylmethylcytosine (Snyder et al., 1976).

In a recent paper appearing after the review, Wu and Geiduschek (1977) point out some additional requirements for T4 true-late gene expression. Protein synthesis early in the late period is required for efficient and abundant transcription of the true-late template DNA strand. Interestingly, a temperature sensitive mutation in gene 55 that is normally thermoreversible is irreversible when T4 true-late gene expression is uncoupled from replication. This is especially interesting because (see the section below discussing T4 induced RNA polymerase modifications) the product of gene 55 binds to the host RNA polymerase, (Ratner, 1974b), which is used throughout T4 development (Haselkown et al., 1969; Mizuno and Nitta, 1969; di Mauro et al., 1969).

T4 Induced Modifications of RNA Polymerase

Because the host RNA polymerase's response to antibiotics is retained by the T4 infected cell throughout infection (Haselkorn et al., 1969; Mizuno and Nitta, 1969; di Mauro et al., 1969), T4 is known to use the host polymerase throughout its development. However, the alpha subunit of the RNA polymerase was altered after infection (Goff and Weber, 1970). Two distinct gene products are responsible for this modification (Horvitz, 1974a, b). Both of these attach an adenine nucleotide to an arginine residue of the alpha subunit (Goff, 1974), and are therefore called ADP-ribosyltransferases (Rohrer et al., 1975). These enzymes are nevertheless not essential for T4's development (Horvitz, 1974b).

Stevens (1972) found that four T4 induced polypeptides bind to RNA polymerase. Confirming her gene assignments, Horvitz, (1973) proved that gene 33 codes for one peptide and Ratner (1974b) proved that gene 55 codes for another. The other two were simply named polypeptides numbers 2 and 4, with no known gene assignments. These polypeptides were distinguished by molecular weight. Some evidence (see below) implies that the *alc* gene may code for polypeptide number 2 (Sirotkin et al., 1977 - appendix 1). The product of gene 45 also binds to the host RNA polymerase, but much more loosely (Ratner, 1974a) than the others.

Is *alc* the structural gene for polypeptide number 2?

Although the RNA polymerase purified from cells infected with some *alc*$^-$ mutants has lacked a polypeptide that might be polypeptide number 2 (Sirotkin et al., 1977 - appendix 1), there is some question

as to whether alc is actually the structural gene for polypeptide number 2. Even assuming that the missing polypeptide from alc$^-$ infection is polypeptide number 2, genetic uncertainties remain. When alc$^-$ T4 are originally selected or propagated, mutations in other genes appear (article 1 and unpublished). The T4 used to infect the cells from which the RNA polymerase was purified may have had second mutations in the actual gene coding for polypeptide number 2. Furthermore, when alc$^-$ T4, that originally did not induce a polypeptide number 2, were propagated they sometimes changed so that polypeptide number 2 could be co-purified with the polymerase from infected cells. Although this may seem to be fairly good evidence that alc is not the structural gene for polypeptide number 2, there were other indications that weaken that interpretation. Not only do these alc$^-$ T4 that changed so that they now induce a polypeptide number 2 that co-purifies with the RNA polymerase usually grow less well, by plaque size, with cytosine containing DNA, they also typically became less defective in their ability to unfold the host nucleoid (see below). That alc$^-$ T4 sometimes tend to become "less alc$^-$" and also to lose some associated phenotypes could be explained by alc being required for a fully normal burst size, at least when T4 contain hydroxymethylcytosine. Alc might even be an essential gene. This could explain the selection of the above mutants, because they were all propagated with hydroxymethylcytosine containing DNA. The question then becomes whether the mutation(s) that "brings back" polypeptide number 2 and causes these other changes is in the alc gene. This question will remain until recombinants from infections with different alc$^-$ T4 that

originally did not induce polypeptide number 2, but now do induce the polypeptide are found (see the recommendations below in a following section). So far (unpublished), when we have attempted to find *alc*$^{+}$ recombinants from crosses with *alc*$^{-}$ parents, they either eluded us or were so infrequent that they may actually have been revertants.

Another technique may lead to an answer to this question. C. Goff (personal communication) is using antibody precipitation to purify RNA polymerase from cells infected with progeny from multifactor genetic crosses. We have sent him some of our mutants to use. There is, however, a potential problem with comparing his results to ours (Sirotkin et al., 1977 - appendix 1). Antibody precipitation may detect a looser association of polypeptide number 2 with the polymerase than would phosphocellulose chromatography. Until such a chromatographic step is added, conclusions regarding the presence or absence of polypeptide number 2 cannot be compared between the different methods. (Of course, the bound antibody would totally distort such a chromatographic step). Thus, difference in methods could account for the difficulty repeating our results that C. Goff has personally communicated to us.

Another approach to this question would not be affected by the above problem. A T4 mutant exists which induces two species of polypeptide number 2 that differ slightly in molecular weight (H. R. Horvitz, D. I. Ratner, and A. R. Poteet, personal communication). C. Goff (personal communication) is using multifactor crosses to locate the responsible mutation. So far, this mutation seems to map between genes 63 and 31, placing it, at least, very close to *alc*

(Sirotkin et al., 1977 - appendix 1).

One model for alc gene product function has it acting as a protease (see the models in a following section). If this is the case, the mutation causing the altered polypeptide number 2 could be in the alc gene and the alc gene would still not be the structural gene for polypeptide number 2. In this case, the recombination studies could also yield similarly deceptive "positive" results. The only way to prove that polypeptide number 2 is coded for by the alc gene would be similar to that used by Horvitz (1973) to prove that gene 33 codes for one of the other polypeptides co-purifying with the polymerase. He used multiple amber mutations in gene 33 and showed that the number of tyrosine residues inserted by an amber suppressing RNA_{tyr} increased as the number of amber mutations. However, this method presupposes that it is possible to get amber mutations in alc that have the "alc^-" phenotype. Since we do not even know if the alc^- phenotype can result from a deficiency, we cannot know if this is possible.

If, as mentioned earlier, alc is an essential gene, an amber alc may not be "alc^-" under any conditions. However, if an amber alc could be found, it would certainly produce an altered polypeptide number 2 when grown on nonsuppressing bacteria, if alc is the structural gene for polypeptide number 2. We have not yet been successful in selecting an amber alc. This, however, may be due to the necessity of forcing such mutants to grow on a nonsuppressor with cytosine containing DNA in order to enrich for them.

The Structure of T4 DNA after Replication has Begun

Altman and Lerman (1970) analyzed normal hydroxymethylcytosine containing T4 DNA from a low salt lysozyme lysis that had been treated with RNAase and trypsin. Because of the low salt and fairly high lysozyme concentration used, it is possible that some artifactual aggregation occurred (see Silberstein and Inouye, 1974). In spite of this problem, their work still merits discussion. They found DNA sedimenting at different rates: 1) "slow," sedimenting at less than 100s--probably genomic size DNA; 2) "fast," sedimenting at about 170-600s; 3) "phage," DNA in virions; 4) "bottom," sedimenting at 950 to 3,000s. Interestingly, the DNA in the fast and bottom categories only partially chased to the slow and phage categories; about one-third of it seems "locked into" the faster sedimenting states.

Gene 49 product, which is required for the packaging of DNA into phage particles, seems to cut single strand gaps in DNA at about genomic intervals (Curtis and Alberts, 1976). Under conditions where $T4^+$ DNA sediments at about 200s, DNA from T4 with an amber mutation in gene 49 sediments at 1,000 to 1,800s (Kemper and Brown, 1976). Obviously, at least some of the differences reported by Altman and Lerman (1970) occurred because of packaging.

Another sedimentation difference is observed that correlates with whether or not T4 true-late genes are being expressed (Snyder and Geiduschek, 1968; Cox and Conway, 1975a, b). Both groups found that a slower sedimenting DNA fraction is absent from cells infected with T4 having an amber mutation in gene 55. (See the discussion of

T4 true-late gene expression). Although it is possible that some true-late genes (gene 49, for example) might be processing the DNA into a slower sedimenting form, it is tempting to speculate that this slower sedimenting state contains the DNA from which true-late mRNA can be transcribed. Consistent with this are the facts that the transcription of the DNA in the slower sedimenting form is sensitive to rifampicin (the faster sedimenting form is not sensitive) and, by immune precipitation, that the RNA transcribed from the slower sedimenting form can be translated into true-late gene products (Cox and Conway, 1975a). Not only that, but Cox and Conway (1975b) also report that mutants in gene 46-47 which express a DNA arrested phenotype, but which express true-late genes normally, created none of the faster sedimenting DNA and an increased amount of the slower form.

It is difficult to compare these results with those obtained with bacterial nucleoids because the conditions used to isolate the T4 DNA were very different from those used to isolate bacterial nucleoids.

T4 nucleoids

Hamilton and Pettijohn (1976) used a lysis procedure similar to that used to obtain isolated bacterial nucleoids to prepare corresponding particles of replicating T4 DNA. By flourescent microscopy, the particles had about the same dimensions as the cells from which they came. There was little effect of nicking by DNAase or RNAase treatment, but about 25% to 40% of the DNA leaves the nucleoids with SDS treatment. The change in sedimentation rate observed with different ethidium bromide concentrations was consistent with about

one-eighth of the nucleoid DNA being supercoiled. Perhaps the DNA that was supercoiled (or that which is removed by SDS treatment) corresponds to the slower sedimenting DNA mentioned earlier. Another possibility is that the DNA lost during nucleoid purification contains this DNA. Mutant studies could answer this point.

Effects of cytosine substitution in T4 DNA

Normally, T4 DNA contains alpha and beta glucosylated (Lehman and Pratt, 1960) hydroxylmethyl cytosine (Wyatt and Cohen, 1952). Except for it being susceptible to bacterial restriction enzymes, T4 with DNA that is unglucosylated because of mutations in the glucosyl transferase genes grows almost as well as T4 with glucosylated DNA (Hattman, 1969). T4 forced to replicate with cytosine in their DNA cannot express true-late genes, even though, as observed on neutral or alkaline sucrose gradients, there is little size difference between it and the glucosylated, hydroxylmethylcytosine containing DNA (Kutter et al., 1975). Some RNA from the strand (r) that codes for late genes was transcribed from cytosine containing DNA (Wu and Geiduschek, 1975), but it obviously has some unknown defect. Snyder et al. (1976) reported that the T4 alc gene product prevents true-late gene expression on cytosine containing DNA. Sirotkin et al. (1977 - appendix 1) report that other phenotypes sometimes occur when this gene is altered. For convenience, throughout the rest of this review, "alc$^-$ T4" means T4 with a mutation in the alc gene that allows T4 with cytosine containing DNA to produce progeny.

T4 Mediated Alterations of Host DNA Organization and T4's Effect upon Host Gene Expression

Although T4 induces a number of nucleases that allow it to break down host DNA and incorporate host nucleotides into its genome (Wiberg, 1966; Warner et al., 1970; Hercules et al., 1971), these will not be discussed further here (see Snyder, 1976 for a review). The changes imposed upon the organization of the host genome will be emphasized rather than interruptions to the host genome's phosphate-sugar backbone.

Host chromosome disruption and unfolding

T4 infection causes a disruption of the location of the host chromosome visable by light microscopy with proper staining (Luria and Human, 1950; Murray et al., 1950), and T4 infection also causes the host nucleoid to be unfolded upon isolation (Tutas et al., 1974). The gene responsible for nuclear "disruption" has been identified as D2b (Snustad and Conroy, 1974), and the gene product responsible for "unfolding," unf, was mapped between genes 63 and 31 by Snustad, et al. (1976). T4 mutants deficient in nuclear disruption still unfold the host chromosome and vice versa (Snustad et al., 1972, 1976; Snustad and Conroy, 1974), although the "unfolding" is more extreme in terms of the resulting viscosity (see the previous discussion of bacterial nucleoids; Snustad et al., 1976; Sirotkin et al., 1977 - appendix 1) if the infecting T4 are also deficient in nuclear "disruption" (unpublished; and D. P. Snustad, personal communication).

Does the alc gene product unfold the host nucleoid?

Appendix 1 (Sirotkin et al., 1977) contains evidence that alc (Snyder et al., 1976) and unf (Snustad et al., 1976) are the same gene. It is clear (Sirotkin et al., 1977 - appendix 1) that many mutants selected for the alc^- phenotype are also unf^-. In addition, D. P. Snustad (personal communication) has recently informed us that the one unf^- mutant selected directly (Snustad et al., 1976) is also alc^-. Although we have not proven that the mutation that leads to the alc^- phenotype also leads to the unf^- phenotype, this seems likely. Finding alc^+ progeny from a genetic cross between to alc^-, unf^- parents could provide additional evidence. As previously mentioned, however, we have not had success obtaining alc^+ recombinants.

The shutoff of host gene expression

There may be two distinct processes which stop host gene expression after T4 infection. One prevents translation of host mRNA sequences (Kennel, 1968, 1970) the other, requiring T4 gene expression, blocks at least some host transcription. It is not clear whether the lack of host protein translation results from the mRNA being defective or from some T4 induced modification to the translation apparatus that discriminates against host mRNA. Kennel (1970), however, presents data indicating that host mRNA may be excluded from polysomes, supporting the latter possibility. The transcriptional shutoff, which is emphasized here, requires T4 gene expression to inhibit host RNA transcription (Duckworth, 1971; Nomura et al., 1962, 1966; Haywood and Green, 1965; Sirotkin et al., 1977 -

appendix 1).

Horvitz (1974b) reported that T4 that did not modify the alpha subunit of the host still shutoff the transcription of stable host RNA species. However, a role in RNA transcriptional shutoff was attributed to the genes responsible for altering the alpha subunit of the host (see the previous discussion) by Mailhammer et al. (1976), because they found that the T4 adenylated RNA polymerase transcribes both bacteriophage lambda and E. coli DNA poorly in vitro. They also found that, in vitro, the presence of T4 DNA blocked transcription of other transcripts, possibly by competing for RNA polymerase (see also Khesin et al., 1972).

Does the alc gene product prevent transcription of host RNA?

Appendix 1 (Sirotkin et al., 1977) contains evidence that at least one alc$^-$ mutant (alc1) allows more transcription of host sequences than T4$^+$. However, we do not know for sure if the alc$^-$ mutation is responsible for this. If a mutation at a second site is responsible for this difference, however, it is likely to be very closely linked to the alc$^-$ mutation, because the mutant used (Sirotkin et al., 1977 - appendix 1) had been backcrossed against its parent. More alc$^-$ mutants and segregants need to be tested for the ability to block host transcription. P. Dennis (personal communication) is testing many of our alc$^-$ T4 for the ability to prevent transcription of host sequences carried by different transducing phage. Technically, it is easier to tell if host sequences carried by a transducing phage are present as RNA after infection than to examine total host RNA sequences. This technique also has the added advantage of

detecting if only certain sequences are affected. In addition, E. Kutter (personal communication) has found that two alc$^-$ mutants (alc1 and alc2) allow the transcription, in vivo, of host mRNA that can later be translated, in vitro. It would be potentially informative to repeat some of Kennel's (1970) in vivo work using these mutants.

Another dramatic and independent confirmation that the alc$^-$ mutant affects host transcription comes from the work of T. Mattson (personal communication). He found that only infecting phage carrying the alc$^-$ (alc1) mutation could have their defective late genes complemented by T4 wild type late genes cloned on host plasmids.

Models for alc and pseT Gene Product Function

If alc is the structural gene for polypeptide number 2, then a simple model can explain most of our data: that the alc gene product, by binding to and altering RNA polymerase, blocks the initiation of transcription (or causes its early termination) on cytosine containing DNA. This primary defect would then cause the other observed phenotypes. The "unfolding" can be explained by way of the cessation of normal transcription, as occurs after rifampicin treatment (Dworsky and Schaechter, 1973; Pettijohn and Hecht, 1973).

The beauty of this model is its simplicity. However, it does not explain why pseT$^-$ mutations appear along with alc$^-$ mutations. Some secondary role for the alc gene product's function on normal hydroxylmethylcytosine containing DNA would have to be invoked to account for the selection of these mutations.

Another model can explain both "unfolding" and host shutoff. We can simply propose that the alc gene product has an "omega-like" (see discussion of enzymes altering DNA supercoiling) or endonucleolytic activity on cytosine containing DNA. Then the "unfolding" of the host nucleoid would be caused by releasing its supercoiling and this lack of supercoiling would then lead to the transcriptional shutoff. Furthermore, it could be postulated that, on hydroxylmethylcytosine containing DNA, the alc gene product may also unwind the helix, and this may be required in some way for normal phage development. This would make its function similar to the function proposed for the nalA gene product (Sugino et al., 1977).

The interaction with the pseT gene product could occur if the two gene products together functioned similarly to DNA gyrase (Gellert et al., 1976a). Then, alc would correspond to nalA and pseT to cou. One prediction of this model would be that E. coli CTr5x (see article 1) has a gyrase deficiency and that, by blocking the gyrase of other E. coli strains with novobiocin, pseT$^-$ T4 would not grow as well as pseT$^+$ T4 in the presence of the antibiotic. This, however, does not seem to occur (L. Snyder, personal communication). To preserve the model, one could presume that novobiocin generates a dominant defect, even in the presence of a functional T4 pseT gene.

A third model would suggest that the alc gene product alters other gene products, perhaps as a protease. There is a precedent for this: the recA gene product, protein X, cleaves bacteriophage lambda's repressor (J. W. Roberts, C. W. Roberts, N. L. Craig, personal communication). This would explain both the pleotrophic

defects of *alc*$^-$ T4 and its interaction with the *pseT* gene product; it could even explain the previously mentioned polypeptide number 2 species observed in cells infected in one mutant (H. R. Horvitz, D. I. Ratner, A. R. Poteet, personal communication). Although this could explain much of the data, this could not be called a simple model.

The first two models predict that infections with *alc*$^-$, but *unf*$^+$ phage would produce nucleoids that would be partially unfolded. They should differ at least slightly from those produced after *alc*$^+$ infections. The third model does not necessarily predict this; the different phenotypes could be totally independent.

Recommendations

Two of the more unsettling and interesting aspects of working with *alc*$^-$ T4 are the various pleotrophic defects different *alc*$^-$ mutants express and the difficulty in obtaining *alc*$^+$ recombinants. If a nonpermissive host for *alc*$^-$ T4, that is permissive for *alc*$^+$ T4, could be found starting with a laboratory strain of *E. coli*, not only could *alc*$^+$ recombinants be directly selected, but by mapping and identifying the responsible *E. coli* gene, an understanding of the *alc* gene's function would most probably be advanced.

Two of the models for *alc* gene product function differed in whether they explained "unfolding" in terms of the shutoff of host RNA transcription or vice versa. R. Pearson in our laboratory is currently studying the effect of T4 infection after lambda induction on lambda supercoiling and gene expression hoping to distinguish between these explanations.

How T4 unfolded E. coli nucleoids compare to the four structures described by Drlica and Worcel (1975; see the discussion of nucleoid structure above), is not known. This should be examined after infection with a variety of alc$^-$ T4, especially those alc$^-$ T4 that still unfold, at least partially, the host nucleoid.

Directly studying the alc gene product's effect on cytosine containing T4 DNA may provide some clues. To test if the alc$^-$, unf$^+$ phenotype arises from a less severe change to the alc gene product, than the alc$^-$, unf$^-$ phenotype, the amount and length of r (late) strand transcripts could be measured: 1) for alc$^+$, unf$^+$ phage; 2) for alc$^-$ but unf$^+$ phage; and 3) for alc$^-$, unf$^-$ phage. If this is done, we would predict increasing amounts or lengths from (1) to (2) to (3).

If the slower sedimenting form of replicating T4 DNA (discussed above) is caused by true-late transcription and not by true-late gene products, its presence or absence from infections might provide useful information. The way to determine if some of the true-late gene products themselves create this slower sedimenting DNA or if the slower sedimenting DNA is responsible for their transcription is with a temperature shift up experiment with a mutant expressing a thermal labile gene 55 product. If enough time at the permissive temperature was allowed so that all the true-late gene products were present, a pulse-chase experiment after shift up would determine if gene 55 is necessary for the production of the slower sedimenting DNA. If gene 55 product is necessary, this would imply that the slower sedimenting form is necessary for true-late transcription and not simply created by the action of true-late genes. Similarly, studying the distribution of the various DNA components described by Altman and Lerman

(1970) may uncover some defects caused by alc gene product function when T4 replicates DNA containing cytosine.

Two aspects of Hamilton and Pettijohn's (1976) work might bear on the effect of alc gene product function on cytosine containing DNA. First, it might affect the yield or the very existence of T4 nucleoids. Second, the distinctive ethidium bromide sedimentation profile, that is consistent with about one-eighth of the DNA in the T4 nucleoids being supercoiled, might be affected by the alc gene product. Of course, any effect of alc on cytosine containing T4 DNA would have to be separated from the effect of the true-late genes themselves. As described above for the slower sedimenting form of T4 DNA, this is not a major problem, especially since we have some mutants that appear to be alc^- only at high temperature (M. Slocum, our laboratory).

Using cells synchronized by membrane binding (Cooper and Helmstetter, 1968) or amino acid starvation (Marunochi and Messer, 1973), a more dramatic shutoff of host mRNA synthesis might be obtained from cells replicating particular areas of their genome. This, of course, presupposes that a parallel to T4's replication coupled transcription exists in E. coli and that these replication coupled transcription units would cluster at certain areas of the genome.

Currently, by looking for "nibbled" colonies, a search for E. coli K12 mutants nonpermissive for alc^- or $pseT^-$ T4, but permissive for $T4^+$, is in progress. Of course, if the alc^- phenotype is due to a partial deficiency, and there is no host counterpart, this is not likely to work for alc. (See appendix 3).

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ARTICLE I

A ROLE IN TRUE-LATE GENE EXPRESSION FOR THE T4 BACTERIOPHAGE 5' POLYNUCLEOTIDE KINASE 3' PHOSPHATASE

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ABSTRACT

The two T4 induced nucleic acid modifying activities, 5' polynucleotide kinase and 3' phosphatase, are both coded by the pseT gene. Therefore, the product of this gene is an enzyme which can remove phosphates from 3' termini and add them to 5' hydroxyl termini and thus could be said to "shuttle" phosphates on polynucleotides. This enzyme is involved in regulating T4 true-late gene expression, probably at the level of transcription, by establishing the required intracellular DNA structure. Our data suggest that a host gene product normally can substitute for the product of the pseT gene making it nonessential for phage multiplication on most laboratory strains of E. coli.

INTRODUCTION

The replication coupled true-late gene transcription of T4 coliphage draws the attention of molecular biologists because it seems to require specific DNA structures. This has been suspected since it was discovered that DNA replication is required for normal T4 true-late gene expression (Epstein et al., 1963). Progress has been made toward the elucidation of these structures (Wu and Geiduschek, 1975; Rabussay and Geiduschek, 1977), but we still need more information concerning *in vivo* requirements for T4 true-late gene expression.

T4 codes for about two hundred gene products of which about seventy are required for development on most laboratory strains. Many of the remaining, so called nonessential, genes are required on other normal T4 hosts. For example, the normally nonessential T4 *pseT* gene product, which is the subject of this publication, is required for phage production on *E. coli* CTr5x (Depew and Cozzarelli, 1974).

Many T4 genes induce enzymes which have been assayed *in vitro*, While a number of these enzymes have been matched with their corresponding genes and probable biological functions, this has not been accomplished for other enzymes, including 5' polynucleotide kinase (E. C. 2.7.1.78) and 3' phosphatase.

The 5' polynucleotide kinase activity, discovered by Richardson (1965) and Novogrodsky and Hurwitz (1966) transfers gamma phosphates of ATP specifically to the 5' hydroxyl termini of nucleic acids.

This enzyme aids in sequencing nucleic acids (Jay et al., 1974; Maxam and Gilbert, 1977) and in synthesizing specific nucleic acids (Khorana et al., 1972; Walker et al., 1975); because of this, the T4 induced 5' polynucleotide kinase can be purchased from many biochemical supply companies. T4 mutants defective in the induction of 5' polynucleotide kinase have been reported (Chan and Ebisuzaki, 1970), but because they expressed no observable phenotype, they were not mapped. No polynucleotide kinase activity has been found in uninfected E. coli, but similar activities have been found in mammalian nuclei (Novogrodsky et al., 1966; Ichimura and Tsukada, 1971; Teraoka et al., 1975; Levin and Zimmerman, 1976).

The T4 induced 3' phosphatase activity, discovered by Becker and Hurwitz (1967), selectively removes 3' phosphate termini from DNA. Depew and Cozzarelli (1974) showed that T4 mutants, in a gene they mapped and named pseT, do not induce 3' phosphatase activity. They also showed that T4 pseT mutations cause no phenotype on normal laboratory strains, but they prevent multiplication on a clinical isolate of E. coli, they called CTr5x. When pseT$^-$ mutants infect E. coli CTr5x, T4 DNA metabolism is altered and the DNA that is made is not packaged efficiently (Depew and Cozzarelli, 1974). The phenotypes of pseT$^-$ mutants are all suppressed by stp$^-$ mutations (closely linked to rIIB) even though the 3' phosphatase still is not induced. Uninfected cells of at least some E. coli strains have 3' phosphatase activity (Becker and Hurwitz, 1967).

The first connection between these two seemingly disparate kinase and phosphatase activities was made by Cameron and Uhlenbeck

(1977) when they reported that a 3' phosphatase activity copurifies with 5' polynucleotide kinase activity; and, by a number of criteria, both activities seem to be associated with the same polypeptide. Thus this enzyme can remove 3' phosphates from DNA termini and add phosphates to 5' hydroxyls at DNA termini; therefore it can be said to "shuttle" phosphates on DNA.

Since the pseT gene induces most or all of T4's 3' phosphatase activity (Depew and Cozzarelli, 1974), we suspected that the activity copurifying with 5' polynucleotide kinase was the same as that discovered by Becker and Hurwitz (1967). Thus, we tested some pseT$^-$ T4 for 5' polynucleotide kinase activity and some T4 mutants that do not induce 5' polynucleotide kinase for pseT gene function. This led us to evidence, which we present, that: 1) the pseT gene codes for the 5' polynucleotide kinase as well as the 3' phosphatase; 2) the pseT gene product is required for normal true-late gene expression on E. coli CTr5x; and 3) the E. coli strain CTr5x is non-permissive for pseT$^-$ T4 because of an amber mutation in one or very few E. coli CTr5x genes.

MATERIALS AND METHODS

a) Materials

Gamma-^{32}P-ATP was made as described by Schendel and Wells (1973) or purchased from Amersham/Searle at a specific activity of about 3 Ci/mmol. Purified polynucleotide kinase was kindly furnished by V. Cameron and O. Uhlenbeck. Deoxynucleoside 3' and 5' monophosphates were purchased from Sigma. Micrococcal nuclease and bovine spleen phosphodiesterase were purchased from Worthington. ^{14}C-leucine was purchased from Schwarz/Mann or Amersham/Searle.

b) Bacteriophage and bacterial strains

Table 1 describes the origins and relevant characteristics of the various bacterial and bacteriophage strains used.

c) Hydroxyalamine (HA) mutagenesis

Hydroxyalamine mutagenesis was performed according to Tessman (1968).

d) Infections for 3' phosphatase, 5' polynucleotide kinase, and β-glucosyl transferase assays

E. coli was grown to an O.D.^{625} of 0.4 in tryptone broth at 37°C and infected at a multiplicity of 5. Ten minutes after infection, the cells were harvested, pelleted, resuspended, and concentrated ten times in 0.05 M tris-HCl (pH 7.5) and 0.1% mercaptoethanol. The resuspended cells were sonicated once or twice for 15 seconds. β-glucosyl transferase was routinely assayed as a control on these infections.

TABLE 1

STRAINS USED

A) T4

Mutation	growth on CTr5x	growth on CTr5x sus$^+$	Mutagenesis	Source	Comments
(T4$^+$)	+	+		our lab	Obtained by crossing am E51 with am E10 (amber in gene 45) and selecting non-amber recombinants.
PseT1	-*	+	HA	T. Snopek and	Described in Depew and Cozzarelli, 1974
PseT2	-*	+	HA	N. Cozzarelli	
7-1	-	+	HA	K. Ebisuzaki	Described in Chan and Ebisuzaki, 1970 Isolated as polynucleotide kinase deficient
PseT4	-	+	spont.	our lab	arose as a second site mutation in an alc2 mutant stock (Sirotkin et al., 1977)
PseTΔ1	-	+	spont.	our lab	Deletion; isolated in original alc$^-$ selection with alc1 (Snyder et al., 1976; Sirotkin et al., 1977)
PseTΔ3	-	+	spont.	our lab	Deletion; isolated in alc$^-$ selection
PseT12,14,47	-	+	HA	our lab	Isolated in alc$^-$ selections, by J. Runnels
5A7 #6	+	ND	HA	K. Ebisuzaki	Described in Chan and Ebisuzaki, 1970 two different 5' polynucleotide kinase deficient segregants
5A7 #11	+	ND	HA		from a cross with E51, DD2 performed in our lab
am E51	+	+			am gene 56, dCTPase
DD2	+	+			Described in Snyder et al., 1976
nd 28	+	+			den A$^-$
tsA56	+				ts in gene 31, obtained from Lindsay Black

B) E. coli

Name	Comments
Be	B strain
B40 Su_I^+	SupD, B. strain
CTr5x	Described in Depew and Cozzarelli, 1974, hybrid between K12 strains and a clinical isolate of E. coli
CTr5x sus$^+$	Selected for a more efficient amber suppressor by Brian Seed (personal communication)

*Failure to complement these two mutants for growth on E. coli CTr5x defines the pseT cistron

e) Infections for labelling proteins for SDS slab gel electrophoresis

E. coli was grown to an $O.D._{625}$ of 0.4 in M9 at 37°C and infected at a M.O.I. of 10. If T4 were not CsCl purified, they were from lysates grown in M9 with no casamino acids.

f) 3' phosphatase assay

The assay mix was the same as reported previously (Depew and Cozzarelli, 1974), except that the assays were 0.5 ml. Exceptions to the infection conditions stated above are noted in the legend to table 2. 3'TM^{32}P was purified by alternating paper electrophoresis in 0.05 M NH_4 acetate (pH 3.5) with descending chromatography in pabst (1.8% NH_4OH; 62% isobuteric acid, 1 X 10^{-3} M EDTA) until a single spot running like 3' TMP was observed by UV absorbance or upon autoradiography.

g) 5' polynucleotide kinase assay

The 0.2 ml assay mix was essentially the same as that used by Richardson (1965) and consisted of 0.1 ml made up of 10 µl 1.0 M tris-HCl (pH 7.5), 10 µl 1.0 M $MgCl_2$, 1.5 µl mercaptoethanol, about 10^6 cpm gamma ^{32}P-ATP, and 50 µl containing 30 to 80 ug hydroxyl terminated DNA prepared by sonication, alkaline phosphatase treatment, phenol extraction and dialysis. After 30 minutes at 37°C, 2 mls of cold calf thymus DNA, 200 µg/ml, was added, followed immediately by 0.5 ml of 7% perchloric acid. The resulting phosphorylated DNA was treated essentially by the method of Richardson (1965), except that the precipitate was dissolved twice with 2% KOH and finally precipitated with 0.5 ml of 50% TCA. The resulting suspensions were collected on glass fiber filters, rinsed with 5%

TCA and then 80% ethanol before being dried and counted with a toluene based scintillation fluid.

Because the assays reported were all done in crude extracts, calculation of specific activities was difficult, so parallel controls were always performed.

h) SDS slab gel electrophoresis

SDS slab gel electrophoresis was performed according to Studier (1973), and the gels were stained routinely with Weber Osborn stain diluted with 7% acetic acid to insure that the amount of total protein per column was uniform. 1 ml of cells infected as in (e) above was labelled with 1 μCi ^{14}C leu (354 mCi/mmol) chilled with ice, centrifuged, and resuspended in 0.1 ml of H_2O + lysozyme (1 mg/ml). Then 0.15 ml of a 2X concentration of Studier's (1973) resuspension buffer was added before the mixture was boiled for 2 min. All viruses used to infect E. coli CTr5x were CsCl purified to prevent ghost exclusion (see below).

i) β-glucosyl transferase assays

The 0.2 ml assay mix consisted of 10 μl of uridine diphospho-^{14}c- glucose at 1 μM/ml and 2 μC/ml, 20 μl 1.0 M tris-HCl (pH 7.5), 10 μl 1.0 M $MgCl_2$, 60 μl containing 3 to 12 μg T4 DNA (lacking β-glucose because it was purified from T4 with a mutation in the β-glucosyl transferase gene) and the equivalent of 0.1 ml of 1X concentrated crude extract.

j) CsCl T4 purification

T4 lysates were treated with chloroform and the aqueous phase was spun to remove debris. The T4 were then concentrated either by

pelleting in an ultracentrifuge (35,000 X g for 1 hr) or precipitated with polyethylene glycol. If the viruses were pelleted, they were allowed to resuspend overnight in a small volumn. A slow speed spin removed any remaining debris and 2 mls of the resulting supernatant was layered on a CsCl step gradient prepared with 6-0.5 ml layers of 70% to 20% saturated CsCl and spun at 147,000 x g for 25 minutes. The lowest band contains infective T4 particles, the next highest band contains ghost (i.e. DNAless) particles. To prevent osmotic shock, the resulting T4 suspension collected from the bottom band is successively dialyzed for 2 hours against 2.0 M NaCl, 1.0 M NaCl, and 0.5 NaCl. The final dialysis is performed overnight in the cold against M9 medium without glucose. These T4 could then be used without the ghost exclusion that interferes with infections of E. coli K12 strains.

RESULTS

a) PseT is the structural gene for a 5' polynucleotide kinase as well as a 3' phosphatase

If 5' polynucleotide kinase is coded by pseT, then many pseT$^-$ T4 mutants should fail to induce kinase activity, and mutants isolated for failing to induce kinase activity should be pseT$^-$. Table 2 shows the kinase and phosphatase activities induced after infection by some T4 mutants. Of all the mutants isolated because they were pseT$^-$ and do not induce 3' phosphatase activity (table 2A) e.g., pseT 1, pseT 2, pseT 4, pseT 12, pseT 14 and pseTΔ1), only pseT 1 induces 5' polynucleotide kinase activity (table 2B). In addition, 7-1, one of two mutants originally isolated for failing to induce 5' polynucleotide kinase activity (Chan and Ebisuzaki, 1970), also fails

TABLE 2

A) 3' phosphatase assays†

Exp.	T4 genotype	CPM	norm. % subtracting background
1*	T4+	293	(100)
	pseT1	103	24
	pseTΔ1	101	23
	uninfected	64	10
2**	T4+	345	(100)
	pseT1	140	22
3@	am E51, DD2, DenA	560	(100)
	pseT2	102	11
	pseT4	101	11
	pseT2 X pseT4		
	pseT+ recombinant	431	74
	E51, DD2, 7-1	115	13.5
	E51, DD2, nd28, pseT12	128	14.5
	E51, DD2, nd28, pseT14	150	23
4@@	T4+	469	(100)
	am E51, DD2, nd28	452	96
	uninfected	148	25
	pseT4	85	11
	pseT2 X pseT4		
	pseT+ recombinant	389	81
	am E51, DD2, nd28, pseT12	85	11
	am E51, DD2, nd28, pseT14	90	12

†0.1 ml of 10 X concentrated crude extract.

*4800 total cpm in each assay. Harvested 15' post infection. Background calculated from exp. 2 (same 3'Tm 32-P).

**8000 total cpm in each assay. Background 80 cpm.

@13,763 total counts in each assay. Background 38 cpm.

@@Cells grown at 30° and harvested 15 minutes post infection. Background calculated as experiment 3.

TABLE 2

B) 5' polynucleotide kinase assays

Exp.	T4 genotype	CPM	norm. % subtracting background
1[++]	T4	11,672	(100)
	pseTΔ1	1,243	11
	am E51, DD2	12,618	108
	uninfected	413	3.5
2[#]	am E51, DD2	9,483	(100)
	am E51, DD2, pseTΔ1	955	9
	am E51, DD2, nd28	5,882	(100)
	am E51, DD2, nd28, pseT12	941	16
	am E51, DD2, nd28, pseT14	1,782	29
3[##]	am E51, DD2	10,929	(100)
	am E51, DD2, pseT1	8,586	78
	am E51, DD2, pseT2	478	2
	am E51, DD2, 5A7 #6	1,302	9
	am E51, DD2, 5A7 #11	320	1
	am E51, DD2, pseTΔ3	411	1
	uninfected	365	1
4[&]	am E51, DD2	8,730	(100)
	am E51, DD2, pseTΔ1	935	4
	7-1	638	6
5[&&]	T4[+]	2,155	(100)
	pseT2	202	5
	pseT4	205	5
	pseT2 X pseT4		
	pseT[+] recombinant	1,865	86

[++]0.01 ml of 10X concentrated crude extracts.

[#]Cells grown at 30° and harvested 15 minutes post infection. Background 100 cpm 0.1 ml of 1X concentrated extracts.

[##]Cells grown and harvested as above, background 300 cpm. 0.1 ml of 1X concentrated extracts.

[&]Background 126 subtracted. 0.03 ml of 1X concentrated extracts.

[&&]B40 used, grown in M9s. Background of 100 cpm assumed. 0.1 ml of 1X concentrated extracts.

to induce 3' phosphatase activity (table 2A) and is pseT$^-$ (table 1). We had difficulty obtaining reproducible results with the other mutant, 5A7. It is defective in inducing 5' polynucleotide kinase activity (table 2B) but does plate on E. coli CTr5x (table 1). The results with these mutants strongly suggest that pseT codes for both activities but it could be argued that they are all double mutants or have deletions spanning two genes.

We can rule out the deletion explanation and render the double mutant explanation unlikely by testing pseT$^+$ recombinants obtained from a cross between two pseT$^-$ parents for the ability to induce kinase and phosphatase activities. As shown in table 2, the two pseT$^+$ recombinants tested induce kinase normally (only one shown) and the one pseT$^+$ recombinant tested induces phosphatase normally. Thus, the most likely explanation is that, in at least one of the pseT$^-$ parents used in the cross, a single mutation in pseT leads to the inability to induce both activities.

This does not rule out polarity as an explanation for our results. However, polarity in T4 has been shown to have only a slight effect on early genes (Stahl et al., 1968) and the frequency of mutations causing defects in both activities is very high.

Additional information concerning one mutant, pseT1, not only indicates that the pseT gene is required for the induction of both activities, but also indicates that pseT is the structural gene for both activities. O. Uhlenbeck and V. Cameron (personal communication) have purified 5' polynucleotide kinase from cells infected with pseT1. The purified enzyme contains no (0.5% or less of wild type)

Figure 1

A T4 induced polypeptide, migrating with the same molecular weight as 5' polynucleotide kinase is missing in extracts from pseT2 infected cells. E. coli B40 was infected with T4+ and pseT2, labelled from four to seven minutes, and the proteins were electrophoresed on a slab gel alongside of purified 5' polynucleotide kinase. The gel was stained with Comassie Blue for total proteins and polynucleotide kinase and used to autoradiographically expose a film for T4 proteins. Column A is a stained pattern showing the total E. coli proteins. Column B is a stained pattern showing the purified polynucleotide kinase (lower band). Columns C and D are autoradiographic patterns from pseT2 and T4+ infected cells, respectively. A T4 band of the same molecular weight as polynucleotide kinase is missing after pseT2 infections. Also identified is the band due to rIIB.

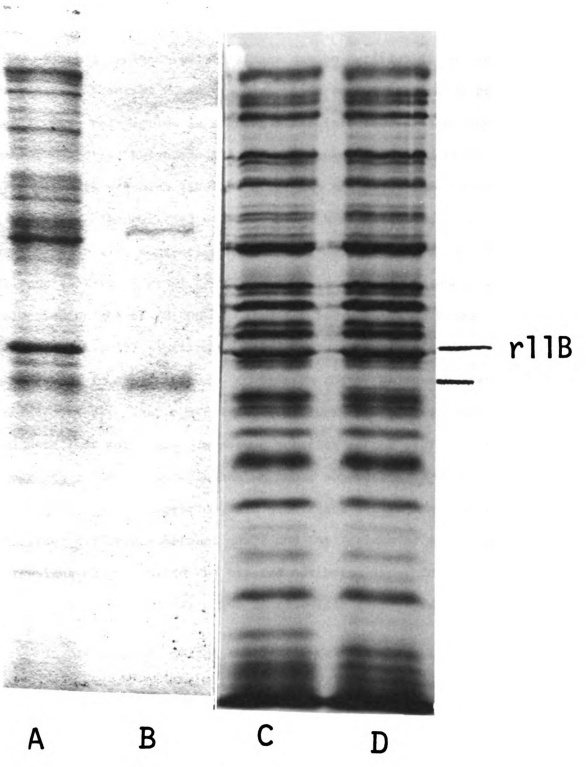

Figure 1

3' phosphatase activity. Because a mutation in the pseT gene alters the purified 5' polynucleotide kinase enzyme, pseT probably codes for it, as well as 3' phosphatase.

Since 5' polynucleotide kinase can be purified it should be possible to identify the pseT polypeptide on SDS acrylamide gels and see if this polypeptide is missing after infection by some pseT mutants. Purified polynucleotide kinase was co-electrophoresed on a slab gel with proteins labelled after T4+ and pseT2 infections (figure 1). The purified polynucleotide kinase (column B) is slightly smaller than rIIB suggesting a molecular weight of about 30,000 daltons (O'Farrell et al., 1973). This is in good agreement with the molecular weight of 33,000 reported for polynucleotide kinase subunits (Panet et al., 1973; Lillehaug, 1977). The higher molecular weight band is due to a contaminant (V. Cameron and O. Uhlenbeck, personal communication). That pseT2 infections lack this band (column C) suggests that pseT2 has a nonsense mutation in the pseT gene (not an amber since the host strain has an amber suppressor). Other mutants lacking the pseT polypeptide, are predictably, pseTΔ1 and pseTΔ3. The other difference between pseT2 and T4+ (column D) at low molecular weight may be due to the strains not being isogenic.

b) PseT$^-$ mutants are defective in true-late gene expression on E. coli CTr5x

In order to understand the biological role of the 5' polynucleotide kinase 3' phosphatase, we decided to reinvestigate why E. coli CTr5x is nonpermissive for pseT$^-$ T4. The rate of DNA synthesis of pseT$^-$ T4 on E. coli CTr5x is 50% of the pseT$^+$ rate

Figure 2

Late proteins are synthesized at a lower rate after pseTΔ1 infection of E. coli CTr5x. Shown is an autoradiograph of a electropherogram of a slab gel of proteins labelled after T4 infection. Infections were as in materials and methods except the temperature was 30°C. Columns A-D are T4+; E-H pseTΔ1. Times after infection of the pulses are: A, E 4-6'; B, F 10-12'; C,G 18-20'; D, H 28-30'. Products of some late genes are identified; p23* is processed from p23.

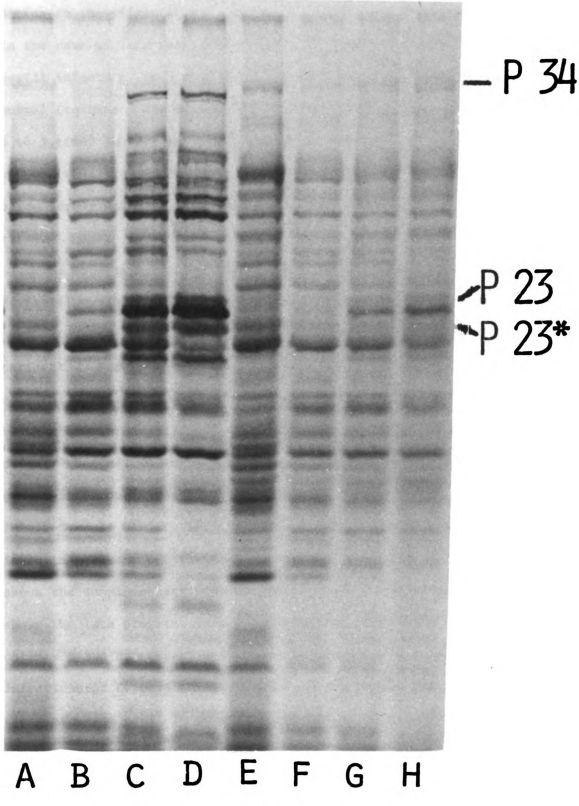

Figure 2

(Depew and Cozzarelli, 1974; our unpublished observations). In addition to the effect on DNA synthesis, there is a dramatic reduction in the rate of late gene expression. As shown in figure 2, after pseTΔ1 infects E. coli CTr5x, the rate of early protein synthesis is normal (columns A and E), but the rate of late protein synthesis is 3 to 5 times lower than normal (compare columns B, C and D to F, G and H); thus, late proteins are specifically affected. Rather than being merely delayed, late gene expression never achieves normal rates.

To show that the deficiency in true-late gene expression that T4 pseTΔ1 exhibits when infecting E. coli CTr5x is due to its being pseT$^-$ and not to some other defect, we performed some additional experiments. First, we examined pseT1, pseT2, and 7-1 (the latter originally isolated as 5' polynucleotide kinase induction deficient by Chan and Ebisuzaki, 1970, see table 1), and they all show the same defect. Second, no complementation is observed (e.g. late protein synthesis is not normal) when pseT1, pseT2, pseT47 and pseTΔ1 mixedly infect E. coli CTr5x. Thus, if the defect is due to a second mutation, it is in the same cistron for all mutants tested so far. Figure 3 shows the complementation experiment for pseT1 and pseT2. Column A shows the late proteins from T4+ infections of E. coli CTr5x and columns C and E, respectively, have the proteins from pseT1 and pseT2 infections of E. coli CTr5x. Columns G and H show the mixed infections of these pseT$^-$ T4 with T4+; the defect in true-late gene expression is recessive. Finally, column I shows that pseT1 does not complement pseT2 for the defect in true-late protein synthesis on

E. coli CTr5x. Thus the 5' polynucleotide kinase 3' phosphatase is required for normal true-late gene expression during T4 infection of E. coli CTr5x.

It is reasonable that the 5' polynucleotide kinase 3' phosphatase is required for normal T4 true-late mRNA synthesis rather than translation because the pseT gene product is also required for normal replication (so acts on DNA) (Depew and Cozzarelli, 1974) and because other gene products required for true-late gene expression regulate transcription (Bolle et al., 1968). To verify this, we have performed "mixed-competitor" hybridization experiments (for methods see Snyder et al., 1976) with RNAs prepared for pseT2 infections of E. coli CTr5x. Our preliminary results indicate that RNA taken at late times from pseT2 infected E. coli CTr5x contains a lower concentration of the sequences which behave in hybridization-competition experiments like T4 true-late mRNA sequences (data not shown). Thus, the 5' polynucleotide kinase 3' phosphatase is probably required for normal T4 true-late transcription.

c) Evidence for a host function which can substitute for the T4 coded 5' polynucleotide kinase 3' phosphatase

A priori, there might be many genetic differences between E. coli CTr5x and other laboratory strains that cause E. coli CTr5x to be non-permissive for pseT⁻T4. However, the following results imply that one or very few genes are involved. E. coli CTr5x has an amber suppressor but it is inefficient. Brian Seed (personal communication) at the California Institute of Technology selected a variant of E. coli CTr5x that efficiently suppresses T4 amber mutants. This efficient suppressing ability alters E. coli CTr5x so that it is

Figure 3

Complementation of pseT1 and pseT2 on E. coli CTr5x and the effect of efficient amber suppression. E. coli CTr5x sus$^+$ were infected as in the materials and methods and the proteins labelled 20-23' post infection. Shown is an autoradiogram as in figure 3. Columns are: A, T4+ infecting E. coli CTr5x; B, T4+ infecting E. coli CTr5x sus$^+$, C, pseT1 infecting E. coli CTr5x; D, pseT1 infecting E. coli CTr5x sus$^+$; E, pseT2 infecting E. coli CTr5x; F. pseT2 infecting E. coli CTr5x sus$^+$; G, T4+ and pseT1 infecting E. coli CTr5x; H, T4+ and pseT2 infecting E. coli CTr5x; I, pseT1 and pseT2 infecting E. coli CTr5x. G, H, I had a multiplicity of 5 of each phage. We cannot explain why the pseT$^-$ infections of E. coli CTr5x sus$^+$ produce late proteins at a greater rate than T4+ infections. This may be a general property of pseT$^-$ mutants on permissive hosts.

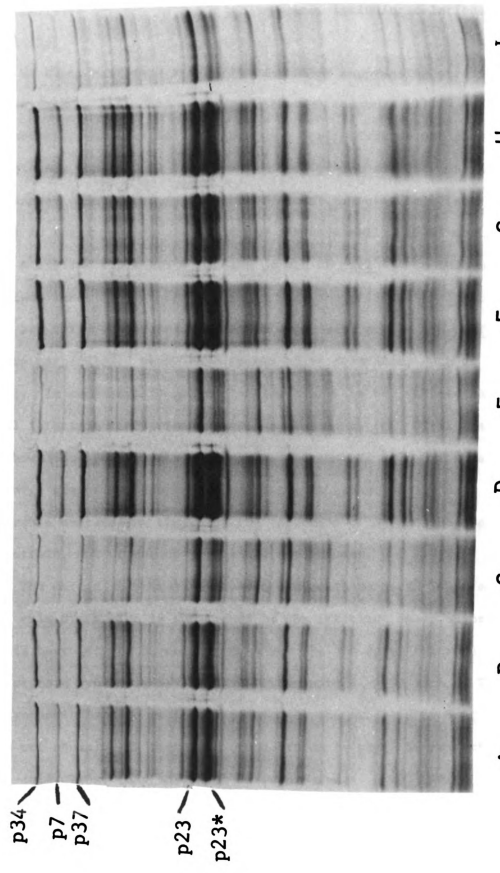

Figure 3

now permissive for all of our pseT$^-$ mutants, including deletions (table 1).

We think that this more efficient amber suppressing strain is derived from E. coli CTr5x for the following reasons: 1) T4 mutated in other cistrons that do not plate on E. coli CTr5x do not plate on it (data now shown), 2) it has an unusually high activity in the 3' phosphatase assay which is characteristic of E. coli CTr5x (see below); and 3) it still carries a str^r (Depew and Cozzarelli, 1974) marker.

A trivial explanation for the growth of pseT$^-$ T4 on this more efficient amber suppressing strain is that the original pseT mutations are being suppressed. This could not be true for all pseT$^-$ mutants particularly those that appear to be deletions, such as pseTΔ1. In addition, 5' polynucleotide kinase is not induced when pseTΔ1 infects the efficient amber suppressing E. coli CTr5x (data not shown) but yet the phage multiply. Because E. coli CTr5x has a very high competing phosphatase activity, we cannot easily draw comparable conclusions regarding the induction of 3' phosphatase on this strain, but we assume it also is not induced.

The defect in true-late protein synthesis in pseT$^-$ T4 infected E. coli CTr5x also is suppressed by the efficient amber suppressor of this host (figure 3, columns B, D and F). In fact, the rate of late protein synthesis may be higher than normal. The rate of T4 DNA synthesis is also no longer reduced on the suppressing host (data not shown). If there are other blocks to pseT$^-$ T4 production on E. coli CTr5x, they must also be suppressed.

DISCUSSION

The work presented here indicates that the product of the *pseT* gene, a 5' polynucleotide kinase 3' phosphatase, has a role in true-late gene expression, probably at the level of transcription. There is good evidence that *pseT* codes for both activities: 1) cells infected with a number of mutants in the *pseT* cistron, discovered by Depew and Cozzarelli (1974), lack both activities when assayed in crude extracts (table 2); 2) two *pseT*$^-$ mutants, which fail to induce both activities, yield *pseT*$^+$ recombinants inducing both activities (table 2); 3) an altered 5' polynucleotide kinase purifies from cells infected with one *pseT*$^-$ mutant (V. Cameron and O. Uhlenbeck, personal communication); 4) cells infected with some *pseT*$^-$ T4 lack a T4 induced polypeptide which comigrates on SDS gels with purified polynucleotide kinase (figure 1). We cannot, however, absolutely rule out other explanations based upon extreme polarity or protein alterations. Because some *pseT*$^-$ mutants lack the *pseT* coded polypeptide, we conclude that inability of *pseT*$^-$ mutants to multiply on *E. coli* CTr5x is simply due to a deficiency in this gene product and is not caused by special mutations in this gene.

Before presenting some possible models to explain the biological role of the *pseT* gene product, it is appropriate to summarize briefly previous research concerning both the 5' polynucleotide kinase and 3' phosphatase. The purified T4 induced 3' phosphatase, as originally reported by Becker and Hurwitz (1967), removed 3' phosphates, but not 5' phosphates, from monomeric and oligomeric deoxynucleotides. They also reported that the 3' phosphatase was

inactive on 3' monoribophosphates and 2'-(3')-P-$(Up)_4$. This last result conflicts with Cameron and Uhlenbeck's (1977) report that the 3' phosphatase activity co-purifying with 5' polynucleotide kinase removed selectively the 3' phosphates of $(Up)_5$, $P(Up)_5$, $(Cp)_2Gp$, and many other short ribonucleotides. We suspect that either those RNAs with 2' phosphates inhibit the enzyme or that the slightly different assay conditions explain the difference. Other similarities between the two reported activities are a pH optimum of about 6 and inhibition by inorganic phosphate.

The 5' polynucleotide kinase has been studied more extensively. The native enzyme has a molecular weight of 140,000 and is composed of 4 subunits of about 33,000 molecular weight (Panet et al., 1973; Lillehaug, 1977). It is most active on oligomeric nucleic acids, does work on 3' monophosphates, and is inactive on ribo- and deoxynucleosides (Richardson, 1965; Novogrodsky and Hurwitz, 1966). The kinase reaction works better on single stranded than double stranded DNA and at gaps in double stranded DNA than at nicks (Lillehaug, 1976). The kinase is freely reversible (van de Sande, 1973), but the reverse reaction has a lower pH optimum than the forward reaction (van de Sande, 1973). An endonuclease activity associated with kinase has been described which introduces single stranded nicks into DNA (Loewen, 1976). This activity is not present in freshly purified kinase preparations; it only appears upon storage, especially storage without ATP or mercaptoethanol. The activity filters through a G-100 column as if it is due to a disassociated kinase subunit (Loewen, 1976). The implications of this endonuclease for models of the biological role of the pseT gene product will be

discussed with the models themselves.

Although we cannot rule out the possibility that the phosphatase and kinase work on different substrates, we prefer the model that they both work on the same substrate *in vivo*. This model is supported by *pseT*47 and *pseT*1's having the same phenotypes even though one fails to induce kinase and the other fails to induce phosphatase. Because the kinase functions best on polymerized nucleic acids, and the phosphatase does not use ribomonophosphates, we think that polymerized DNA, rather than RNA, is probably the substrate *in vivo*, although this is not proven. It may be relevant to this discussion that many of our *pseT*$^-$ mutations arose in *alc*$^-$ mutant selections (see table 1). Many of these are second site mutations and may be selected because they enhance the multiplication of *alc*$^-$ T4. Since *alc* has been shown to affect the structure of the bacterial nucleoid (Sirotkin et al., 1977), by association, *pseT* is also implicated in DNA structure. Before examining how this enzyme might change DNA to affect transcription, we shall discuss what is known regarding DNA structural requirements for T4 true-late transcription.

Concommitant T4 DNA replication is required for normal T4 true-late transcription (Riva et al., 1970a). However, true-late transcription can be genetically uncoupled from replication by inactivating T4 DNA polymerase, T4 DNA ligase and a T4 promoted exonuclease (Riva et al., 1970b). The antagonistic effect of ligase implies that nicks or gaps must be present in T4 DNA before it can serve as a template for true-late transcription. Apparently, the exonuclease must be inactivated to prevent degradation of the unligated DNA. The question remains of how these facts relate to our observations

that this form of transcription might be altered by the product of the pseT gene which in vitro, at least, alters the orientation of phosphates at termini. Since DNA synthesis and true-late transcription of pseT$^-$ T4 are both defective on E. coli CTr5x, a trivial explanation for the defect in true-late transcription is that, because this transcription relies normally on DNA synthesis, the lessened DNA synthesis causes the lessened true-late transcription. Then the only effect of pseT would be on replication. We think that this explanation is unlikely because other mutations, which have an even more dramatic effect on DNA synthesis, do not affect true-late gene expression. Therefore, the following models are presented assuming both defects stem from a common problem of DNA structure or of DNA interaction with some other cell constituent.

All of the direct models for the biological activity of 5' polynucleotide kinase 3' phosphatase on DNA fall into three categories. The first includes those models in which the reactions are concerted. By this we mean that the kinase and phosphatase activities which we assay in vitro are actually components of an overall reaction in vivo. This reaction could involve the DNA's tertiary structure. Examples of a change in tertiary structure include DNA winding such as performed by DNA gyrase (Gellert et al., 1976) and DNA unwinding such as performed by omega proteins (Wang, 1971). There is evidence that some supercoiling may exist in replicating T4 DNA (Hamilton and Pettijohn, 1976). The three known activities (nickase, kinase, phosphatase) together with a ligating activity that can only be postulated, may work together concertedly. If the final DNA structure is stable or if the ligating protein forms a tight complex with the 5'

polynucleotide kinase 3' phosphatase, the ligating activity need not reside in the pseT gene product. In our view of this theoretical event: a nick leaves a 5' hydroxyl and a 3' phosphate; the phosphate is removed; with the phosphate and energy from ATP, some change in DNA structure occurs; the phosphate is attached to the 5'-hydroxyl; and finally, either by itself or utilizing DNA ligase activity from another polypeptide, the nick seals. In the process, the DNA strands are rotated 360° around one another. The intermediates in omega protein and gyrase action have not been isolated and these reactions could involve "phosphate shuttling."

Another category of model includes the binding of DNA to some other cell constituent, such as a protein or membrane phospholipid. In these models polynucleotide kinase forms the bonds between some phosphorylated compound and a 5' hydroxyl terminus in DNA. The apparent lack of specificity for the donor of phosphate in the kinase reaction (Novogrodsky et al., 1966) is consistent with these models. In vivo, in T4 infected cells, the bonds might be transient and therefore hard to detect. These models have to be elaborated to account for the 3' phosphatase activity.

One final model category assumes that the phosphate shuttling observed in vitro is biologically significant in vivo. These models imply that, in DNA with single strand nicks or gaps, the orientation of the phosphates, or their absence, accounts for the observed biological effects. The presence or absence of a 5' phosphate or 3' phosphate, respectively, might alter the terminus so that it can be used by some other enzyme, for example, DNA polymerases, ligases or exonucleases. As previously mentioned, nicks, at least, are already

implicated as being necessary for DNA to be competent for T4 true-late gene expression, since T4 DNA ligase is antagonistic to T4 true-late transcription. Since a 3' phosphate interferes with the filling of a single stranded gap in DNA by DNA polymerase and either a 3' phosphate or a 5' hydroxyl interferes in the ligation of nicks by DNA ligase, if gaps or nicks are simply required for true-late transcription, then the lack of the combined 3' phosphatase 5' polynucleotide kinase activities would increase true-late transcription. This is the opposite of what is observed on E. coli CTr5x; the lack of the combined activities decreases T4 true-late transcription. Two explanations could be offered to preserve these models in the face of this apparent contradiction: 1) T4 ligase acts at different times or places on the DNA than T4 5' polynucleotide kinase 3' phosphatase; 2) there is a third activity required for T4 true-late transcription which requires the phosphate orientations established by the pseT gene product. Ligase is antagonistic to this third activity rather than to the 5' polynucleotide kinase 3' phosphatase. This third activity could be an exonuclease. There is a precedent for exonucleases affecting gene expression. The T5 exonuclease (Frenkel and Richardson, 1971a and 1971b), is necessary for the expression of T5 late genes (Chinnadurai and McCorquodale, 1973). Perhaps for T4 true-late transcription, one DNA strand must be unzipped or degraded before RNA polymerase will act, and, either the 5' polynucleotide kinase 3' phosphatase unzips or degrades it itself or, by changing the DNA termini, allows another protein to do it. Gaps in intracellular T4 DNA have been reported by Curtis and Alberts (1976). However, the gene 49 product is required for their formation

Figure 4

The model categories depicting possible pseT gene product functions. The figure is explained in the text.

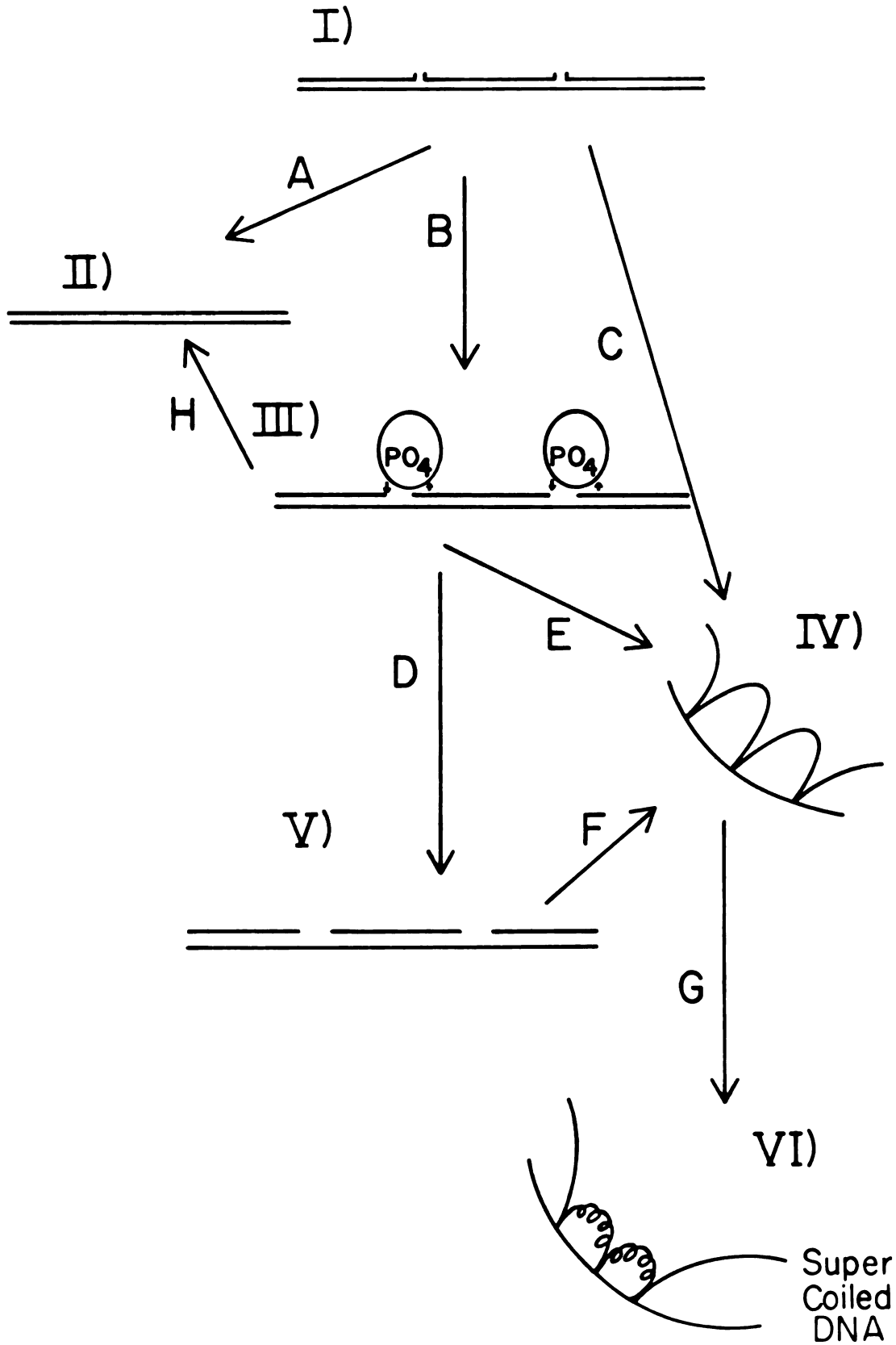

Figure 4

and mutants in gene 49 are normal in terms of gene expression, and only defective in packaging.

One way of presenting all three types of models appears in figure 4. Different DNA states are represented by Roman Numerals (I through VI) and different alterations are represented by letters (A through H). State I represents freshly replicated DNA that is assumed to contain nicks with unspecified phosphate orientations. Alteration A, performed by DNA ligase, leads to State II which is inactive for T4 true-late transcription. "Phosphate shuttling" performed by the 5' polynucleotide kinase 3' phosphatase, alteration B, could lead to state III that has 5' phosphate and 3' hydroxyl termini. The nicks might be sealed by DNA ligase (alteration H) or "gapped" by an exonuclease as in alteration D. If the pseT gene product binds the DNA to some cell constituent, either before or after the phosphates are shuttled (C or E), or after some third activity acts (F), this would lead to state IV. Supercoiling is alteration G leading to state VI. All alterations except D (gapping) and A and H (ligating) might, according to the models, be performed by the pseT gene product. In the discussion of the models, states III through VI are considered as possible required DNA structures for T4 true-late transcription.

To choose between the models, it may be relevant that pseT1 and pseT47 do not complement each other even though their activities are complementary. It could be argued that this supports the concerted reaction model but the fact that the isolated enzyme is a tetramer weakens the argument. More mutants should help in distinguishing between the models.

If we knew why E. coli CTr5x is nonpermissive for pseT⁻ mutants of T4, we might know how universal such functions are, as well as the details of the reaction they promote. Mutating E. coli CTr5x to an efficient amber suppressor changes it so that it becomes permissive for pseT⁻ T4. The simplest explanation for this is that a single locus in E. coli CTr5x contains an amber codon that, when suppressed efficiently, allows growth of pseT⁻ T4. Presumably the deficiency in this gene product makes E. coli CTr5x nonpermissive. Note that, since the original strain has an inefficient suppressor, there is probably some of this gene product in the cell. This leads to the conjecture that, with no amber suppression, no T4 true-late proteins would be made in pseT⁻ infections. It also leaves open the possibility that the gene product is required for growth of the host.

We imagine three possibilities as to how a host gene product can make a phage gene product nonessential: a) T4 "commandeers" a host gene product, which had a different function in uninfected cells, and uses it in its late mRNA synthesis; b) the host gene product is used in a pathway to make true-late mRNA which is different from the one in which the pseT gene product functions; c) the host gene product is an analogue of the T4 coded 5' polynucleotide kinase 3' phosphatase.

At present, we cannot distinguish between these possibilities. However, precedents exist for host proteins being able to substitute, under some conditions, for analogous phage proteins. For example, T4 can use the host DNA ligase in lieu of its own DNA ligase when the T4 RII gene is inactivated (Ebisuzaki and Campbell, 1969; Berger and Kozinski, 1969; Karam, 1969). If the parallel is accurate, this

could correspond to: 1) the host counterpart is normally able to substitute; 2) it is either unavailable or reduced because of an amber mutation in E. coli CTr5x; 3) inactivating stp (the extracistronic suppressor of the pseT$^-$ phenotype; Depew and Cozzarelli, 1974) either makes less of it necessary or increases the availability of that of it that is made. That no 5' polynucleotide kinase has been detected in uninfected cells may merely mean that the host enzyme assays differently.

If there is a host counterpart to the T4 5' polynucleotide kinase 3' phosphatase, it may also be involved in transcription in uninfected bacteria. This transcription may share requirements with the true-late replication-coupled transcription of T4. In any case, only one or few differences are causing E. coli CTr5x to be non-permissive for pseT$^-$ mutants of T4, raising the possibility that similar restricting mutants can be isolated starting with other E. coli strains and the responsible mutations mapped.

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APPENDICES

APPENDIX I

T4 Bacteriophage-coded RNA polymerase subunit blocks host transcription and unfolds the host chromosome

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T4 Bacteriophage-coded RNA polymerase subunit blocks host transcription and unfolds the host chromosome

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T4 bacteriophage mutants selected for their ability to grow with cytosine in their DNA are defective in host transcriptional shutoff and host chromosome unfolding. The host RNA polymerase purified from cells infected by such a mutant lacks a small T4-coded subunit.

T4 BACTERIOPHAGE shuts off host transcription efficiently and rapidly but the molecular mechanism behind this remains a mystery. One reason for the mystery may be that no T4 mutants defective in host shutoff have yet been isolated. Since T4 probably uses the host RNA polymerase for all its transcription[1,2], inactivation of the polymerase cannot be involved in shutoff. During infection however, various T4 coded polypeptides bind to the host RNA polymerase. These include the products of T4 genes *55* (refs 3 and 4) and *33* (refs 3 and 5) and of two other unidentified genes. The latter two polypeptides, molecular weights 15,000 and 10,000, were assigned the numbers 2 and 4, respectively, by Stevens[3]. There are other polypeptides which bind loosely to the RNA polymerase[6]. In addition, the α subunit of the RNA polymerase is adenylated[7] by two distinct T4-coded activities, *alt* and *mod* (ref. 25), one of which, *alt*, is carried in the virus particle[8,9]. The adenylated RNA polymerase transcribes *E. coli* genes poorly *in vitro*[29]. Neither *alt* nor *mod* mutants affect the shutoff of host protein or r RNA synthesis *in vivo*[8], however, and none of the T4-coded RNA polymerase subunits has been implicated in host shutoff.

Besides changing the host RNA polymerase, T4 infection alters the host "chromosome". The host DNA changes its intracellular location[31] (nuclear disruption), a process requiring the T4 *ndd* gene product[10]. The highly compact structure of the host nucleoid is unfolded[12], requiring the product of the T4 *unf* gene[11]. The host DNA is degraded to mononucleotides chiefly by the products of genes *denA endoII* (refs 13 and 14) and *46, 47* (ref. 15). All of these phenomena can proceed independently of each other[10,11]. None of them has been related in a simple way to the shutoff of the synthesis of at least the major host proteins[11].

T4 has hydroxymethylcytosine in its DNA instead of the usual cytosine[16]. It is tempting to speculate that the shutoff of host transcription and/or replication occurs when T4 causes the cell to reject DNA with cytosine for some aspect of transcription and/or replication. If this is true, T4 with cytosine in its DNA might "shut itself off". T4 mutations which permit the multiplication of cytosine-containing T4 would occur in genes normally involved in host shutoff. To test this hypothesis, and to find T4 regulatory genes, we have begun the search for T4 mutants which can produce T4 with cytosine in their DNA.

The first such mutants we have found are deficient in the function of a gene which we call *alc* (ref. 17). When cytosine-containing T4 DNA is made after infection, the *alc* gene product almost completely prevents true-late transcription. True-late mRNAs are those which require T4 DNA replication[18] to have occurred and the products of genes *33, 55* (ref. 18) and *45* (ref. 19) to be present. Here we present evidence that the product of the *alc* gene becomes an RNA polymerase subunit, probably the polypeptide no. 2 of Stevens[3]. It is also required to unfold the host nucleoid after infection and is likely to be the *unf* gene product of Snustad and his collaborators[11]. We discuss the implications of this for the structure of the host nucleoid, and the relationship of the structure to transcriptional regulation.

Missing T4-coded polypeptide in *alc* RNA polymerase

In the original report on *alc*$^-$ mutants, we proposed that the *alc* gene product, itself non-essential, interacts with an essential part of the transcription apparatus. We proposed that this may be one of the activities which adenylate the α subunit of the RNA polymerase or may be one of the T4-coded RNA polymerase subunits. Since mutants are available which lack one or the other adenylating activity[8], we tested these first. Neither *alt*$^-$ nor *mod*$^-$ mutants were able to confer the *alc*$^-$ phenotype after recombination with T4 which makes cytosine-containing DNA. Thus, we

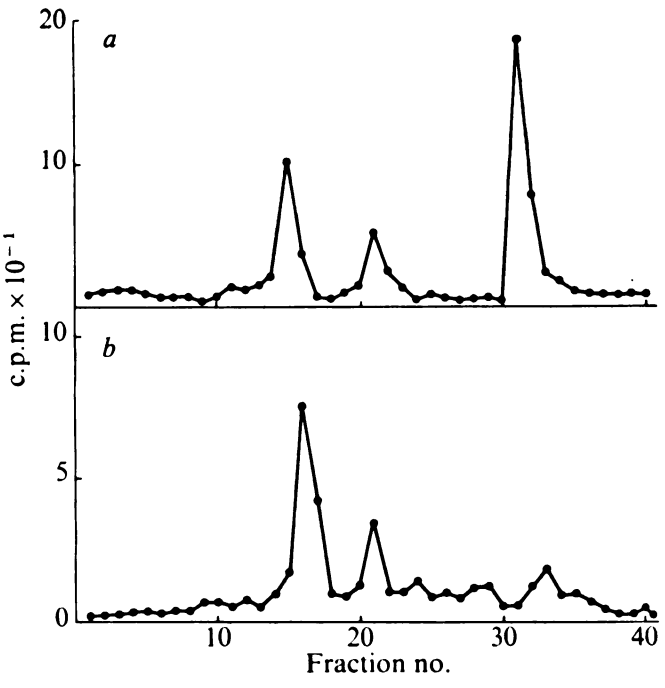

Fig. 1 An *alc*$^-$ mutant is lacking a T4-coded polypeptide on the RNA polymerase. *E. coli* B^E (100 ml) growing in M9 without amino-acids at 30 °C were labelled with ^{3}H-leucine 5–15 min after infection. They were then mixed with 1 g of uninfected cells, sonicated, and the RNA polymerase purified by the method of Burgess[21] through phosphocellulose chromatography. The results of slicing and counting 12% SDS gels (procedure of Fairbanks *et al.*[22]) is shown. The dye front was on the right. *a*, T4-coded polypeptides on the RNA polymerase from the parent of the spontaneous *alc*$^-$ (alc1) mutant (*b*). The *alc*$^-$ mutant is clearly missing the first peak from the right in about fraction 34. We think this peak is due to Stevens' polypeptide no. 2.

Table 1 Map position of *alc*⁻ mutations

Cross	*am*H21,	*alcI*	× *am*H39	*am*H39,	*alcI*	× *am*H54	*am*N54,	*alcI*	× *am*M69	*am*M69,	*alcI*	× *am*N54
Crossover occurring	54		alc	30		alc	31	alc			alc	63
		30			31				63	31		
Expected frequency *alc*⁻		~1			~1			~½			~½	
Observed frequency		50			57			43			56	
		56			65			112			97	
Map order: 54	30 31		*alc* 63									

Double mutants *am. alc*⁻ were constructed by crossing and screening *am* progeny for temperature sensitivity by spotting on K803 Rif^r-2[23] at 42 °C. These double mutants were then crossed with single amber mutants in the region of gene 31. The *am*⁺ recombinants were spotted on K803 Rif^r-2 to determine their temperature sensitivity. Shown is the fraction of the total *am*⁺ progeny from one plate which are *alc*⁻. The amber mutants used were *am* H21 (gene *54*), *am* N54 (gene *31*) and *am* M69 (gene *63*). The *alc*⁻ mutant is *alc1*, the same mutant used in the RNA polymerase experiment in Fig. 1. *alcI* is also *unf*⁻. We have also analysed other crosses, the results of most of which were also consistent with this map order.

tentatively concluded that *alc*⁻ mutants are not simply *alt*⁻ or *mod*⁻. We then purified the RNA polymerase from cells after infection by an *alc*⁻ mutant to test for the presence of the T4-coded polypeptides. Figures 1*a* and *b* show polyacrylamide gels of RNA polymerase from *alc*⁺ and *alc1* (*alc*⁻) infected cells respectively. The *alc*⁻ mutant is conspicuously lacking the major peak of radioactivity in about fraction 33, but has all the others. By molecular weight determinations with known markers and by comparison with the published results of Stevens[3], we think that the missing peak is the 15,000 molecular weight polypeptide no. 2. *Alc1* was a spontaneous mutant of the *alc*⁺ parent shown in Fig. 1*a*. Thus, we are quite sure that the *alc*⁻ mutation caused the absence of the polypeptide in the RNA polymerase. In addition, we have done this experiment using 'mixed labels'. The *alc1* mutant infected cells were labelled with ^{35}S-methionine and the *alc*⁺ parent with ^{3}H leucine. The infected cells were then mixed and the RNA polymerase purified as before. The polypeptide in question was only labelled with ^{3}H offering additional evidence that, after infection by the *alc1* mutant, the polypeptide is not bound to the RNA polymerase. This is not true for all apparent *alc*⁻ mutants, however. We shall return to this point later. The *alc1* mutation does not seem to affect the synthesis of any other T4 early gene products. Thus, *alc* is probably the structural gene for this RNA polymerase-bound polypeptide.

Map position of *alc*⁻ mutations

The *alc* gene product seems to be relatively non-essential in most laboratory conditions. We have found, however, that the multiplication of some *alc*⁻ mutants, including *alc1*, is temperature sensitive. This effect is amplified in certain RNA polymerase mutants of *E. coli* of the Rif^R-2 type[24] and *alc1* will not form plaques at 42 °C on these strains. We think the temperature sensitivity is due to the *alc*⁻ mutation because (1) a fairly high percentage (~10%) of spontaneous *alc*⁻ mutants are temperature sensitive, and (2) we have not been able to separate the temperature sensitive and *alc*⁻ phenotypes by recombination.

Even though we do not know the molecular basis of the temperature sensitivity we have used it to map *alc*⁻ mutations. First, we isolated the *alc1* mutation by crossing out the amber mutation and the rII deletion which were used in its selection[17]. We then crossed this temperature-sensitive mutant with amber mutants in genes around the T4 map and selected for non-temperature sensitive, *am*⁺ recombinants. We obtained the lowest recombination frequencies with mutants in the region of gene *31*. To localise the mutation more precisely, we constructed double mutants of the temperature-sensitive mutant and amber mutants in genes in this region. We then crossed these against single amber mutants and tested the *am*⁺ progeny for their temperature sensitivity (Table 1). We have shown the map order which is consistent with the data and the crossover occurring in each case. The data support the map order gene *63*-*alc*-gene *31*.

There are four other types of mutations which have been reported to map between genes *63* and *31*. These are *r*III, *cd*, *pset* and *unf* (ref. 11). Of these, *unf* may be related to *alc* since they both might be expected to play a part in host shutoff, *unf* because it is required for the unfolding of the bacterial nucleoid and *alc* because it prevents a type of transcription on cytosine-containing T4 DNA. Thus, we studied the ability of *alc*⁻ mutants to unfold the host nucleoid after infection.

alc⁻ Mutants defective in host nucleoid unfolding

In 1971, Stonington and Pettijohn reported a lysis procedure which leaves *E. coli* DNA highly folded, perhaps in a similar state to its intracellular structure[20]. These compact structures, nucleoids, have a high sedimentation rate and contribute little to to the viscosity of lysates. If they are unfolded, however, after T4 infection[12] for example, their S value drops markedly and the lysates are extremely viscous.

To see whether *alc*⁻ mutations affect the T4-induced unfolding of the host nucleoid, we made sucrose gradients of nucleoids from uninfected, *alc*⁻ infected and *alc*⁺ infected cells (Fig. 2). In agreement with others, our nucleoids from uninfected cells sedimented heterogenously with an S value of about 2,000. This S value was largely unchanged by infection with the *alc*⁻ mutant while it dropped to about 1,000S after infection by *alc*⁺ T4. Thus, the *alc*⁻ mutant is defective in host nucleoid unfolding.

We also determined the time course of unfolding by *alc*⁻ mutants by measuring the viscosity of lysates of cells taken at different times after infection. The results for two independent *alc*⁻ mutants and their parent are shown in Fig. 3. Both *alc*⁻ mutants were defective in host nucleoid unfolding compared with the parent. One of them, *alc2*, probably never unfolds the host nucleoid whereas the other, *alc4*, is merely delayed about 1–2 min in unfolding. Some *alc*⁻ mutations do not measurably affect the timing or extent of host nucleoid unfolding.

Are *alc* and *unf* the same gene?

Several considerations support the conclusion that *alc* and *unf* code for the same gene product. A fairly high percentage (> 10%) of *alc*⁻ mutants are also measurably *unf*⁻. Those *alc*⁻ mutants which are not measurably *unf*⁻ are also those which make reduced amounts of late gene prducts when cytosine-containing T4 DNA is made (data not shown). We have also obtained what seem to be partial revertants of the *alc1* mutation (that is, they are less *alc*⁻). These have also become *unf*⁺ to varying degrees. The temperature-sensitive mutation which we have mapped is inseparable from *alc* by recombination and maps in the same region as the *unf* mutation of Snustad *et al.*[11]. So far, those *alc*⁻ mutations which confer the most temperature sensitivity have been the most completely *unf*⁻. The *alc2* mutant used was back-crossed against its parent three times at a ratio of 1:10, selecting the *alc*⁻ phenotype each time; it was still *unf*⁻. All of these observations

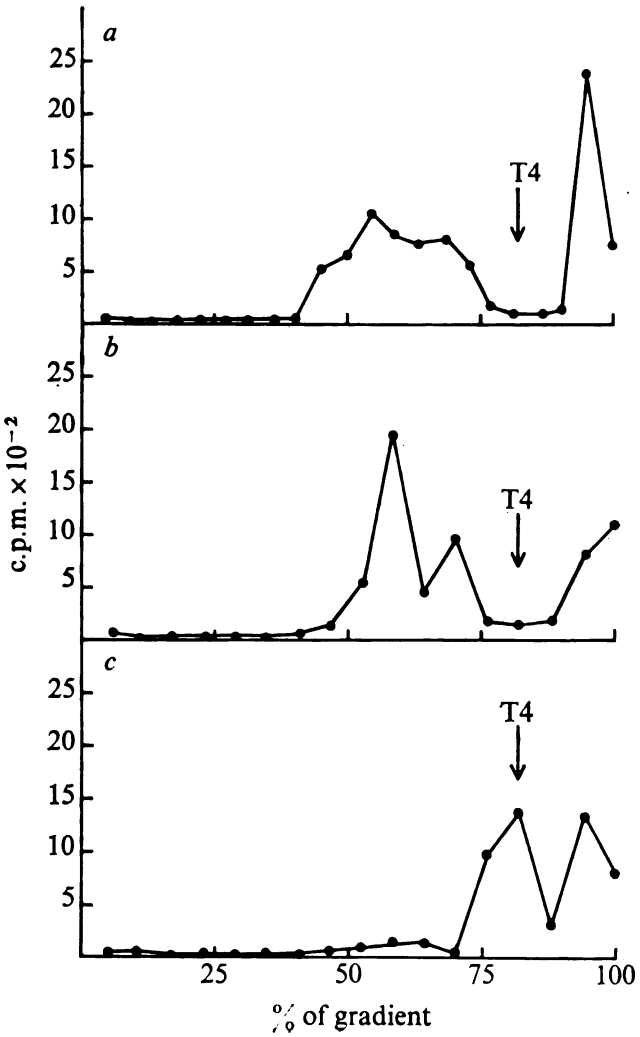

Fig. 2 Sucrose gradients of nucleoids after infection by an *alc*⁻ mutant. *E. coli* B^E (5 ml) growing at 30 °C in M9S were labelled for 1 h before infection with 16 μCi ml^{-1} thymidine (5.5 mCi mg^{-1}). They were infected at a concentration of 4×10^8 ml^{-1} at a multiplicity of infection of 5 in 10 μgml^{-1} tryptophan. At 5 min after infection, 1.0 ml was poured into a chilled tube containing 0.1 ml of 1 mg ml^{-1} chloramphenicol. The lysis procedure of Snustad *et al.*[11] was used except that the solutions were made up as follows. Solution I: 0.1 m Tris, 6.5 mg sodium azide, 1.0 g sucrose, 0.58 g NaCl, and 9 ml H_2O. Solution II: 1.2 ml Tris, 2.0 ml EDTA, 4 mg lysozyme, and 6.8 ml H_2O. Solution III: 0.1 ml Sarkosyl, 1.16 g NaCl, 0.5 ml EDTA, and 9.0 ml H_2O. Tris was 1 M adjusted to *p*H 8.15 with HCl. EDTA was 0.25 M adjusted to *p*H 7.0 with NaOH. After 5 min for lysis, two additional steps were performed before layering on gradients. 0.1 ml of 20.0 mg ml^{-1} lysozyme dissolved in solution II were added for 10 min and then the lysate was diluted with 2.0 more ml of solution III. Five minutes later the lysate was chilled and 0.15 ml were layered on 4.7 ml 10–30% w/v sucrose gradients made up with 0.5 ml Tris, 0.25 ml EDTA, 0.25 ml Sarkosyl, 5.8 g NaCl, and 10 μl mercaptoethanol per 50 ml. There was a 0.5-ml step at the bottom made from a solution containing 0.1 ml Tris, 6.0 g sucrose, 1.0 g NaCl, 3.0 ml saturated CsCl and 3.0 ml H_2O. They were centrifuged at 4 °C for 25 min at 17,000 r.p.m. in a Spinco SW50L rotor. Fractions were collected from the bottom which is at the left. *a*, Uninfected; *b*, *am*E51, DD2, *alc2* (ref. 17), an *alc*⁻ mutant induced by hydroxylamine and back crossed three times against its parent at a ratio of 1 : 10; and *c*, the *alc*$^+$ parent of *alc2*. The arrows show the peak of sedimentation of a T4 phage marker.

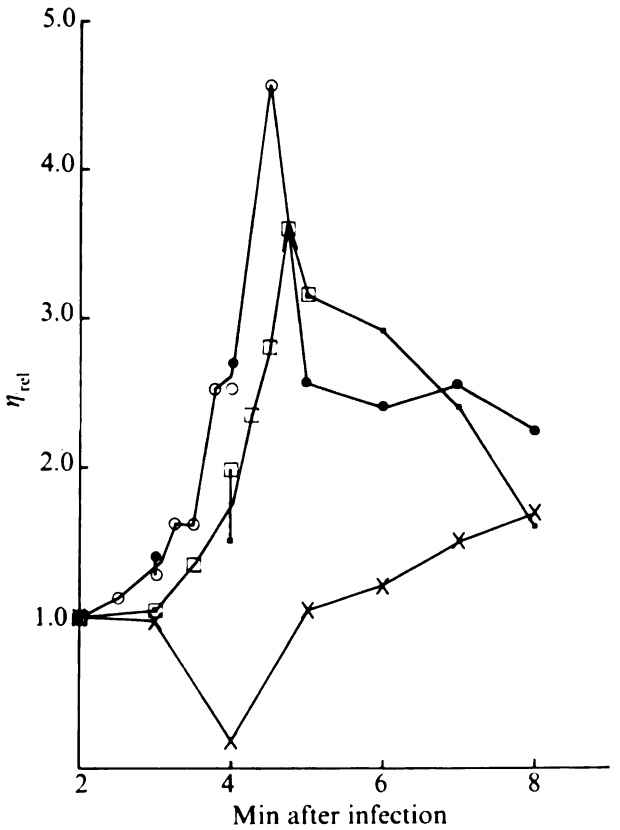

Fig. 3 Time course of unfolding of host nucleoids after infection by *alc*⁻ mutants. Cells were infected and all 5 ml lysed as in Fig. 2. 12 min after solution III was added, the viscosity of the lysate was measured without previous centrifugation by measuring the rate of movement in mg s^{-1} through a 20-gauge needle 4 cm long. The ratio of this number to that obtained from lysates of cells at 2 min after infection is plotted. Lysates of uninfected cells flowed at close to the same rate as cells collected 2 min after infection and both were only slightly slower than H_2O. The process of measuring the viscosity of all the samples took less than 15 min and the uninfected cell lysates were still not viscous at this time. The closed and open figures of each type represent different experiments. We drew the line through the midpoint when the results were slightly different for the same time after infection. (○), *am*E51, DD2 (ref. 17); □, *am*E51, DD2, *alc4*; ×, *am*E51, DD2, *alc2*.

support the conclusion that *alc* and *unf* are the same gene. However, we have not rigorously excluded two other possibilities. One of these is that *alc* and *unf* are neighbouring genes and those *alc*⁻ mutants which are also *unf*⁻ are deletions spanning these two genes. The existence of partially *unf*⁻ mutants such as *alc4*, however, which is also intermediate in its temperature sensitivity and *alc*⁻ phenotype, argues against the deletion hypothesis. Also, the high frequency of *alc*⁻,-*unf*⁻ mutants has already been given as evidence for point mutants. The other possibility is that *alc*⁻, *unf*⁻ mutants are double point mutants, *alc* and *unf* are adjacent genes, both the *alc*⁻ and *unf*⁻ mutations enhance the multiplication of cytosine-containing T4, and the temperature sensitivity is due to the *unf*⁻ mutation. However, we think these alternative explanations are very unlikely and that *alc* and *unf* are the same gene.

If *alc* and *unf* are the same gene why are not all *alc*⁻ mutations measurably *unf* deficient? The most likely explanation is that T4 mutants which are only partially defective in *alc* function can still multiply with cytosine in their DNA to make a plaque; but are not as temperature sensitive and are scored as *unf*$^+$ in our unfolding test.

Implications for host shutoff and *E. coli* nucleoid structure

Although *alc* might be expected to play a part in host shutoff, *alc*⁻ and *unf*⁻ mutants do not affect the timing of shutoff of synthesis of the host proteins one sees on polyacrylamide gels (ref. 11 and our unpublished observations). Since there could be a translational shutoff superimposed on the transcriptional one, however, we looked at the shutoff of host RNA synthesis directly by testing to see whether any RNA synthesised after infection by an *alc*⁻ mutant can hybridise to *E. coli* DNA. The results are shown in Table 2. It is clear that the *alc*⁻ mutation

Table 2 Hybridisation of RNA labelled after infection by an *alc*$^-$ mutant to *E. coli* DNA

RNA ^{3}H	Labelling time	c.p.m. Input	c.p.m. Hybridised	% of RNA Hybridised	% of RNA which is *E. coli*
Uninfected	2-min pulse	8,397	1061	12.7	(100)
T4+	4–6	4,736	107	2.26	17.8
	8–10	3,033	31	1.04	8.2
	14–16	2,557	103	4.0	31.5
alc1	4–6	5,048	224	4.4	34.6
	8–10	3,495	196	5.5	43.0
	14–16	2,840	261	9.2	72.5

RNA was pulse labelled at the times indicated in M9 medium at 30 °C with 10 μCi ml^{-1} ^{3}H-uridine (5 Cimmol^{-1}). The phage were *alc1* which had been crossed against the wild type (T4$^+$) to remove the *am*E51 and DD2 mutations[17]. The multiplicity of infection was 10 and tryptophan was added at 10 μg ml^{-1} before infection. Surviving bacteria were less than 0.1 % at 2 min after infection. The RNA was extracted three times with phenol at 60 °C by the Method I of Bolle *et al.*[18], ethanol precipitated, treated with DNase (5 μg ml^{-1}) in 0.1 M Tris-Cl *p*H 8.0, 0.01 M $MgCl_2$ for 15 min at 37 °C, extracted twice and reprecipitated with ethanol. Hybridisation was with 20 μg ml^{-1} *E. coli* DNA[30] for 40 h at 60 °C in 2SSC. The hybrids were treated with RNase (12 μg ml^{-1} RNase A at 37 °C for 15 min), collected on presoaked Millipore filters, and washed with 0.5 M KCl, 0.01 M Tris, *p*H 7.5. The specific activities of the RNAs were from top down, 10.6, 6.76, 4.0, 3.32, 7.8, 5.85, and 4.27 c.p.m. ng. The percentage of RNA which is *E. coli* was determined by dividing the percentage of input counts which hybridised by the percentage of the uninfected labelled RNA which hybridised. This number is affected by changes in the relative concentrations of *E. coli* RNA species so should only be considered qualitative. It is perhaps surprising that *E. coli* transcription can continue so late into infection when the host DNA is being degraded. This RNA is presumably being made on fragmented DNA.

affects the shutoff of the synthesis of at least some *E. coli* transcripts. Thus, selecting T4 mutants which could multiply with cytosine in their DNA has led to the discovery of a host shutoff gene, *alc*.

If *alc*$^-$ mutants are defective in the shutoff of host transcription, why are they normal in the shutoff of the host proteins one sees on gels? One possibility is that there is a translational shutoff which is independent of transcriptional shutoff, and evidence has been presented for such a translational shutoff by T4 (refs 27 and 28).

Host RNA is still being made after infection even by *alc*$^+$ phage, as also observed by Kennel[27]. In fact, at late times the percentage of RNA synthesis attributable to the host apparently increases (see Table 2). Either *alc* is inefficient in its shutoff of host transcription or it is only responsible for shutting off a subset of the host RNA synthesis. Accordingly, we are undertaking experiments to determine whether all types of host transcription are shut off equally by *alc*.

In conclusion, we have presented evidence that an RNA polymerase subunit can cause the host nucleoid to unfold. Since mutants which lack the function of this subunit allow increased transcription of the host genome after infection, perhaps the *alc* gene product prevents the synthesis of RNA which holds the nucleoid together. In this connection, it has been reported that treating cells with rifampin causes the unfolding of the nucleoid[26]. Other types of experiment also suggest that RNA is important in maintaining the highly folded state of the host nucleoid since RNase treatment can cause it to unfold[20]. According to one model, then, the unfolding of the host chromosome is merely a consequence of shutting off host transcription by *alc*. However, another possibility should be considered: the unfolding of the host chromosome by *alc* may cause host transcription to cease. If so, perhaps T4 does not make RNA on cytosine-containing T4 DNA because *alc* causes it to "unfold itself". It should be possible to design experiments to test these, and other, hypotheses. Because of this, the T4 *alc* gene product promises to be an important tool in the study of the modes and mechanism of prokaryotic transcription. It also promises to be useful in the study of the roles of RNA and RNA polymerase in maintaining the structure of the prokaryotic chromosome.

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APPENDIX II

Fine structure map of pseT and its position relative to other nearby genes

The following work was performed by Wendy Cooley, Karl Sirotkin and Larry R. Snyder.

APPENDIX II

Fine structure map of pseT and its position relative to other nearby genes.

To orient the pseT gene relative to other genes in this region and to locate mutations within the pseT gene, we have performed three factor crosses. We constructed double mutants consisting of tsA56 (gene 31) and some pseT$^-$ mutations. These double mutants were crossed against other pseT$^-$ mutants and pseT$^+$ recombinants were tested for temperature sensitivity. We also tried to determine the endpoints of pseTΔ1 and pseTΔ3 by crossing them against other pseT$^-$ mutants, but both deletions fail to recombine with all of those that we used. The relative position of another gene in this region, alc (-unf?) (Snustad et al., 1976; Sirotkin et al., 1977), was determined by crossing multiple mutants containing pseT$^-$ mutations with or without the alc$^-$ mutation in the necessary genetic background to test for the alc$^-$ phenotype (Snyder et al., 1976), selecting pseT$^+$ recombinants, and scoring for the alc$^-$ phenotype. The map positions are shown in figure 1; the amino and carboxyl termini orientations are included, assuming these genes are transcribed with the same orientation as other early genes. It is of interest that pseT1, which only fails to induce 3' phosphatase activity (table 2) maps close to pseT47 which by a preliminary datum induces 3' phosphatase activity but which certainly fails to induce 5' polynucleotide

kinase activity (data not shown). This suggests that the active centers for these activities are not located on opposite ends of the polypeptide chain.

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Figure 1

Fine structure map of pseT and its position with respect to other nearby genes. All distances are arbitrary. The map orders were obtained with "three factor crosses" using tsA56 (gene 31). PseT12 could not be mapped because it plates on E. coli CTr5x at low temperature. We do not know the endpoints of pseTΔ1 and pseTΔ3; this is represented by dotted lines.

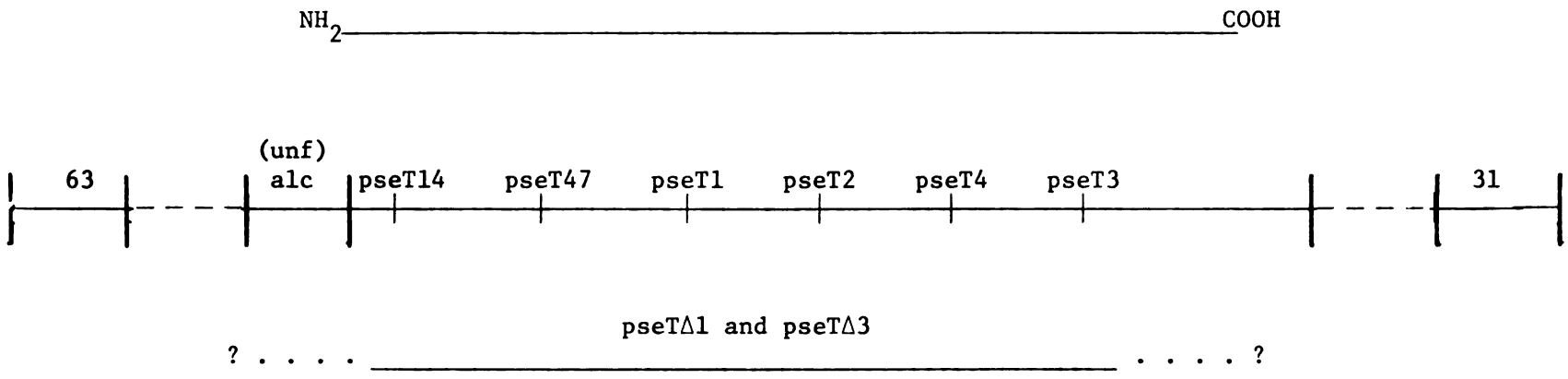
NH2
COOH
63
(unf)
alc
pseT14
pseT47
pseT1
pseT2
pseT4
pseT3
31
pseTΔ1 and pseTΔ3
? . . . .
. . . . ?

Figure 1

APPENDIX III

Isolation of a *E. coli* K12 mutant selectively nonpermissive for some *pseT*⁻ T4

Work performed by K. Sirotkin and L. Snyder during typing of this thesis.

APPENDIX III

Isolation of an E. coli K12 mutant selectively nonpermissive for some pseT⁻ T4

A mutant of E. coli AB2495, a multiple auxotrophic female, isolated after NTG mutagenesis plates $T4^+$ at 37°C with an efficiency of about 10% and pseTΔ1 (see article 1) with an efficiency of about 0.1%. This E. coli mutant was selected as a "nibbled" colony in the presence of pseTΔ1. We estimate its frequency, after mutagenesis, at $5 \times 10^{-5 \pm 1}$. The growth of some $pseT^-$ T4, such as pseT2, is not well supported by this strain, but the growth of, for example, pseT12 is about the same as $T4^+$. This difference between plating efficiencies might arise from differences in the degree of inactivation of the pseT gene product. However, very recent evidence shows that some other mutants also do not plate on this strain. Experiments are in progress to determine if the mutation causing the low plating efficiencies maps near pseT.

It is also interesting to note that this E. coli strain plates pseT2 normally at 42°C and is totally nonpermissive for even $T4^+$ at 27°C. We do not yet know if the reason this mutant does not support $T4^+$ at 27°C is the same as the reason it is less permissive for some $pseT^-$ T4 at 37°C.