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THE TRANSCRIPTION OF CYTOSINE T_4 DNA _IN VITRO_ AND THE EFFECTS OF THE T_4 _ALC_ GENE ON TRANSCRIPTION

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ABSTRACT

THE TRANSCRIPTION OF CYTOSINE T_4 DNA *IN VITRO* AND THE EFFECTS OF THE T_4 *ALC* GENE ON TRANSCRIPTION

By

Robert Edwin Pearson

The bacteriophage T_4 normally synthesizes DNA with 5-hydroxymethylcytosine in place of cytosine. When T_4 replicates with cytosine no phage are produced because no T_4 true late mRNA is synthesized demonstrating that 5-hydroxymethylcytosine is required for normal T_4 true late transcription. Recently, T_4 mutants, termed *alc* mutants, were isolated because they allow T_4 to grow with cytosine containing DNA. *Alc* mutants permit cytosine T_4 development by allowing almost normal T_4 true late transcription from cytosine T_4 DNA. Experiments were performed to investigate transcription from cytosine T_4 DNA *in vitro* and the *alc* gene product *in vivo*.

The first article describes studies of transcription from cytosine T_4 DNA, 5-hydroxymethylcytosine T_4 DNA, and calf thymus DNA *in vitro*. Experiments were performed to determine if T_4 induced modifications of the host RNA polymerase prevent transcription from cytosine T_4 DNA. The results demonstrate that the T_4 modified RNA polymerase transcribes cytosine T_4 DNA *in vitro*. Thus, the T_4

induced modifications did not prevent transcription from cytosine T_4 DNA. In addition, the T_4 modified RNA polymerase synthesizes more RNA from cytosine T_4 DNA or calf thymus DNA suggesting that hydroxymethylcytosine impedes transcription.

The second article describes studies of the *in vivo* affects of the *alc* gene product on the bacteriophage Lambda. It was found that the *alc* gene product shut off late Lambda transcription but did not affect the supercoiling of intracellular Lambda DNA. Therefore, the *alc* gene product does not act by directly removing supercoils from DNA. Furthermore, the results demonstrate that normal molecular weight Lambda mRNA is synthesized after T_4 superinfection, but not translated. Thus, a T_4 mechanism blocks translation of non-T_4 mRNAs.

DEDICATION

This project is dedicated
to my mother and father
who have supported me in many ways
throughout my life.

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INTRODUCTION AND LITERATURE REVIEW

The regulation of gene expression is central to many complex cellular processes including cell division and cell differentiation. An approach to understanding such complex phenomena is to first investigate a simple model. The model selected for analysis was the development of the bacteriophage T_4 because it is genetically and biochemically more pliable than other systems. It is hoped that a better understanding of T_4 development will provide insight to other complex biological processes.

T_4 is a large virulent bacteriophage of E. coli with enough DNA to code for approximately 200 genes of which around 60% have been identified. A number of T_4 genes are directly involved in regulating transcription during infection. Recently, a T_4 mutant termed alc has been shown to regulate both host and T_4 transcription from cytosine T_4 DNA. This gene is also involved in unfolding the nucleoid and may be associated with an alteration of the host RNA polymerase. Clearly, a better understanding of the T_4 alc gene would provide useful information about the regulation of transcription.

The following discussion will be separated into three major sections. The first section reviews literature pertinent to the alc gene and the bacteriophage Lambda which was used to investigate the alc gene. The second section describes experiments which show that T_4 induced RNA polymerase modifications do not prevent transcription from cytosine T_4 DNA. Also, the experiments suggest that 5-hydroxymethylycytosine directly impedes transcription from T_4 DNA. The final section will examine the affects of the T_4 alc gene product on the development of the bacteriophage Lambda. These experiments demonstrate that the alc gene product shut off Lambda late period transcription, but did not affect the supercoiling of intracellular Lambda DNA. Therefore, the alc product does not block transcription by directly removing the supercoils from DNA. In addition, other experiments show that normal length Lambda mRNAs are made during an alc mutant superinfection, but not translated. Thus, a T_4 mechanism exists which prevents translation of non-T_4 mRNAs.

The Bacteriophage T_4

The T_4 Genome

The T_4 genome contains about 170 kilobases and has a molecular weight of approximately 120 x 10^6 daltons. T_4 DNA is terminally redundant with approximately two percent of the total DNA repeated at both termini of the molecule (Kim and Davidson, 1974; Thomas and Rubenstein, 1964). In a population of molecules, the terminally redundant sequences are not identical, but consist of a permuted collection of all T_4 sequences (Streisinger et al., 1967; Thomas and Rubenstein, 1964). Therefore, some genes may be adjacent to each other on some molecules and located at the antipoles on other molecules. Consequences of the circularly permuted terminal redundancies include a circular genetic map and intramolecular heterzygotes.

T_4 DNA is unusual because it contains the base 5-hydroxymethylcytosine in place of cytosine (Wyatt and Cohen, 1953). This base protects T_4 DNA from at least one host restriction system (Snyder et al., 1976) and more than likely protects T_4 from other restriction systems as well. In addition to this function, hydroxymethylcytosine is involved in T_4 gene expression because it is required for normal T_4 true late gene expression (Kutter et al., 1975; Wu and Geiduschek, 1975). Another effect, as shown by experiments to be presented below, is an alteration of the template activity of phage DNA in vitro.

T_4 DNA contains glucose as well as hydroxymethylcytosine. Glucose is attached to hydroxymethylcytosine through either an α or β linkage (Lehman and Pratt, 1960). Glucosylation protects T_4 DNA from several host-mediated restriction systems suggesting that it is involved in extending the host range of T_4 (Revel, 1967; Revel and Georgopoulos, 1969) and it effects T_4 gene expression on host strains with the rifR2 mutation (Snyder, 1972; Snyder and Montgomery, 1974). The rifR2 mutation, a mutation that confers resistance to the antibiotic rifampicin, causes an alteration in the β subunit of the host RNA polymerase. In addition, glucosylation may alter phage transcription directly, since it effects the template activity of T_4 DNA in vitro (Cox and Conway, 1973).

T_4 Transcription

The T_4 genome can be separated genetically and biochemically into four transcriptional segments (Wood, 1974). Two segments are transcribed before replication and two after. Transcription of these segments depends on the host RNA polymerase which is modified during development. For the purposes of this discussion, T_4 RNA will be separated into early and late RNA. The early RNAs are synthesized before DNA replication and code for products involved in T_4 DNA replication, T_4 DNA recombination, T_4 host shutoff functions, and T_4 late gene expression. The late RNAs are synthesized after the onset of DNA replication and they code for functions involved in maturation of DNA and synthesis of the virion.

T_4 Induced RNA Polymerase Modifications.--T_4 uses the host RNA polymerase for its transcriptional program (di Mauro et al., 1969; Haselkorn et al., 1969). During the course of infection, the phage induces several modifications of the host RNA polymerase. These modifications include adenylation of the RNA polymerase and addition of T_4 induced polypeptides to the RNA polymerase.

Two separate adenylation reactions, termed "alteration" and "modification," are induced during infection. Both reactions result in the covalent addition of adenosine-phosphate moeities to subunits of the host RNA polymerase (Goff, 1974; Seifert et al., 1971). In the absence of protein synthesis, alteration very rapidly causes adenylation of subunits of the host RNA polymerase (Horvitz, 1974a; Seifert et al., 1969) in a reaction requiring the alt gene product

(Horvitz, 1974b). This product has been found to be part of the phage particle (Rohrer et al., 1975), so it is probably injected into the host along with T_4 DNA. The second adenylation reaction, termed "modification", occurs approximately three minutes after infection (Horvitz, 1974a; Seifert et al., 1969). Modification requires the *mod* gene product and results in further adenylation of the host RNA polymerase (Goff, 1974). *In vivo*, neither alteration nor modification is essential for T_4 development (Horvitz, 1974b). However, *in vitro* experiments suggest that either alteration or modification might be involved in the shutoff of some host transcription. For example, Mailhammer et al. (1975) found that adenylation of the polymerase reduces the transcriptional activity of the RNA polymerase. They proposed that the reduction in activity was sufficient to account for the shutoff of host transcription. Whether either the *alt* or *mod* gene product is involved in the shutoff of host transcription *in vivo* is not known.

A number of T_4 induced polypeptides bind to the RNA polymerase of the host. As an example, Ratner (1974a) found that several T_4 induced polypeptides bound to RNA polymerase immobilized on a column. Among these polypeptides were the gene 45, 33, and 55 products, all of which are required for T_4 late gene expression. Employing more stringent purification criteria, Stevens (1972) reports that four T_4 induced polypeptides bind tightly enough to copurify with the RNA polymerase. These polypeptides have been numbered 1, 2, 3 and 4 in the order of decreasing molecular weight. Of these polypeptides, polypeptides number 1 and number 3 have been identified as the products of genes 55 and 33 respectively (Horvitz, 1973; Ratner, 1974; Stevens, 1972). Both of these products are required for T_4 true late expression, so, presumably, they alter the specificity of the host RNA polymerase. Recently, polypeptide number 2 of Stevens (1972) has been found to be associated with the T_4 alc gene. The alc gene of T_4 is involved in shutting off transcription from host DNA and T_4 cytosine DNA. Sirotkin et al. (1977) have reported that polypeptide number 2 was missing from the RNA polymerase of cells infected with an alc mutant. However, the relationship of the alc gene with polypeptide number two is not known. At present, polypeptide number 4 of Stevens (1972) has not been associated with any T_4 gene.

Regulation of T_4 Early Transcription.--The early transcripts of T_4 are synthesized asymmetrically from T_4 DNA originating predominantly from the l stand of DNA (Guda et al., 1971; Notani, 1973). Based on the kinetics of transcription, the early transcripts have been separated into immediate early, delayed early, and quasi-late transcripts (Salser et al., 1970).

Immediate early RNA is synthesized at the beginning of infection and its synthesis continues until it is shut off during T_4 DNA replication (O'Farrell et al., 1973; Salser et al., 1970). Immediate early transcription proceeds in the absence of T_4 protein synthesis. Thus, it is resistant to chloramphenicol added at the time of infection (Lemback and Buchanan, 1970; Petterson et al., 1972; Salser et al., 1970). Furthermore, immediate early transcription is resistant to the antibiotic rifampicin added one minute after infection (O'Farrell and Gold, 1973a). O'Farrell and Gold (1973a) have concluded that the immediate early promoters are recognized almost immediately after infection. In support of these observations are reports that transcription of the immediate early T_4 genes *in vitro* resembles immediate early transcription *in vivo*. Both asymmetric transcription of the immediate early genes and resistance to the antibiotic rifampicin added after the start of transcription can be demonstrated *in vitro* (Brody and Geiduschek, 1970; Black and Gold, 1971; O'Farrell and Gold, 1973b; Milanesi et al., 1969).

The delayed early transcripts first appear 1.5 minutes after infection at 30°C (Salser et al., 1970) and are separable into two classes. One class is like the immediate early transcripts because it is shut off during phage replication, while the other class of delayed early transcripts is different because it is synthesized throughout infection (Salser et al., 1970; O'Farrell et al., 1973). Transcription of both classes of genes is blocked by inhibitors of protein synthesis, such as puromycin and chloramphenicol, added at the time of infection (Grasso and Buchanan, 1969; Lembach and Buchanan, 1970; Petterson et al., 1972; Salser et al., 1970). In addition, prior amino acid and potassium starvation block delayed early transcription (Boros and Witmer, 1975; Petterson et al., 1972). One interpretation of these observations is that delayed early transcription requires a T_4 gene product. However, other experiments suggest that at least some delayed early transcription is independent of T_4 protein synthesis. For example, both Black and Gold (1971) and Lemback and Buchanan (1970) report that some delayed early transcripts are synthesized in the presence of amino acid analogues which presumably cause protein dysfunction. As an alternative to a T_4 gene product being required for delayed early transcription, Black and Gold (1971) proposed that the inhibition of protein synthesis causes premature termination of T_4 transcription. The precedent for this is a report that chloramphenicol induces premature termination of transcription of the tryptophan operon (Morse, 1971). Whether chloramphenicol, puromycin, potassium starvation, and amino acid starvation cause premature termination of T_4 transcription has not been directly tested. Transcriptional termination aside, several reports suggest that some delayed early transcripts are synthesized as part of large polycistronic RNA molecules containing immediate early sequences. For example, Black and Gold (1971), Brody and Geiduschek (1970), and Milanesi et al. (1969) report that some delayed early transcripts contain immediate early sequences. In accord with these experiments, Sauerbier et al. (1970) report that the ultraviolet light target size of the delayed early genes was greater than might be expected if the promoters for the delayed early transcripts were located close to the delayed early genes. Furthermore, O'Farrell and Gold (1973a) found that the promoters for some delayed early RNAs were recognized almost immediately after infection and Milanesi et al. (1970) and Black and Gold (1971) found that the promoters for some delayed early RNAs could be physically separated from the delayed early genes by shearing. In summary, a number of experiments suggest that a subclass of delayed early genes are regulated like the immediate early genes and are more than likely transcribed by extension of the immediate early RNAs. The other subclass of delayed early genes is apparently transcribed independently of the immediate early genes suggesting that it has a different regulatory control.

The third class of early RNA, the quasi-late RNA, first appears in low amounts after 1.5 minutes of infection at 30°C. Like some delayed early transcripts, this class is synthesized throughout infection (O'Farrell and Gold, 1973a) making it kinetically analogous to a subclass of delayed early RNAs. The distinctive feature of quasi-late transcription is its sensitivity to the antibiotic rifampicin added one minute after infection. By following the synthesis of T_4 gene products with polyacrylamide gel electrophoresis, O'Farrell and Gold (1973a) observed that the quasi-late gene products failed to appear when rifampicin was added one minute after infection at 30°C. In contrast, the immediate early and some delayed early gene products were refractive to the same antibiotic treatment. They concluded that the promoters for the immediate early transcripts and for some delayed early transcripts were used almost immediately after infection, while the quasi-late promoters were used after one minute of infection. In addition, they proposed that the quasi-late promoters were made accessible or opened by transcription of the immediate early and certain delayed early genes.

In the past, several limitations have restricted the investigation of T_4 early transcription. One limitation has been the lack of a suitable *in vitro* system for classes of some early transcription. For example, only recently has a suitable system been developed for quasi-late transcription *in vitro* (Thermes et al., 1976). A second limitation has been the dearth of genes which have been shown to alter early transcription. Only one gene, termed *mot*, has been shown to reduce delayed early transcription (Mattson et al., 1974). Clearly, the discovery of additional mutations affecting early transcription and more useful *in vitro* systems would be helpful in understanding the regulation of early transcription.

Regulation of T_4 Late Transcription.--Late T_4 RNA is synthesized after the start of DNA replication. This RNA synthesis begins approximately seven minutes after infection at 30°C and new RNA sequences appear that are not synthesized early. Unlike the early transcripts, the late transcripts originate predominantly from the *r* strand of DNA (Guda et al., 1971; Notani, 1973). These molecules can be separated into quasi-late RNA, anti-late RNA, and true late RNA. A discussion of quasi-late RNA has been presented above.

Anti-late RNA first appears approximately two minutes after infection at 30°C and is synthesized from the *l* strand of DNA making it complementary to the true late RNA (Notani, 1973). As yet, the function of anti-late RNA is unknown, but its regulation is similar to those delayed early transcripts which are analogous to the quasi-late transcripts. For example, Frederick and Snyder (1977) report that anti-late transcription was blocked by rifampicin added one minute after infection and it was altered by a mutant in the mot gene.

Normal true late transcription requires continuous T_4 DNA replication, a number of T_4 gene products, and the unusual base hydroxymethylcytosine. The dependence of true late transcription on continuous replication has been investigated with temperature sensitive phage DNA polymerase mutations (Riva et al., 1970a). When shifted to a non-permissive temperature, both DNA replication and true late transcription abruptly stop suggesting that T_4 true late transcription was coupled to T_4 DNA replication. A number of years ago, Riva et al. (1970b) and Cascino et al. (1970, 1971) found that true late transcription could be uncoupled from viral DNA replication and reported that uncoupling requires a T_4 DNA ligase mutation and a mutation in a T_4 induced exonuclease. Since these mutations might be expected to stabilize gaps, they proposed that the template involved in T_4 late transcription contained gaps. Furthermore, they proposed that replication was required to continuously synthesize the gapped template. Very recently, Sirotkin et al. (1978) reported that the T_4 induced 3' phosphatase-5' polynucleotide kinase, an enzyme that might be expected to act at gaps, was required for true late gene expression on certain E. coli strains, again, supporting the role of gaps in T_4 true late transcription. Besides a competent template, a number of T_4 gene products are essential for T_4 true late transcription. One essential gene product is the gene 45 product (Epstein et al., 1963; Bolle et al., 1968). This gene product binds to the host RNA polymerase (Ratner, 1974) and is continuously required for both T_4 DNA replication and true late transcription (Wu and Geidushek, 1975) suggesting that some of the replication machinery is involved in T_4 true late transcription. Besides the gene 45 product, the gene 33 and gene 55 products, although not required for DNA replication, are required for true late transcription (Bolle et al., 1968; Epstein et al., 1963; Pulitzer and Geidushek, 1970). Both of these gene products bind tightly to the host RNA polymerase (Horvitz, 1973; Stevens, 1972) and they may alter the specificity of the host RNA polymerase. Regardless of their function, normal true late transcription requires the unusual base 5-hydroxymethylcytosine (Kutter et al., 1975; Wu and Geidushek, 1975). The base is not required for normal length viral replication (Kutter et al., 1975), but it has a direct role or roles in T_4 true late transcription. One role is protective because it blocks the T_4 alc gene mediated shutoff of transcription from cytosine T_4 DNA (Snyder et al., 1976). Also, some hydroxymethylcytosine may be directly required for T_4 transcription (Morton et al., 1978).

Perhaps the greatest limitation to the investigation of T_4 late transcription has been the development of suitable in vitro systems. Currently, only crude in vitro systems are available for examining late transcription (Snyder and Geiduschek, 1968; Rabussary and Geiduschek, 1979) As a consequence, the template necessary for true late transcription, the activities of the T_4 gene products, and the function of hydroxymethylcytosine all remain ill defined.

Synthesis of T_4 With Cytosine T_4 DNA.--T_4 synthesizes DNA containing 5-hydroxymethylcytosine. The primary mechanism excluding cytosine from T_4 DNA involves a viral induced dCTPase. The enzyme is responsible for degrading both dCTP and dCDP to dCMP (Warner et al., 1966) and is coded for by the T_4 gene 56 (Wiberg, 1967). Because of this activity, dCTP is effectively removed from the nucleotide triphosphate pool used for DNA replication. With a mutation in gene 56, some cytosine will be incorporated into T_4 DNA. However, most, if not all, cytosine T_4 DNA is degraded by T_4 induced nucleases involved in degrading host DNA (Kutter and Wiberg, 1968). These nucleases consist of two phage encoded endonucleases that fragment cytosine DNA and an exonuclease that degrades the fragments (Kutter et al., 1975; Kutter and Wiberg, 1969; Wu and Geiduschek, 1975). With mutations in the genes responsible for these nucleases, T_4 will synthesize cytosine containing T_4 DNA (Kutter et al., 1975; Kutter and Wiberg, 1968; Wu and Geiduschek, 1975), some of which is of normal length (Kutter et al., 1975). Even though apparently normal cytosine containing T_4 DNA is made, no phage are produced because no true late gene products are synthesized (Kutter et al., 1975; Wu and Geiduschek, 1975).

When T_4 replicates with cytosine in its DNA, spontaneous mutations accumulate that allow T_4 to develop with cytosine T_4 DNA (Snyder et al., 1976; Takahashi et al., 1979; Wilson et al., 1977). These mutations, called alc mutations, allow T_4 development with cytosine DNA because they permit almost normal T_4 true late gene transcription from cytosine T_4 DNA (Snyder et al., 1976). Therefore, the normal alc gene product must block transcription from cytosine containing T_4 DNA. At present, it is not known if the alc gene product affects early T_4 transcription nor is its of action understood. In regards to the synthesis of T_4 with cytosine DNA, it has been reported that at least some hydroxymethylcytosine is required for T_4 development (Snyder et al., 1976; Morton et al., 1978) which may, or may not, be significant.

T_4 Induced Alterations of Host Nucleic Acid Metabolism

During the course of infection, a number of gene products are induced that alter host nucleic acid metabolism. Examples of these alterations include disruption of the host nucleus, degradation of the host nucleus, unfolding of the host nucleoid, and shut off of host protein synthesis. Several of the T_4 gene products responsible for altering the host nucleic acid metabolism are related to the alc gene product. Therefore, it is pertinent to review some relevant T_4 gene products.

Nuclear Disruption.--In the uninfected host, the DNA, or chromosome, is located in the middle of the cell. After five minutes of T_4 infection, the chromosome position has changed from its central location to a more peripheral position adjoining the cell wall (Luria and Human, 1950; Murray, 1950). This T_4 induced phenomena, termed "nuclear disruption," occurs in the absence of the phage nucleases that degrade host DNA, but requires viral gene expression (Snustad et al., 1972).

Several years ago, Snustad et al. (1974a) isolated mutants which fail to disrupt the nucleus. These mutations, termed *ndd*$^-$ mutants, fail to induce the change in chromosome position (Snustad et al., 1974b). *Ndd*$^-$ mutants have two additional phenotypes which may be related to the failure to disrupt the nucleus. One phenotype is the failure of *ndd*$^-$ mutants to shut off host DNA replication normally and the second phenotype is enhancement of T_4 induced host degradation (Snustad et al., 1976a). As yet, it is not known whether the *ndd* gene product affects either cytosine T_4 DNA replication or T_4 gene expression from cytosine containing DNA. However, it should be pointed out that *ndd*$^+$ T_4 synthesize normal length cytosine T_4 DNA (Kutter et al., 1975). Thus, the gene product does not completely block phage cytosine replication, but it might affect the kinetics of replication.

Degradation of Host DNA.--T_4 degrades the DNA of the host to mononucleotides which are re-utualized for phage DNA replication. This process is not essential because mononucleotides are synthesized *de novo* after infection. Host DNA degradation is a two step process. During the first step, phage coded endonucleases degrade host DNA into fragments, while during the second step a phage coded exonuclease degrades the fragments to mononucleotides. As mentioned above, the nucleases involved in the breakdown of host DNA are also involved in the breakdown of cytosine T_4 DNA.

Two different T_4 induced endonucleases are involved in fragmenting host DNA. One endonuclease, termed endonuclease II, is the product of the *den A* gene (Hercules et al., 1971; Warner et al., 1970) and it has a major role in the breakdown of host DNA, while playing only a minor role in the breakdown of cytosine T_4 DNA (Hercules et al., 1971; Kutter et al., 1975; Warner et al., 1970). As characterized *in vitro* by Sadowski and Hurwitz (1969), endonuclease II breaks native double stranded and single stranded *E. coli* and Lambda DNAs into fragments with 3' OH and 5' PO_4 termini. The second endonuclease involved in the breakdown of host DNA, termed endonuclease IV, requires the *denB* gene product (Bruner et al., 1972; Sadowski and Vetter, 1973) and has only a minor role in the breakdown of host DNA, except when the infecting phage also has an *ndd*$^-$ mutation. However, in contrast to endonuclease II, endonuclease IV plays a major role in the breakdown of cytosine T_4 DNA (Kutter et al., 1975; Wu and Geiduschek, 1975). *In vitro*, the nuclease breaks native double stranded and single strand *E. coli* and Lambda DNAs leaving fragments with 3' OH and 5" PO_4 termini and it is ten times more active with single stranded gaps suggesting that it acts at gaps (Sadowski and Hurwitz, 1969b).

Exonuclease degradation of fragments is the second step in the breakdown of host DNA. Exonuclease breakdown depends on the gene 46 and the gene 47 products because mutations in either gene 46 or gene 47 fail to solubilize host DNA (Kutter and Wiberg, 1968; Wiberg, 1967). Not only do these gene products degrade host DNA. but they affect T_4 DNA metabolism because mutations in either gene 46 or gene 47 cause premature termination of T_4 DNA replication (Epstein et al., 1963).

Unfolding the Host Nucleoid.--The chromosome of the uninfected cell can be isolated as a highly folded complex called the nucleoid (Stonington and Pettijohn, 1971). The isolated complex has dimensions resembling those of the chromosome in vivo (Hecht et al., 1975) and contains approximately 80% DNA by weight with nascent RNA and protein accounting for the remainder (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972). The DNA of the nucleoid is arranged in supercoiled domains which are rotationally independent of each other (Pettijohn and Hecht, 1972; Worcel and Burgi, 1972). As an aside, some observations suggest that host DNA may be supercoiled in vivo. Drlica and Snyder (1978) report that the drug coumermycin, a drug which inhibits the host DNA gyrase (Gellert et al., 1976a, 1976b), reduces the superhelical content of the DNA of the nucleoid suggesting that host DNA is supercoiled. In any case, the supercoiled domains of DNA are stabilized by a core which more than likely contains RNA. The RNA bound to the nucleoid is composed in part of nascent rRNA and mRNA, some of which be associated with the core (Hecht and Pettijohn, 1976). When the RNA of the nucleoid is either released or destroyed, the supercoiled domains are destroyed causing the nucleoid to become much less compact (Pettijohn and Hecht, 1973; Worcel and Burgi, 1972). One interpretation of these observations is that RNA stabilizes the supercoiled domains of the nucleoid. Other observations support this role for RNA in maintaining the nucleoid. For example, Dworsky and Schachter (1973) and Pettijohn and Hecht (1973) found that nucleoid is unfolded by rifampicin, again, suggesting that RNA has an important structural role. In contrast to RNA, the proteins of the nucleoid do not have an essential role in maintaining the supercoiled domains (Stonington and Pettijohn, 1971; Worcel and Burgi, 1971), although they may be involved in attaching the nucleoid to the cellular membrane (Portalier and Worcel, 1976).

After T_4 infection, the nucleoid becomes much less compact or "unfolded". As reported by Tutas et al. (1974), the sedimentation coefficient of the nucleoid decreased from greater than 1000s to less than 300s after T_4 infection. Unfolding requires neither the T_4 induced nuclear disruption nor the T_4 nucleases which degrade host DNA (Tutas et al., 1974). Recently, mutations in the alc gene have been shown to be defective in unfolding the nucleoid (Sirotkin et al., 1977). Another mutation, termed unf 39, also fails to unfold the nucleoid after infection (Snustad et al., 1976b). Alc mutations and unf 39 map in the same region and are allelic, so they are more than likely mutations in the same gene (Sirotkin et al., 1977); Tigges et al., 1977). Thus, the normal alc gene product is required for unfolding the host nucleoid, although the mechanism for unfolding the nucleoid is not known.

Shutting Off Host DNA Synthesis.--The bacteriophage T_4 shuts off host DNA replication within the first five minutes of infection at $30^{o}C$ (Duckworth, 1971; Nomura et al., 1966. Snustad et al (1976a) have shown that the ndd gene product is required for the shutoff of host DNA synthesis. It is unclear how the ndd gene product functions, nor is it clear whether the ndd gene product is the only phage product which shuts off host DNA synthesis. In fact, some evidence suggests that a second viral mechanism inhibits cytosine DNA replication. For example, Snustad et al. (1976a) report that host DNA replication is blocked at ten minutes during ndd^{-} mutant infection. This delayed shutoff may represent a second shut off mechanism. Additional evidence, which is presented below, supports this interpretation. Since the ndd function, and perhaps other functions, alters cytosine DNA metabolism, it might interact with cytosine T_4 DNA replication and it may effect gene expression from cytosine DNA.

Shutting Off Host RNA Synthesis.--Host RNA synthesis is blocked after T_4 protein synthesis (Rouviere et al., 1968; Nomura et al., 1966; Terzi, 1967). Transcription of soluble RNA, ribosomal RNA and messenger RNA is shut off (Nomura et al., 1962; Hayward and Green, 1965). As first reported by Sirotkin et al. (1977), the alc gene of T_4 is involved in the shutoff of some host transcription.

They observed that alc mutants were defective in shutting off at least some host transcription. Recently, a similar observation was reported by Tigges et al (1977). At present, the mechanism for the alc-mediated shutoff is not known, nor is it clear if the alc gene product blocks all host transcription or just some host transcription. Besides the alc-mediated shutoff, other T_4 functions may shut off host RNA synthesis because even during an alc mutant infection some host RNA synthesis is shut off (Sirotkin et al., 1977; Tigges et al., 1977). Further support for alternate T_4 RNA shutoff mechanisms from cytosine DNA will be presented below.

Shutting Off Host Protein Synthesis.--T_4 prevents the synthesis of host proteins after infection. Explanations for this shutoff include the possibilities that either the T_4 induced transcriptional shutoff prevents host protein synthesis or, alternatively, a T_4 coded function prevents translation of host mRNA. In support of the first explanation, Kaempfer and Magasanik (1967) report that after T_4 infection the decay rate of B-galactosidase was similar to the rate of decay of B-galactosidase after actinomycin D treatment. The authors conclude that the transcriptional shutoff by T_4 probably accounts for the shutoff of host protein synthesis. Others have concluded that a T_4 mechanism disrupts translation of host mRNA. For example, Rouviere et al. (1968) and Kennel (1970), from experiments similar to Kaempfer and Magasanik's, concluded that a T_4 mechanism disrupts translation of host mRNAs. Recently, Svenson and Karlstrom (1976), in experiments analogous to

Kaempfer and Magasanik's, concluded that a T_4 mechanism dependent on the multiplicity of infection disrupted host translation. In conjunction with these reports, Kennel (1970) showed that after T_4 infection the mRNA for B-galactosidase was present, but excluded from polysomes suggesting that a phage function blocks translation. Experiments that are presented below provide additional support for a phage induced translational shutoff function.

The Bacteriophage Lambda as a Model System for Investigating the *alc* Gene Product

The *alc* gene product of T_4 is interesting because it shuts off transcription from cytosine T_4 DNA as well as some host transcription. This the gene product is involved in unfolding the host nucleoid and may be associated with a T_4 coded RNA polymerase binding protein. One approach to investigating the *alc* gene product and its associated phenotypes is to investigate the effects of the *alc* gene product on a more pliable model. The model selected was the bacteriophage Lambda. This bacteriophage was chosen because a great deal of genetic and biochemical information is available and because Lambda DNA is smaller and more tractable biochemically than either T_4 DNA or *E. coli* DNA.

The bacteriophage Lambda is a temperate bacteriophage of *E. coli* with both a lysogenic life cycle and lytic life cycle. Only the lytic cycle will be reviewed because it more closely resembles the development of T_4. For the purposes of this discussion, the lytic cycle will be separated into early and late periods. The early period begins with the initiation of lytic development and terminates with the end of monomeric circle replication. This period includes the first fifteen minutes of lytic development at 37^oC. The late period extends from the early period to cellular lysis which begins approximately 45 minutes after the initiation of lytic development at 37^oC.

The Early Period of Lambda Development

During the course of the early period, Lambda DNA replicates as monomeric circles and RNA is synthesized from both strands of DNA by the host RNA polymerase (Takeda et al., 1969). The early period transcripts code for products that are responsible for Lambda mediated recombination, Lambda DNA synthesis, Lambda late gene expression and Lambda late gene products. Based on the kinetics of early transcription, early RNA can be separated into immediate early RNA, delayed early RNA, and late RNA.

Early Period Transcription.--Immediate early RNA is synthesized almost immediately following the initiation of lytic development. This RNA, originating from both strands of Lambda DNA, codes for the *N* and *cro* gene products (Kourilsky et al., 1968; Taylor et al., 1967). Immediate early RNA is controlled by two regions of Lambda DNA and Lambda gene products. One region of Lambda DNA is involved in regulating *r* strand transcription, while the other segment is involved in regulating *l* strand transcription. Both control segments have promoter and operator sites. Mutations in either promoter site cause a *cis*-dominant reduction in transcription from the control segments both *in vivo* and *in vitro* (Cohen and Hurwitz, 1968; Roberts, 1970; Taylor et al, 1967), while other mutations in either operator cause *cis*-dominant constitutive transcription from the control segments (Sakakibara et al., 1971, 1972). In the absence of protein synthesis, immediate early transcription is limited to regions of DNA coding for the *N* and *cro* gene products (Heineman and Speigelman, 1970; Kourilsky et al., 1970; Kumar et al., 1969). Very recently, Salstrom and Szybalski (1979) identified three sites on Lambda DNA involved in the termination of immediate early transcription. Besides the control segments, at least two Lambda gene products regulate immediate early transcription. Both the Lambda repressor protein and the *cro* gene product block immediate early transcription by acting at the operators (Sakakibara et al., 1971; Takeda et al., 1975). The repressor physically binds to the immediate early operators preventing transcription (Ptashne and Topkins, 1968) as does the *cro* gene product (Folkmanis et al., 1976; Takeda et al., 1977).

Delayed early RNA is synthesized from both strands of Lambda DNA beginning approximately two minutes after initiation of lytic development (Kourilsky et al., 1968; Taylor et al., 1967). These transcripts code for proteins that are involved in DNA replication, DNA recombination, and late gene expression. Delayed early transcription requires the Lambda *N* gene product. Also, several observations suggest that delayed early transcription is controlled directly by the immediate early control segments. These observations include the reduction of delayed early transcription caused by immediate early promoter mutations (Cohen and Hurwitz, 1969; Nijkamp et al., 1970; Taylor et al., 1967) and constitutive delayed early transcription caused by immediate early operator mutations (Sakakibara et al., 1971, 1972). Furthermore, delayed early transcription is blocked by the Lambda repressor (Thomas, 1970; Wu et al., 1972) and by the _cro_ gene product (Takeda et al., 1975). Besides being controlled by the immediate early control segments, the delayed early transcripts contain immediate early sequences (Portier et al., 1972). One interpretation of these observations is that delayed early RNAs are initiated at the immediate early promoters and synthesized as part of large polycistronic mRNAs. After a series of transcription experiments _in vitro_, Roberts (1969) proposed that the _N_ gene product was responsible for extending immediate early transcription into the delayed early genes. He also proposed that the _N_ gene product acts as an antagonist to _rho_ dependent transcription termination, thereby allowing extension of immediate early transcription. The _rho_ factor of the host is involved in some types of transcription termination (Roberts, 1970; Korn and Yanofsky, 1976). A number of reports support the anti-termination model for the gene product activity. For example, Korn and Yanofsky (1976) and Das et al. (1976) report that $\underline{N}^-$ mutants propagate lytically on _SuA_ mutant hosts. Host strains with _SuA_ mutations have altered _rho_ factors and are defective in termination of some transcription. Along with these observations, Adhya et al. (1974) report that the _N_ gene product suppresses a rho dependent termination signal in an insertion sequence. They conclude that the N gene product acts at the promoters because the N gene product suppressed rho dependent termination only when transcription was initiated from Lambda promoters and not when it was initiated from other promoters.

Lambda late RNA is synthesized beginning approximately 8 minutes after initiation of lytic development. Late RNA is synthesized predominantly from the r strand of DNA (Oda et al., 1969; Taylor et al., 1967) and codes for products involved in maturation of DNA and synthesis of the virion. Normal late transcription depends on a segment of Lambda DNA, the Q gene product, and DNA replication. The segment involved in late transcription is different than the immediate early control segments (Thomas, 1970). Herskowitz and Singer (1970) found that a deletion of the segment of DNA caused a cis-dominant reduction of late transcription. They termed the segment pR2 and concluded that it acts as a promoter for late transcription. Because deletion of pR2 pleiotropically reduces late transcription, they proposed that all of the late genes are transcribed as part of one large polycistronic mRNA. Supporting this hypothesis, are reports by Green (1970), who showed that an unusual amount of time was required for rifampicin inhibition of late transcription, and by Gariglio and Green (1973), who isolated an unuaually large polycistronic mRNA containing late RNA sequences. However, studies on the polarity of amber mutations suggest that there are multiple promoters for late transcription (Murialdo and

Siminovitch, 1972). Promoter of promoters aside, the Q gene product is required for normal late transcription because Q mutants synthesize reduced amounts of late RNA (Dove, 1966; Eisen et al. 1966; Joyner et al., 1966; Oda et al., 1969). Explanations for the failure of Q^- mutants to completely block late transcription include the possibilities that either all Q mutants are leaky or, alternatively, there are Q independent pathways for late transcription. Some experiments by Herskowitz and Singer (1970), which show that Q independent transcription is regulated like delayed early transcription, support the second alternative. For the stimulatory effect of the Q gene product on late transcription, Roberts (1975) proposed that Q acts as an anti-terminator like the N gene product. However, other modes of action are also possible. In addition to the Q gene product and pR2, Lambda DNA replication is required for normal late transcription. Replication at least increases the number of genomes available for Lambda late transcription (Stevens et al., 1970). But, normal late transcription may depend on, or be coupled to, continuous DNA replication. Observations supporting this proposal include experiments by Takeda (1970) which showed that thymine deprivation blocked late transcription and experiments by Grzesiuk and Taylor (1977) which showed that late transcription was blocked in minicells. Whether Lambda phage replication coupled transcription is similar to T_4 phage replication coupled transcription is not known.

Early Lambda DNA Replication.--Upon initiation of lytic development, Lambda DNA first cyclizes (Ogawa and Tomizawa, 1968;

Young and Sinsheimer, 1968) and replicates as monomeric circles beginning approximately 8 minutes after initiation of lytic development at 37^{o}C (Carter et al., 1969; Ogawa and Tomizara, 1968; Young and Sinsheimer, 1968). Monomeric circle replication is initiated in the O gene of Lambda at a site on DNA termed ori (Furth et al., 1977) which can be mutated resulting in cis-dominant reduction of monomeric circle replication (Dove et al., 1971; Rambach, 1973). Recently, ori has been isolated and sequenced (Denniston-Thompson et al., 1977). It contains palindromic sequences that may form hairpin loops involved in the initiation of replication. From this site, most monomeric circular replication proceeds bidirectionally (Schnos and Inman, 1970). The replication machinery responsible for DNA replication is composed in part of both host and phage proteins (Fangman and Feiss, 1969; Georgopoulos and Herkowitz, 1971) and the entire complex of proteins and DNA is associated with the cellular membrane (Hallick et al., 1969; Nishimota and Matubara, 1972). The two Lambda gene products essential to monomeric circular replication are the O and P gene products (Eisen et al., 1966; Joyner et al., 1966). As yet, the role of both proteins is undefined, although they may be involved in nicking DNA (McMachen et al., 1975).

The Late Period of Lytic Development

The late period of lytic development consists of the maturation of Lambda DNA, synthesis of virus, and release of virus beginning around 45 minutes after initiation of lytic development.

During the late period, Lambda late transcription predominantes and Lambda DNA replicates as concatamers.

Late Period Transcription.--During the course of the late period, immediate early RNA, delayed early RNA and late RNA are all synthesized. But, in contrast to the early period, immediate early transcription and delayed early transcription are drastically reduced during the late period. The Lambda function responsible for this reduction is the cro gene product. The cro product reduced immediate early and delayed early transcription by binding to the operator sites of the immediate early control segments (Takeda, 1975; Folkmanis et al., 1976; Takeda et al., 1977). As a consequence, Lambda late RNA synthesized from the r strand predominants (Oda et al., 1969; Taylor et al., 1967).

Late Lambda Replication.--Late period Lambda DNA replicates as fast sedimenting concatameric DNA (Ogawa and Tomizawa, 1968; Smith and Skalka, 1966; Young and Sinsheimer, 1968). Concatameric molecules can be formed by either recombination or by DNA replication (Enquist and Skalka, 1973; Greenstein and Skalka, 1975). The structures contain a mixture of linear concatamers, linear concatamers with circular termini and concatameric circular molecules (Takahashi, 1974). To account for the variety of concatamers, it has been proposed that late period DNA replicates by a rolling circle mechanism, although other mechanisms are also feasible. Whatever the replication mechanism, the fast sedimenting DNA behaves as if it contains single stranded regions because the replicative intermediate is more sensitive to shearing than linear DNA and it binds to columns which retain gapped DNA (Kiger and Sinsheimer, 1969). Consistent with the presence of single stranded regions is the extreme sensitivity of the replicative intermediate to the *rec BC* nuclease *in vivo*. This nuclease must be inactivated by the Lambda *gam* gene product during the synthesis of concatameric DNA (Enquist and Skalka, 1973; Greenstein and Skalka, 1975). As yet, it has not been determined whether the single stranded regions have an essential role during development. Whatever their role, concatameric replication appears to be associated with the membrane (Hallick et al., 1959; Niskimato and Maturbara, 1972) and depends on a composite structure of host and Lambda proteins (Fangman and Feiss, 1969; Georgopoulos and Herskowitz, 1971. Part of the replicative complex contains the *O* and *P* gene products (Eisen et al., 1966; Joyner et al., 1966), although the *P* gene product is not essential for concatameric replication during the later stages of replication (Klinkert and Albercht, 1978).

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ARTICLE I

THE TRANSCRIPTION OF CYTOSINE T_4 DNA, 5-HYDROXYMETHYLCYTOSINE T_4 DNA, AND CALF THYMUS DNA *IN VITRO* BY T_4 MODIFIED RNA POLYMERASES

ABSTRACT

When T_4 replicates DNA using cytosine, no phage are produced because no T_4 true late mRNAs are synthesized from cytosine T_4 DNA. An explanation accounting for the dependence of T_4 true late gene expression on hydroxymethylcytosine is that hydroxymethylcytosine is required as a template for transcription after T_4 modification of the host RNA polymerase. To investigate this possibility, transcription from cytosine T_4 DNA was examined *in vitro*. The experiments demonstrated that the T_4 modified RNA polymerase transcribed cytosine T_4 DNA *in vitro*. Thus, hydroxymethylcytosine is not required in the template for transcription. In addition, we found that cytosine T_4 DNA is transcribed more efficiently than either hydroxymethylcytosine T_4 DNA or calf thymus DNAs.

INTRODUCTION

T_4 DNA contains the base 5-hydroxymethylcytosine in place of cytosine (Wyatt and Cohen, 1953). This base protects T_4 DNA from host restriction systems (Snyder et al., 1976) and is involved in normal T_4 true late gene expression because no T_4 true late gene products are synthesized when T_4 replicates DNA with cytosine (Kutter et al., 1975; Wu and Geiduschek, 1975). One possible explanation accounting for this observation is that hydroxymethylcytosine protects against a T_4 gene product which prevents transcription from cytosine DNA. Another possible explanation is that the base is required by the T_4 modified RNA polymerase for transcription of T_4 DNA.

T_4 uses (di Mauro et al., 1969; Haselkorn et al., 1969) and modifies the host RNA polymerase during infection. One form of modification is adenylation of the RNA polymerase and another form is addition of T_4 induced polypeptides to the host RNA polymerase. Adenylation occurs in two separate reactions which are dependent on the _alt_ and _mod_ gene products (Horvitz, 1975a). Although neither adenylation reaction is required for T_4 development (Horvitz, 1975b), adenylation of the polymerase reduces transcription from cytosine DNAs _in vitro_ (Mailhammer et al., 1975). Besides adenylation, four T_4 induced polypeptides bind tightly enough to copurify with the host RNA polymerase (Horvitz, 1973; Stevens, 1972). These polypeptides have been termed numbers 1, 2, 3 and 4 in the order of decreasing molecular weight (Stevens, 1972). Polypeptides #1 and #3 have been identified as the products of T_4 genes 55 and 33 (Horvitz, 1973; Stevens, 1972) and both are required for T_4 true late transcription (Bolle et al., 1968; Epstein et al., 1963). Presumably, both gene products alter the transcriptional characteristics of the host RNA polymerase enabling T_4 true late transcription. Recently, another RNA polymerase binding polypeptide has been associated with a T_4 gene named alc (Sirotkin et al., 1977).

T_4 alc mutants were isolated because they allow transcription from cytosine T_4 DNA and, therefore, they permit production T_4 phage containing cytosine. As might be expected, because the host has cytosine DNA, T_4 alc mutants permit some host transcription after infection (Sirotkin et al., 1977; Tigges et al., 1977). In addition, alc mutants fail to "unfold" the host "nucleoid" (Sirotkin et al., 1977). The term "nucleoid" refers to the isolated compact complex of host nucleic acids (Stonington and Pettijohn, 1971). After T_4 infection, the nucleoid normally becomes much less compact or "unfolded" (Tutus et al., 1974). Besides unfolding the nucleoid, the alc gene product may be related to an RNA polymerase binding polypeptide because the RNA polymerase of cells infected with an alc mutant lacks an RNA polymerase binding polypeptide (Sirotkin et al., 1977). This polypeptide corresponds to polypeptide #2 of Stevens (1972).

There are two interesting questions about transcription from cytosine T_4 DNA. The first is, does the T_4 modified host RNA polymerase transcribe cytosine T_4 DNA? The second is, do T_4 induced modifications affect transcription from cytosine T_4 DNA. One approach to answering these questions is to investigate the T_4 modified host RNA polymerase *in vitro*.

The following experiments show that the T_4 modified RNA polymerase transcribes cytosine T_4 DNA *in vitro*. Therefore, hydroxymethylcytosine is not required by the modified RNA polymerase transcription for transcription of cytosine T_4 DNA. In addition, the experiments show that more RNA is synthesized from cytosine T_4 DNA than from hydroxymethylcytosine T_4 DNA suggesting that hydroxymethycytosine directly impedes transcription.

MATERIALS AND METHODS

Bacteriophage and Bacteria.--Four different bacteriophage strains were employed in the following experiments. One strain was wild type T_4D and it was from a stock maintained in this laboratory. This second strain was an alc mutant isolated in this laboratory by K. Sirotkin. The RNA polymerase of cells infected with this mutant fails to have the T_4 induced RNA polymerase binding polypeptide #2 of Stevens (1972). The third strain was an alt^- mod^- mutant T_4. This mutant T_4 was from the stock of R. Horvitz and it fails to adenylate the host RNA polymerase. The final strain was a multiple mutant T_4 with gene 56^-, endo II^-, endo IV^- and alc mutations and was constructed by K. Sirotkin. When this strain propagates on appropriate host strains, it will develop with cytosine in its DNA.

The bacterial strains employed were E. coli B^e (Su^-) from the stock of L. Gold and E. coli B834 (Su^-, r^-m^-) from the stock of H. Revel.

Synthesis of Cytosine T_4 and 5-Hydroxymethylcytosine T_4.--For growing cytosine T_4, the T_4 strain with gene 56^-, endo II^-, endo IV^-, and alc mutations was propagated on B834 in liquid culture (Snyder et al., 1976). For growing hydroxymethylcytosine T_4, wild type T_4D was propagated on B834 in liquid culture.

Purification of Bacteriophage.--Both hydroxymethylcytosine T_4 and cytosine T_4 were purified by procedures outlined by Pearson (this dissertation).

Extraction of DNA.--DNA was extracted from purified bacteriophage with cold phosphate buffered phenol (Bolle et al., 1968) and the concentration determined spectrophotometrically.

Medium.--The medium used for growing cells was M9S. A recipe for M9S has been published by Snyder and Montgomery (1974).

Isolation of RNA polymerases from Uninfected and T_4 Infected Cells.--*E. coli* B^e was grown at 37°C in M9S supplemented with 10 ug./ml. tryptophan to a cell concentration of $4X10^8$ cells/ml. Two liters of cells were either mock infected or infected with T_4 (m.o.i. 8) for 15 min. at 37°C. Fewer than 1% of the cells survived the infection. The infected cells were concentrated, resuspended in 50 ml. of Buffer A, and sonicated. Buffer A is 0.01 M Tris 7.9, 0.01 M $MgCl_2$, 1mM EDTA, 0.1 mM dithiothreital, 5% glycerol. The method of Burgess (1969) was used to purify RNA polymerases. The procedure includes ammonium sulfate precipitation, DEAE cellulose chromotography, low salt glycerol gradient centrifugation and high salt glycerol gradient centrifugation. Peak fractions from the high salt glycerol gradients were dialyzed overnight against buffer A at 4°C. After dialysis, glycerol was added to 50% for storage.

In vitro Transcription Assay.--The RNA polymerases were assayed with the method of Burgess (1969). *In vitro* reactions were performed in 0.4 ml. solution (0.01 M Tris-Hcl pH 7.5, 0.01M MgCl) containing 0.125 mM CTP, 0.125 mM ATP, 0.125 mM GTP, 0.1 mM UTP, 10uC 5' -H^3 UTP, and 20 ug./ml. DNA. The reactions were performed at 37°C for 10 minutes. Following the incubation, the reactions were TCA precipitated, collected on filters and counted with toluene base scintilation fluid.

Chemicals.--CTP, ATP, GTP, and UTP were purchased from Sigma and 5' -H^3 UTP (1 mCi./0.025 mg) was purchased from Schwarz/Mann.

RESULTS

In Vitro Transcription of Cytosine T_4 DNA, 5-Hydroxymethylcytosine T_4 DNA, and Calf Thymus DNA by the Bacterial and T_4 Modified RNA Polymerases

As a control for the wild type T_4 modified RNA polymerase, we first decided to investigate the RNA polymerase of uninfected cells. The results of the experiment are presented in Figure 1A. It is clear from the data that the two types of T_4 DNAs resemble each other as templates for transcription. This was not a suprising result because both DNAs contained T_4 sequences. Also, the bacterial polymerase synthesizes less RNA from calf thymus DNA than from either of the T_4 DNAs. This result is in accord with those reported by others (Bautz and Dunn, 1969).

To examine the wild type T_4 modified RNA polymerase, the RNA polymerase was extracted from cells 15 minutes after phage infection. We choose this time because both adenylation of the polymerase (Horvitz, 1974a) and addition of the T_4 induced polypeptides to the RNA polymerase are completed during this period (Stevens, 1972). After purification, the enzyme was studied in an experiment analogous to that done with the uninfected RNA polymerase. The results of the experiment are presented in Figure 1B. The data demonstrate that unlike the uninfected polymerase, the wild type T_4 modified polymerase synthesizes more RNA from cytosine T_4 DNA than from either

Figure 1.--*In vitro* transcription of cytosine, hydroxymethylcytosine and calf thymus DNAs by uninfected RNA polymerase (A) and wild type T_4 modified RNA polymerase (B). *E. coli* B^e was grown and either mock infected (A) or infected with wild type T_4D (B) as described in the text. The RNA polymerases were extracted and assayed as described in an earlier section. Each assay contained either cytosine T_4 DNA (●———●), hydroxymethylcytosine T_4 DNA (o———o), of calf thymus DNA (△———△). Background counts following incubation on ice were 300 c.p.m. and 168 c.p.m. for uninfected and T_4 modified RNA polymerases respectively. Background counts after incubation without DNA were less than 98 c.p.m. In the figure, one relative unit of polymerase is equal to 10 ul. of enzyme solution added to the reaction.

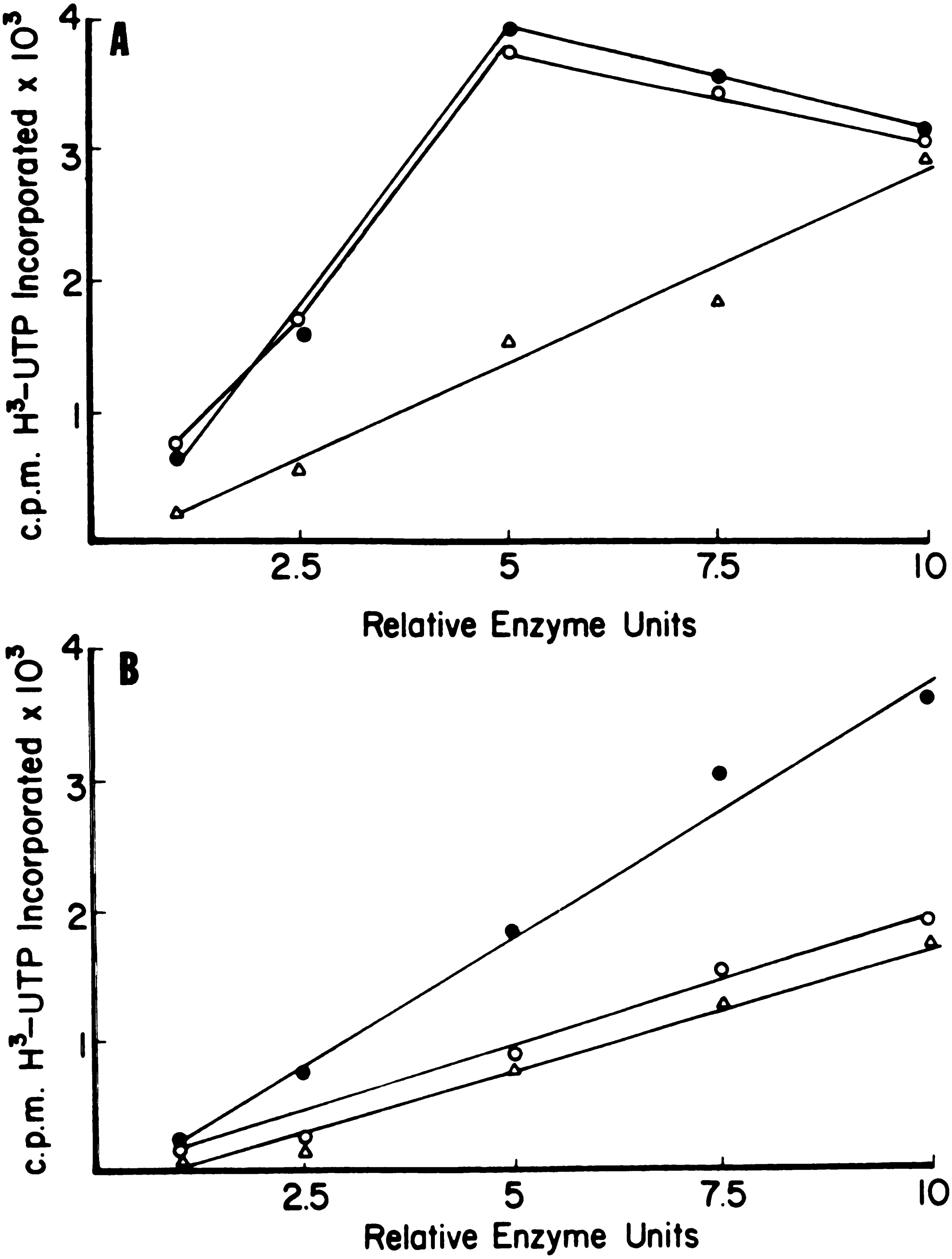
A
c.p.m. H^3–UTP Incorporated x 10^3
4
3
2
1
2.5
5
7.5
10
Relative Enzyme Units
B
c.p.m. H^3–UTP Incorporated x 10^3
4
3
2
1
2.5
5
7.5
10
Relative Enzyme Units

Figure 1

5-hydroxymethylcytosine T_4 DNA or calf thymus DNA. Therefore, 5-hydroxymethylcytosine is not directly required for transcription by the T_4 modified RNA polymerase. Indeed, the wild type T_4 modified RNA polymerase synthesizes less RNA from the T_4 DNAs than did the uninfected RNA polymerase. Some of this result was in part expected because others have reported similar observations about the activity of T_4 modified RNA polymerases on 5-hydroxymethylcytosine T_4 DNA and on calf thymus DNA (Bautz and Dunn, 1969). However, the activity of the modified RNA polymerase on cytosine T_4 DNA was not expected because the uninfected RNA polymerase synthesized similar amounts of RNA on the two T_4 DNAs.

In Vitro Transcription of Cytosine T_4 DNA, 5-Hydroxymethylcytosine T_4 DNA and Calf Thymus DNA by the T_4 *alt*⁻ *mod*⁻ Mutant RNA Polymerase and an *alc* Mutant RNA Polymerase

Explanations for the differences in activity of the T_4 modified RNA polymerase on the two types of T_4 DNAs include the possibility that either some of the T_4 induced modifications affect the activity of the modified RNA polymerase or, alternatively, hydroxymethylcytosine directly impedes transcription. These alternatives can be investigated *in vitro* by employing T_4 RNA polymerases that lack modifications. One T_4 employed for these experiments was an *alt*⁻ *mod*⁻ double mutant. This mutant T_4 fails to adenylate the host RNA polymerase during infection (Horvitz, 1974a). The second

T_4 used was an alc mutant. After infection with this alc mutant, the RNA polymerase extracted from the cells lacks polypeptide #2 of Stevens (Sirotkin et al., 1977).

Experiments analogous to that done with the wild type RNA polymerase were performed with these two T_4 mutant polymerases. The results of the experiment with the alt^- mod^- mutant RNA polymerase are presented in Figure 2A and the results of the experiment with the alc mutant RNA polymerase are presented in Figure 2B. The data demonstrate that both the alt^- mod^- mutant and the alc mutant RNA polymerases synthesize more RNA from cytosine T_4 DNA than from either hydroxymethylcytosine T_4 DNA or calf thymus DNA. Also, similar amounts of RNA are synthesized from both hydroxymethylcytosine T_4 DNA and calf thymus DNA by the T_4 mutant RNA polymerases. It is clear from the data, that neither adenylation of the RNA polymerase nor addition of the alc mutant associated RNA polymerase binding polypeptide are responsible for the activity differences of the wild type T_4 modified RNA polymerase on the two types of T_4 DNA.

Figure 2.--*In vitro* transcription of cytosine, hydroxymethylcytosine and calf thymus DNAs by *alt*$^-$ *mod*$^-$ mutant RNA polymerase (A) and *alc* mutant RNA polymerase (B). *E. coli* B^e was grown and either infected with *alt*$^-$ *mod*$^-$ mutant T_4 (A) or infected with *alc* mutant T_4 (B) as described in the text. The RNA polymerases were extracted and assayed as described earlier. Each assay contained either cytosine T_4 DNA (●———●), hydroxymethylcytosine T_4 DNA (○———○) or calf thymus DNA (△———△). Background counts following incubation on ice were less than 180 c.p.m. and background counts after incubation without DNA were less than 100 c.p.m. As in Figure 1, one relative unit of polymerase is equal to 10 ul. of polymerase solution added to the reaction.

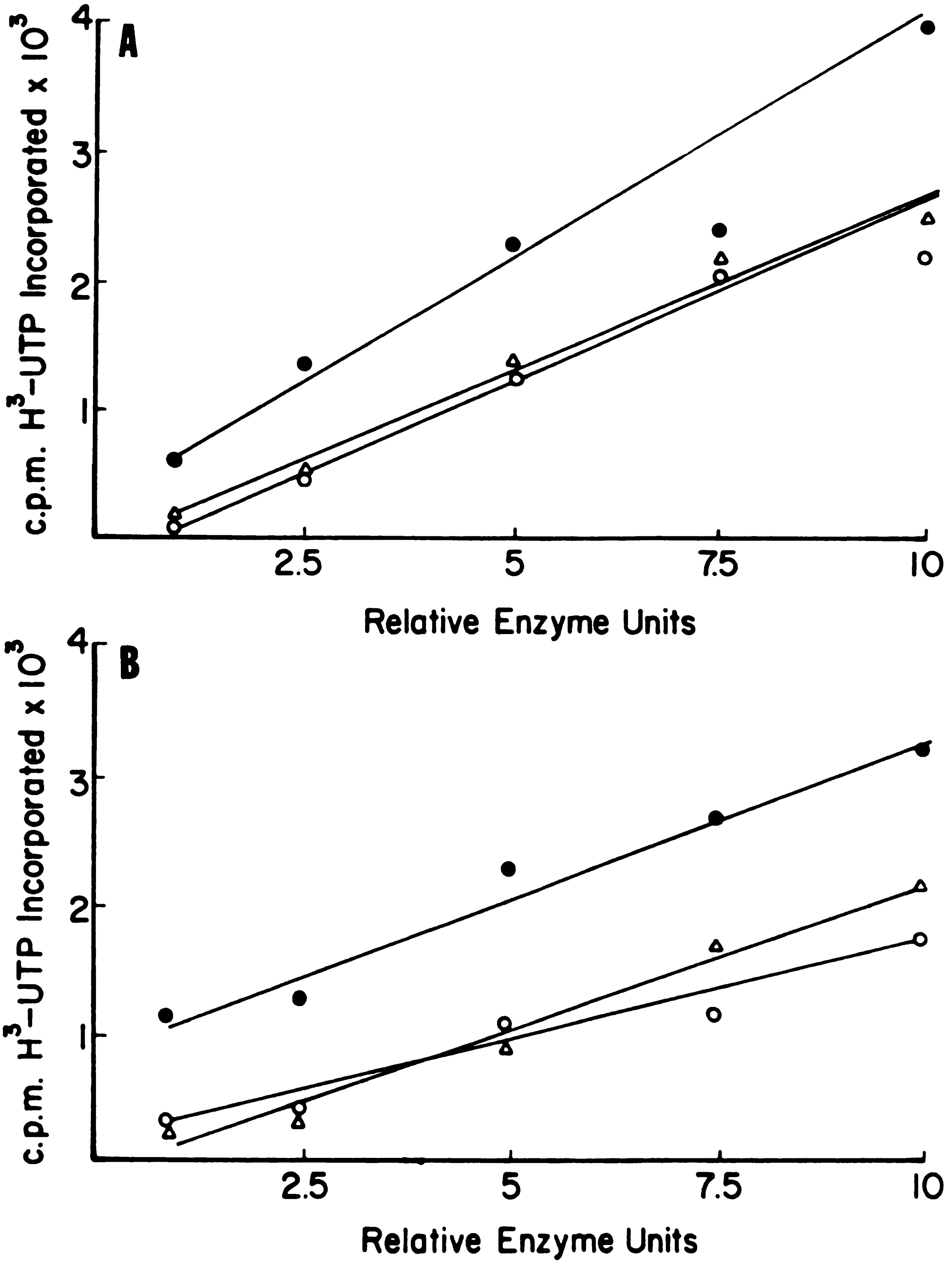
A
B
c.p.m. H^3-UTP Incorporated x 10^3
4
3
2
1
2.5
5
7.5
10
Relative Enzyme Units

Figure 2

DISCUSSION

The experiments reported here demonstrate that the T_4 modified RNA polymerase transcribes cytosine T_4 DNA. Thus, hydroxymethylcytosine is not a template requirement for cytosine T_4 DNA transcription by the T_4 modified RNA polymerase. As alternative explanations, either other undetected RNA polymerase modifications prevent transcription from cytosine T_4 DNA *in vivo* or hydroxymethylcytosine protects against functions, such as the *alc* gene product, that shut off cytosine DNA transcription. In addition, the experiments demonstrate that the T_4 modified RNA polymerase synthesizes more RNA from cytosine T_4 DNA than from hydroxymethylcytosine T_4 DNA. This property of the T_4 polymerase is probably not dependent on either adenylation of the RNA polymerase or addition of one of the T_4 coded RNA polymerase binding polypeptides. However, it should be pointed out that the RNA polymerases were not examined for adenylated subunits nor the addition of T_4 proteins to the polymerase. Nevertheless, the results suggest that either other undetected T_4 induced modifications are responsible for the differences in activities of the polymerases on the DNAs or hydroxymethylcytosine directly impedes transcription. Because hydroxymethylcytosine is normally glucosylated, either the base itself or the glucose residues could be responsible.

All of the T_4 modified RNA polymerases examined synthesized less RNA from the three types of DNAs than the unmodified RNA polymerase. This result might have been anticipated, because others have shown that the T_4 modified enzyme synthesizes less RNA from the three types of DNA than does the unmodified RNA enzyme (Bautz and Dunn, 1969). This loss of RNA polymerase synthetic activity after T_4 infection has been correlated with the loss of some sigma subunits from the RNA polymerases during purification. Presumably, the loss of some of the sigma subunits during purification of the T_4 modified RNA polymerases used in the experiments is responsible for the loss of activity of the T_4 modified RNA polymerases on cytosine, hydroxymethylcytosine T_4 DNA and calf thymus DNA reported here.

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THE SHUTOFF OF LAMBDA TRANSCRIPTION BY BACTERIOPHAGE T_4

ROLE OF THE T_4 ALC GENE

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ABSTRACT

Bacteriophage T_4 normally has 5-hydroxymethylcytosine instead of cytosine in its DNA. Mutants of T_4 which synthesize DNA with cytosine do not transcribe their late genes due to the function of the T_4 alc gene (Snyder et al., 1976) which is also responsible for shutting off at least some of the host transcription and for unfolding the host nucleoid after T_4 infection (Sirotkin et al., 1977; Tigges et al., 1977). In order to examine how alc works, we have studied the affects of the T_4 alc function on λ bacteriophage late transcription and on the structure of intracellular λ DNA. After T_4 superinfection, the T_4 alc function shuts off the late transcription of λ but does not affect the supercoiling of intracellular circular λ DNA. Therefore, alc does not unfold the nucleoid by directly removing supercoils from DNA. We also conclude from these experiments that T_4 infection alters the translational machinery in a way which causes almost 100% discrimination against non-T_4 messenger RNA. Those late λ mRNAs which are made after superinfection by a T_4 alc mutant are of normal length but are not translated into λ late proteins.

INTRODUCTION

The shutoff of host RNA synthesis by bacteriophage T_4 is an example of general genic control because many genes, regulated in many different ways, are affected. The molecular basis for the shutoff is not understood. Possible explanations include either altering the transcriptional specificity of the host RNA polymerase to prevent host transcription or altering the structure of the bacterial chromosome so it is no longer functional for transcription.

Recently, a T_4 gene involved in the shutoff of host transcription has been identified and named alc (Snyder et al., 1976). T_4 alc mutations were first isolated because they permit the synthesis of true late RNA from cytosine containing T_4 DNA (Snyder et al., 1976). Thus, they allow the production of T_4 with cytosine in place of the normal T_4 base hydroxymethylcytosine (Snyder et al., 1976; Takahashi et al., 1979; Wilson et al., 1977). In contrast to wild type T_4, T_4 alc mutants permit the synthesis of at least some host RNA after infection (Sirotkin et al., 1977; Tigges et al., 1977) which is perhaps expected, since the host DNA contains cytosine. Also, T_4 alc mutants often fail to "unfold" the host nucleoid after infection. To explain what is meant by unfolding the host nucleoid, from uninfected bacteria, the nucleoid of the host can be isolated as a folded complex of nucleic acids and proteins (Stonington and Pettijohn, 1971). The structure of the nucleic acids in the nucleoid consists of DNA arranged in supercoiled domains around a core which probably contains RNA (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972). Each supercoiled domain is stabilized by the core and is rotationally independent of every other supercoiled domain (Worcel and Burgi, 1972). After T_4 infection, the host nucleoid becomes much less compact or "unfolded" (Tutas et al., 1974). A T_4 mutation, unf 39, prevents the unfolding of the nucleoid (Snustad et al., 1976b) and is allelic with alc mutations (Sirotkin et al., 1977; Tigges et al., 1977). Therefore, the alc gene product, which is required for the shutoff of at least some host transcription, is required for the unfolding of the host nucleoid after infection. Considering the role of RNA in nucleoid structure and the possible dependence of some types of transcription on nucleoid structures, it seems likely that the above phenotypes are causally related.

There are two questions we would like to answer concerning the affects of the alc gene product on transcription and nucleoid structure. The first question is, does the alc gene function shut off all RNA synthesis from cytosine containing DNA or only those types of transcription analogous in some way to T_4 true late RNA synthesis? The second question is, does the alc gene product act either on the promoters for RNA synthesis or on the structure of DNA required for transcription? The first question could be examined either by comparing the affects of the alc gene product on early T_4 RNA synthesis with the affects on T_4 true late RNA synthesis, to see if only late transcription is affected, or by analyzing the affects of alc mutations on the shutoff of specific host transcripts. There are difficulties with both approaches. For example, studying the affect of alc product on T_4 early RNA transcription from cytosine DNA is complicated since the alc gene product itself is an early function. To examine specific host transcripts, either individual *E. coli* genes must be isolated or care must be taken to assure that only one type of transcript is being studied. To answer the second question, how alc acts, we could examine the affect of the alc gene product on either the intracellular structure of T_4 cytosine containing DNA or host DNA. However, the intracellular structure of both DNAs is very complex. To avoid the problems mentioned above, we decided instead to investigate the action of the T_4 alc gene product on λ transcription and on the intracellular structure of the DNA of the bacteriophage λ. It has been shown that T_4 superinfection shuts off λ transcription (Hayward and Green, 1965; Kennel, 1970) and alc could be involved.

For simplicity throughout the following discussion, the lytic cycle of λ will be segregated into two periods, early and late. The early period begins with the initiation of lytic development and extends through the first 15 minutes at 37°C. The late period extends from the end of the early period to cellular lysis. During the early period, λ DNA replicates as monomeric circles

(Schnos and Inman, 1970; Ogawa et al., 1968; Young and Sinsheimer, 1968) and the transcripts synthesized, the "early" RNAs, code for products involved in viral recombination, viral DNA synthesis and late gene expression (Dove, 1966; Eisen et al., 1966; Joyner et al., 1966. At 15 minutes, the early RNA molecules are originating from both strands of the virus and can be separated into three classes: the immediate early, the delayed early, and the late RNA molecules (Oda et al., 1969; Taylor et al., 1967). During the late period, DNA replicates as concatamers (Takahashi, 1974, 1975; Young and Sinsheimer, 1968) and, like the early period transcription, three classes of RNA molecules are synthesized from both strands. However, one class of transcripts now predominates: the late RNA molecules transcribed from the r strand (Herskowitz and Singer, 1970; Nijkamp et al., 1970; Oda et al., 1969; Taylor et al., 1967).

The following experiments demonstrate that the T_4 alc gene product is required for the shutoff of λ late period RNA synthesis, but does not alter the structure of closed circular supercoiled λ DNA. We also found that those λ late mRNAs which were being synthesized after infection by a T_4 alc mutant were not being translated into λ proteins. This is direct evidence for a very efficient T_4 translational shutoff function for non-T_4 mRNA.

MATERIALS AND METHODS

Bacteriophage and Bacteria.--Two types of T_4 bacteriophage were used in the following experiments. One type, subsequently referred to as $\underline{alc}^+$ T_4, was constructed in this laboratory and was the parent of the alc mutant T_4. The $\underline{alc}^+$ parent was deficient in two phage endonucleases which nick cytosine containing DNA; in a function that degrades dCTP to dCMP; and in a function which shuts off host DNA synthesis. The T_4 alterations responsible for the deficiencies are amE51 (dCTPase$^-$), SAΔ9 (endo IV$^-$, ndd$^-$), and den A$^-$ (endo II$^-$). The second type of T_4, subsequently referred to as alc mutant T_4, was alc 95. It was a spontaneous mutant derived from the $\underline{alc}^+$ parent on B834 galU56 (Runnels and Snyder, 1978) by a published procedure (Snyder et al., 1976) and it had all of the other mutations of the parent.

The bacteriophage λ has the mutations cI857 and sus S7. The cI857 mutation makes the prophage thermally inducible and sus S7 prevents lysis.

Several different bacterial strains were used in the experiments to follow. E. coli 594 cI857susS7 was from the stock of Gerald Smith and is called S655. E. coli 594 was an isolate of S655 cured of the prophage λ. E. coli A19 with a Col II plasmid was from the stock of Robert Brubaker. E. coli DG75, from Worcel and Burgi (1972), is leu$^-$, thy$^-$ and E. coli DG75 cI857susS7 is a λ lysogen of DG75 which was constructed in this laboratory.

Media and Buffer.--Three types of media were employed in the experiments. A recipe for M9 media was given previously by Snyder and Montgomery (1974). M9A is M9 medium supplemented with 10 ug./ml. of thiamine and 50 ug./ml. of each amino acid except methionine. KMT is KM medium of Ross and Howard-Flanders (1977) supplemented with 10 mg./ml. of tryptone (Difco).

Lambda buffer was described by Ross and Howard-Flanders (1977).

Purification of Bacteriophage.--To purify λ, a λ lysogen (S655) was induced with heat; concentrated; lysed with lysozyme and chloroform; and treated with RNAse and DNAse. To purify T_4, a lysate was concentrated at 4°C with polyethylene glycol (10% w/v) at 0.5 M NaCl. Both λ and T_4 were further purified on cesium chloride step gradients. After centrifugation, λ was dialyzed in the cold against two changes of λ buffer. T_4 was dialyized against successive changes of NaCl (2M-1M-0.5M-0.25M) followed by a final dialysis against M9 salts with $MgSO_4$.

Radioisotope labelling of T_4 infected induced lysogens.--A similar protocol was employed for labelling RNA with 5-H^3 uridine, DNA with methyl-H^3 thymidine, and proteins with S^{35} methionine. After the cell concentration had reached 4 X 10^8 cells/ml., a lysogen and the strain cured of the prophage were heat pulsed at

42^oC for 15 minutes. Aliquots of cells were infected with T_4 (m.o.i. of 12) at either an early time (10 min.) or a late time (50 min.) after the initial elevation of temperature. Radioisotope was added 4.5 min. after T_4 infection and the incorporation was stopped 3 min. later with ice. The efficiency of T_4 infection was monitored by the survival of the non-lysogen in the culture 2 min. after T_4 infection, since the λ lysogen will be killed by the induction of λ. Unless otherwise noted, fewer than 1% of the non-lysogen survived the T_4 infection.

Nucleic acid extractions.--RNA was extracted with hot phenol by the technique of Frederick and Snyder (1977). Concentrations were determined spectrophotometrically (Bolle et al., 1968).

DNA for hybridization was extracted from CsCl purified bacteriophage with phosphate buffered phenol in the cold (Bolle et al., 1968). Pulse labelled DNA was extracted by TCA precipitation, boiling in NaOH and neutralization with HCl (Oda et al., 1969).

Nucleic acid hybridizations.--RNA-DNA hybridizations were performed in 0.01M Tris-citrate pH 7.5 with 0.25M EDTA pH 7.5, 0.5M NaCl, and 10% of saturated phenol. After five hours at 60^oC, the hybrids were diluted with 0.5M NaCl, 0.2M EDTA pH 7.5; treated with RNAse A; collected on nitrocellulose membrane filters (Schleicher and Schuell); and washed with 0.5M NaCl, 0.2M EDTA pH 7.5 (Wu et al., 1972). RNA-DNA hybridizations of sucrose gradient fractions were performed in 6mM Tris-HCl pH 7.5. with 2XSSC (0.30M NaCl, 0.03 M Na Citrate). Hybrids were treated with RNAse A, collected on nitrocellulose membrane filters, and washed with 0.5M KCl, 0.01M Tris Cl pH 7.5 (Bolle et al., 1968).

DNA-DNA hybridizations were done on nitrocellulose membrane filters by the methods of Warnaar and Cohen (1966).

Protein slab gel electrophoresis.--Samples were treated as described by Frederick and Snyder (1977) and 12% slab gel electrophoresis was performed according to Studier (1973). The gels were stained with Comassie Blue to ensure that the total protein per column was uniform.

Preparing T_4 infected cells containing supercoiled Lambda DNA.--A λ lysogen (S655) was used to repress the lytic development of the superinfecting λ and allow the accumulation of supercoiled circular λ DNA. Cells were grown in KMT at 32°C to 4 X 10^8/ml., centrifuged, and resuspended at 10^9 cells/ml. in 1.5 ml. of λ buffer with H^3-thymidine labelled λ (m.o.i. of 5). After 20 min., the superinfected lysogen was diluted with 13.5 ml. of KMT and incubated at 32°C for an additional 40 min. before infecting with T_4. The conditions for infecting 5 ml. aliquots with T_4 were the same as those employed for infecting a heat induced λ lysogen. Six min. after T_4 infection, the cells were chilled and washed twice with 10 mM Tris-HCl pH 8 and resuspended in 0.2 ml. solution of 12% sucrose in 10 mM Tris-HCl, 0.25 mM EDTA pH 7.5.

The following procedures, which are similar to those of Ross and Howard-Flanders (1977), were used to lyse the cells. First, 0.1 ml. of a lysozyme solution (1 mg./ml. lysozyme in 10 mM Tris-HCl pH 8) was added. The extract was incubated for 10 min. on ice. Proteinase K (0.05 ml. of 1 mg./ml. preincubated for 1 h. at 37°C) and sarkosyl (0.2 ml. of a .5% solution V./V.) were added to the extract before incubating at 37°C for 1 hr. A portion of the lysate (0.2 ml.) was subjected to centrifugation as described below.

Preparation of Col II DNA.--A supercoiled DNA marker was prepared by the method of Blair et al (1971). An overnight culture of A19 was diluted one to twenty into 10 ml. of M9S supplemented with 10 uCi. methyl-C^{14} thymidine. After 3 h. of incubation at 37°C, solid chloramphenical (Sigma) to a final concentration of 150 ug./ml., and 10 uCi. methyl-C^{14} thymidine were added before continuing the incubation for another hour. The supernatant fraction from a Brij-deoxycholate lysate of the labelled cells was added to each centrifugation tube.

Ethidium bromide-cesium chloride-gradient centrifugation.--Density gradient centrifugation was performed as follows. Each 5 ml. gradient was made up in 10 mM Tris, 2.25 mM EDTA pH 7.5 and contained 0.33 mg./ml. ethidium bromide with cesium chloride to a final density of 1.54 g./ml. Centrifugation was carried out in a Beckman SW 50.1 rotor for 60 h. at 35,000 r.p.m. at 15°C.

Fractions of approximately 0.075 ml. were collected after centrifugation from the bottom of the tube and counted in PCS (Amersham/ Searle).

Chemicals and Enzymes.--Amino acids, ethidium bromide, and sucrose (RNAse free) were purchased from Sigma. S^{35}-methionine was purchased from Amersham/Searle. Cesium chloride (optical grade), methyl-C^{14} thymidine, methyl-H^3 thymidine, and 5-H^3 uridine were all purchased from Schwarz/Mann. Proteinase K was purchased from Beckman and lysozyme from Worthington Biochemicals.

RESULTS

The effect of the T_4 alc gene product on late period and early period λ RNA snythesis.--Because the T_4 alc gene product is required for the shutoff of T_4 late RNA snythesis from DNA with cytosine, we investigated the effect of the alc gene product on λ RNA synthesis late in λ development. We needed first to decide at what time after T_4 superinfection to label the λ RNA. Previous evidence has demonstrated that the nucleoid has been unfolded and host transcription has been shut off by 4.5 min. after T_4 infection (Sirotkin et al., 1977; Tigges et al., 1977), so, we assume, that the alc protein is functioning by this time. Accordingly, RNA was pulse labeled from 4.5 to 7.5 min. after T_4 infection of a heat induced λ lysogen. These RNAs were then hybridized to λ DNA. As indicated in Fig. 1A, the synthesis of λ RNA is barely detectable after an alc^+ T_4 infection. In contrast, substantial amounts of λ RNA are synthesized after the alc mutant infection. Therefore, the alc function is required for the shutoff of λ late transcription. The experiment has been repeated with two other independent alc mutants with essentially the same results (R.E.P., unpublished observations). Infection by the alc mutant did reduce the efficiency of hybridization of late λ RNA. Presumably, at least some of this reduction is due to labelled T_4 RNA which does not hybridize to DNA.

Since λ early period transcription and late period transcription may differ in their requirements, it is of interest to determine if λ early period transcription is affected by the _alc_ gene product. RNA was pulse labelled and hybridized to λ DNA in an experiment like that shown in Fig. 1A, but with λ in the early period of development (Fig. 1B). Both _alc_$^+$ T_4 and _alc_ mutant T_4 superinfections substantially reduce early period RNA synthesis. There is a difference in the amount of λ RNA synthesized during the _alc_$^+$ and _alc_ mutant T_4 infections which may represent the _alc_-mediated shutoff of the late λ RNA, since it is already being synthesized by time time after λ induction (Nijkamp et al., 1970; Oda et al., 1969; Skalka et al., 1967; Taylor et al., 1967). Since both _alc_$^+$ and _alc_ mutant T_4 shut off most of the λ transcription in the early period, some other mechanism independent of _alc_ blocks early λ transcription. Because of this other mechanism, the effect of the _alc_ gene product on early period transcription could not be determined unequivocally.

The alc gene product did not alter the superhelical content of closed circular λ DNA.--There is evidence that supercoiling of DNA enhances at least some types of transcription _in vivo_ (De Wyngaert and Hinkle, 1979; Puga and Tessman, 1973; Ryan, 1976). It is conceivable that the T_4 _alc_ function shuts off host transcription and unfolds the host nucleoid by directly removing supercoils from DNA. If so, then the _alc_ function may shut off λ late RNA synthesis, by directly removing supercoils from λ DNA. The DNA of λ becomes

Figure 1.--Hybridizations of λ RNA synthesized after T_4 infection to λ DNA. Heat induced λ lysogen (S655/594) in M9S supplemented with 10 μg./ml. tryptophan was infected with T_4 at 50 (A) and 10 min. (B) after induction as described in the text. The RNA was pulse labelled with 5 μCi./ml. 5-H^3 uridine, extracted, and hybridized to 25 μg. of λ DNA. Each point represents the amount of RNA hybridized to λ DNA minus the amount of RNA hybridized without DNA which was always less than 70 c.p.m. The specific activities of the input RNAs were in part A, uninfected late RNA at 6.6 c.p.m./ng.; *alc*$^+$ infected RNA at 2.9 c.p.m./ng.; *alc* mutant RNA at 3.4 c.p.m./ng.; and in part B, uninfected early RNA at 1.54 c.p.m./ng.; *alc*$^+$ infected RNA at .9 c.p.m./ng.; and *alc* mutant infected RNA at 1.15 c.p.m./ng. In both A and B, uninfected RNA is represented by (●———●), *alc*$^+$ RNA by (o———o), and *alc* mutant infected RNA by (•———•).

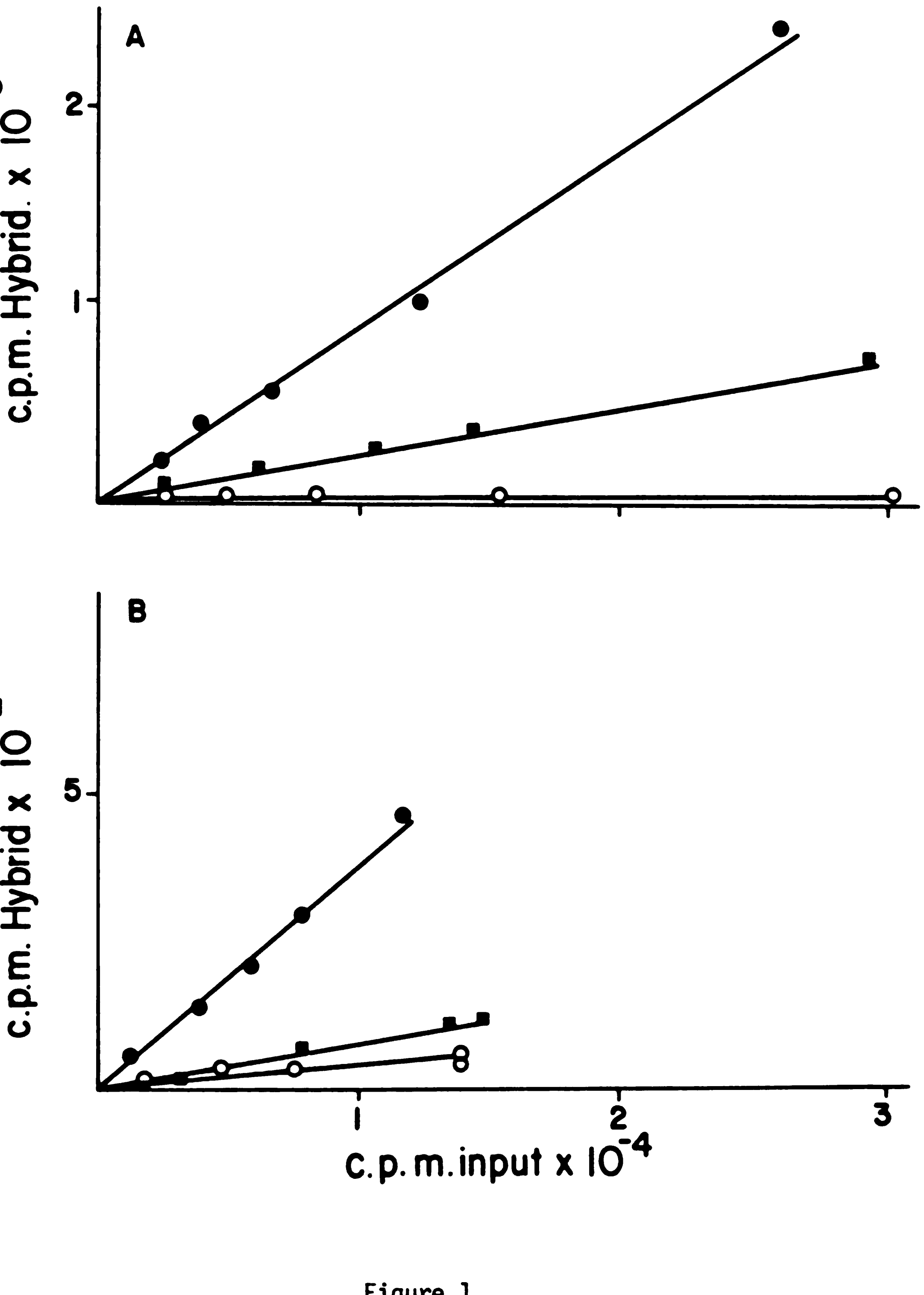


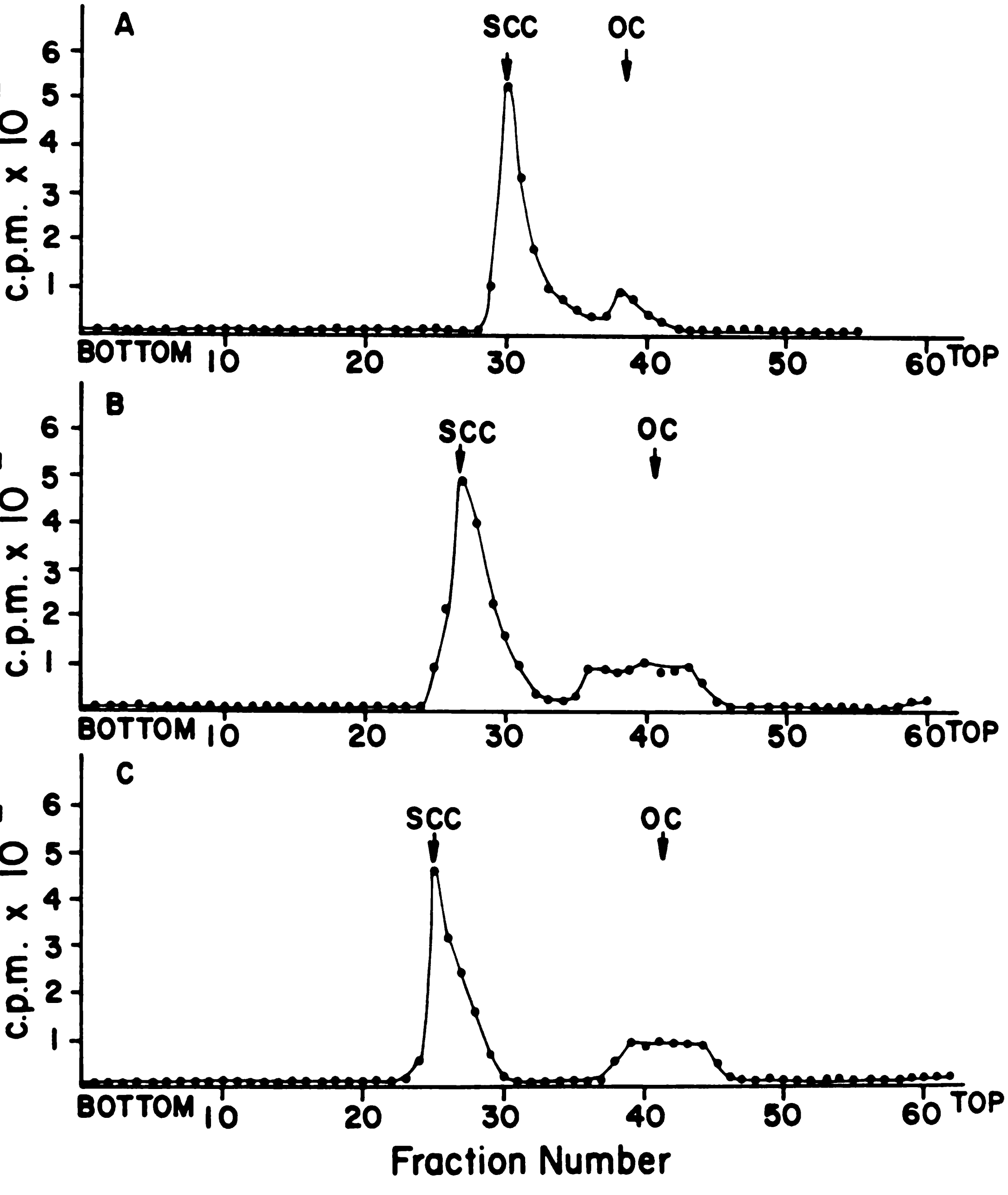
Figure 1

circular and some of it becomes supercoiled soon after infection. Lambda DNA which is covalently closed circular and supercoiled can be distinguished from that which is covalently closed, but relaxed, and from linear or nicked circular DNA by ethidium bromide-cesium chloride-gradient centrifugation.

To determine if the alc gene product destroys the supercoiling of circular λ DNA, a λ lysogen containing radioactive λ supercoiled DNA was infected with alc^+ T_4 and alc mutant T_4. The affect of the T_4 infections is shown in Fig. 2. Neither the alc^+ T_4 (B) nor the alc mutant T_4 (C) alters the supercoiled peak of λ DNA. There was also no increase in either the relaxed circular, open circular, or linear DNA peaks. As an aside, the above result indicates that there are no additional T_4 nicking activities for cytosine DNA, other than those of the den A (Hercules et al., 1971; Warner et al., 1970) and den B (Bruner et al., 1972; Sadowski and Vetter, 1973) gene products, at this time after T_4 infection because there are no significant increases in either relaxed open or linear λ DNA molecules during either infection.

The alc gene product did not inhibit late period λ DNA replication.--It has been reported that normal late λ RNA synthesis depends on viral DNA synthesis (Dove, 1968; Joyner et al., 1968; Skalka et al., 1967). Since the alc gene product might affect late λ RNA synthesis by preventing λ DNA synthesis, we decided to examine the effect of the alc gene product on λ DNA replication by hybridizing pulse labelled DNA synthesized after T_4 infection to λ DNA

Figure 2.--Ethidium bromide cesium chloride gradient centrifugation of supercoiled DNA without T_4 infection (A), with *alc*$^+$ infection, and with T_4 *alc* mutant infection (C). Extracts of a λ lysogen which has been infected with labelled λ, then superinfected by T_4 were lysed, centrifuged, and counted as described earlier. Tritiated λ DNA is represented by (●———●), the position of the marker Col II DNA on the same gradient is represented by the arrow. SCC indicates closed supercoiled circular molecules and OC indicates open or linear DNA molecules.


A
SCC
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SCC
OC
c.p.m. x 10^-2
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BOTTOM
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50
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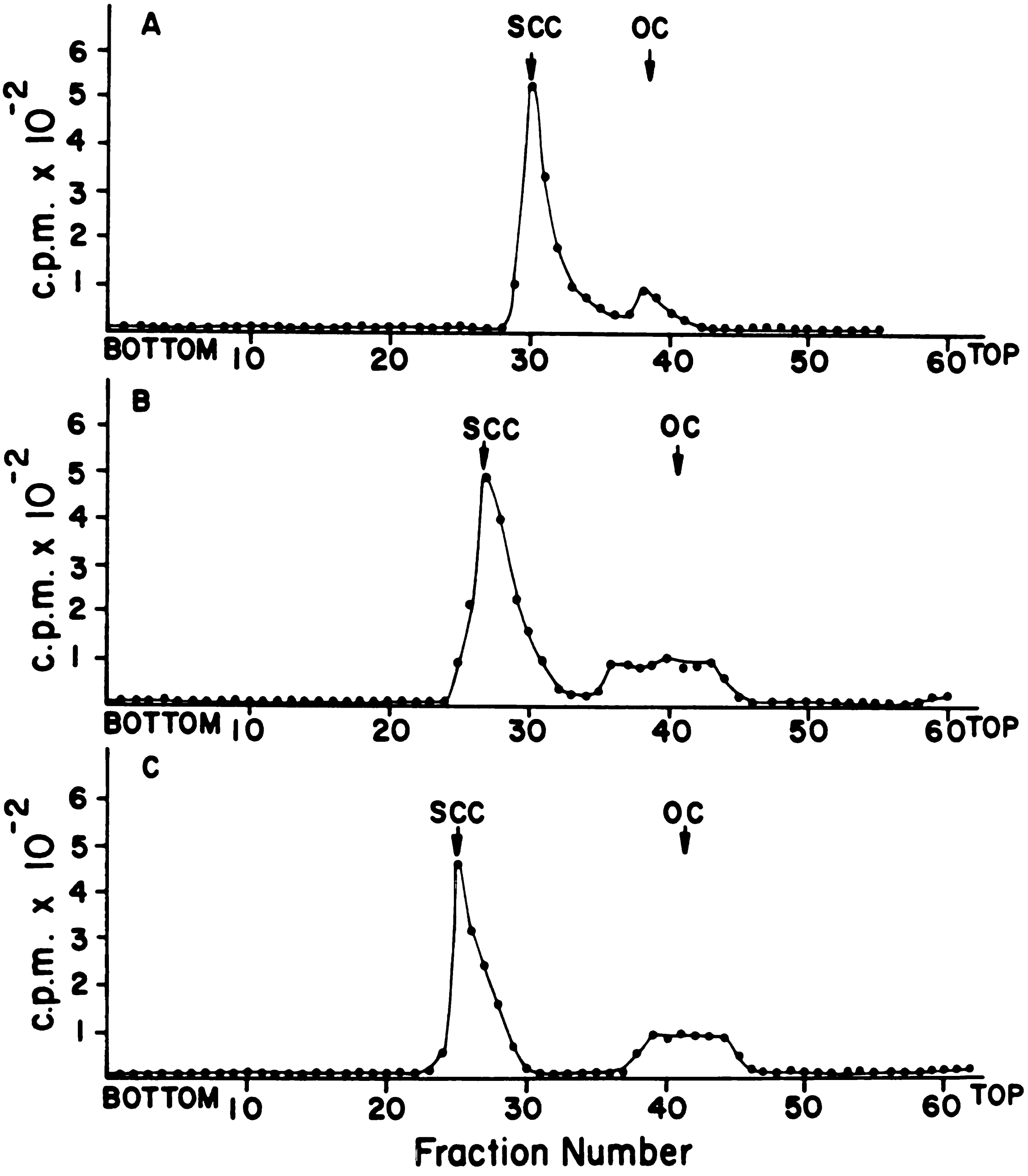
TOP
Fraction Number

Figure 2

Figure 2.--Ethidium bromide cesium chloride gradient centrifugation of supercoiled DNA without T_4 infection (A), with alc$^+$ infection, and with T_4 alc mutant infection (C). Extracts of a λ lysogen which has been infected with labelled λ, then superinfected by T_4 were lysed, centrifuged, and counted as described earlier. Tritiated λ DNA is represented by (●——●), the position of the marker Col II DNA on the same gradient is represented by the arrow. SCC indicates closed supercoiled circular molecules and OC indicates open or linear DNA molecules.


A
SCC
OC
B
SCC
OC
C
SCC
OC
c.p.m. x 10^{-2}
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Fraction Number

Figure 2

(Table 1). As the data indicates, λ DNA replication continues but at a reduced rate during the alc$^+$ and alc mutant T_4 infections. Somewhat higher rates of λ DNA replication are observed after the alc$^+$ infection. We observed this difference both times we performed the experiment and are unsure as to the explanation.

The affect of T_4 infection on the translation of λ late period RNA.--Over the years, a number of indirect experiments have suggested the existence of a T_4 mechanism for inhibiting the translation of host mRNA which is independent of the transcriptional shutoff (Kennel, 1970; Svenson and Karlstrom, 1976). Because λ late RNA synthesis continues during an alc mutant infection, it is possible directly to determine if T_4 has a mechanism to prevent translation of non-T_4 RNA molecules.

The effects of alc$^+$ T_4 and alc mutant T_4 on λ late protein synthesis were studied by slab gel electrophoresis and autoradiography. The results are shown in Fig. 3. Also shown, for comparison, are the labeled proteins of an induced and uninduced λ lysogen. Proteins which were labelled in the induced but not the uninduced culture are assumed to be λ proteins. It can be seen that no λ proteins were synthesized after either an alc$^+$ or alc mutant T_4 infection. Thus, the late λ RNAs made after the alc mutant T_4 infection are not translated.

The size of the λ late period RNA synthesized after T_4 infection.--One possible explanation for the failure to translate

TABLE 1.--Lambda DNA synthesis after infection by T_4. Heat induced cells (DG 75λc1857susS7/DG75) in M9S supplemented with 5 μg./ml. thymidine and 10 μg./ml. tryptophan were infected with T_4 and pulse labelled with 10 μCi./ml. methyl-H thymidine as described. Pulse labelled DNA from 5 ml. aliquots was extracted and hybridized to 20 μg. of λ DNA per filter.

	T_4	Time After Induction	Input c.p.m.	Filter + λ DNA c.p.m.	Filter - λ DNA c.p.m.	% Input Hybridized
A	None	Uninduced	1092	23	22	.09
B	None	Late	8958	2085	86	22.3
C	Alc^+	Late	3042	551	45	16.6
D	Alc^-	Late	4373	436	31	9.3

Figure 3.--Slab gel electropherograms of proteins synthesized in an uninduced culture of a λ lysogen and in heat induced lysogen infected by T_4. The columns contain, A: induced late period proteins, B: Uninduced host proteins, C: alc$^+$ T_4 infected proteins, and D: alc mutant T_4 infected proteins. Tentative identification of some late λ protein bands and T_4 bands was by comparison to published data (Murialdo and Siminovitch, 1972; O'Farrell and Gold, 1973).

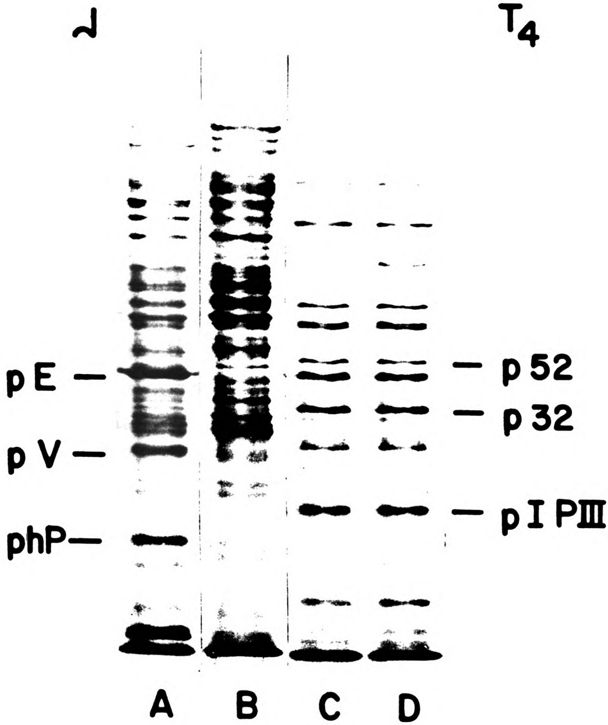

Figure 3

the λ late mRNAs made after a T_4 alc mutant superinfection could be that they are non-translatable fragments. Accordingly, the in vivo size of late period λ RNA synthesized during an alc mutant infection was examined. Fig. 4A shows the size of the λ RNA made late in the λ cycle without T_4 infection. Lambda RNA is detected throughout the gradient, but particularly in the high molecular weight region and most sedimented faster than the 16S ribosomal marker. This was expected, because λ late mRNA has been shown to be polycistronic (Garilglio and Green, 1973). Like the λ late RNA synthesized without T_4 infection, the bulk of the λ RNA synthesized during an alc mutant infection sediments faster than the 16S ribosomal marker (Fig. 4B). Therefore, the failure to translate the λ late RNAs made after an alc mutant T_4 infection is not due to the size of the λ RNA.

In the same experiments we examined the size of T_4 RNA by hybridizing the fractions of the same gradients to T_4 DNA. As expected, a considerable amount of RNA synthesized during the alc mutant infection hybridized to T_4 DNA (Fig. 4B). The sedimentation profile of the T_4 RNA corresponds with previous profiles of T_4 RNA (Ricard and Salser, 1975). Thus, there did not appear to be extensive degradation during the extraction of RNA. Without T_4 superinfection, the RNA in the gradient fractions hybridized at background levels to T_4 DNA (Fig. 4B), as expected, since there is little sequence homology between λ RNA and T_4 DNA (Kennel, 1968; Skalka et al., 1967).

Figure 4.--Sucrose gradient centrifugation of heat induced late period RNA synthesized without and with T_4 infection. Late induced λ RNA (A) and late induced RNA synthesized during an alc mutant superinfection (B) were isolated as in Fig. 1A and heat denatured for 3 min. before layering on 5 ml. 5-20% sucrose gradients. The methods for disaggregation and sucrose gradient centrifugation were described by Ricard and Salser (1975). Centrifugation was performed in an SW 50.1 rotor at 45,000 r.p.m. for 5 h. at $4^{o}C$. The gradient in part A contained 33 μg. of RNA. At the end of centrifugation, 0.2 ml. fractions were collected from the top with an ISCO fractionator. The positions of the ribosomal subunits were determined with a u.v. monitor. Each fraction was subjected to hybridization as described earlier to one of the following: 10 μg. of λ DNA (●———●), 10 μg. of T_4 DNA (o———o), no DNA (▽). The yield of hybridized counts in A and B were 98% and 95% of the input hybrid radioactivity.

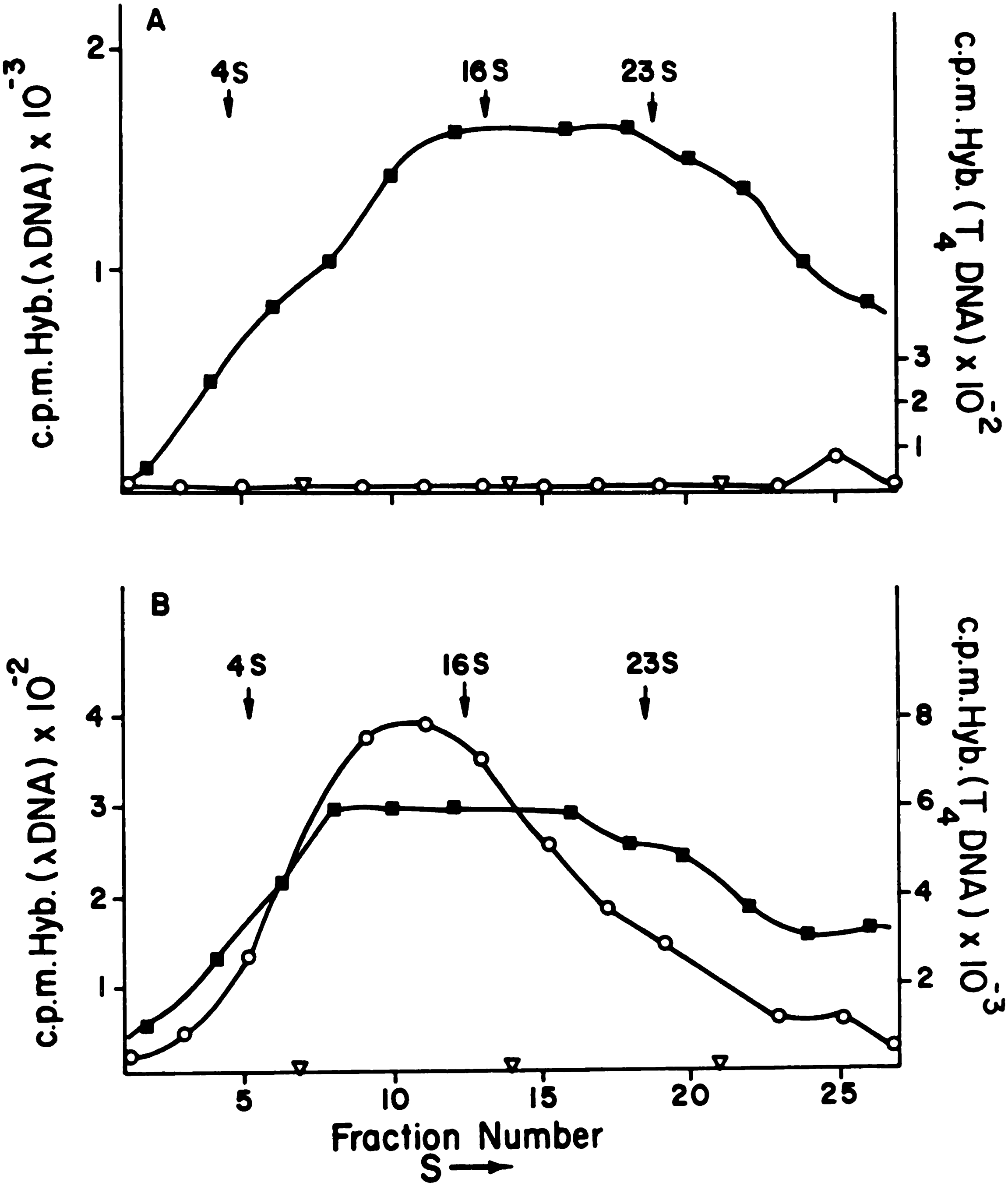
A
c.p.m.Hyb.(λDNA) x 10^{-3}
2
1
4S
16S
23S
c.p.m.Hyb.(T$_4$DNA) x 10^{-2}
3
2
1
B
c.p.m.Hyb.(λDNA) x 10^{-2}
4
3
2
1
4S
16S
23S
c.p.m.Hyb.(T$_4$DNA) x 10^{-3}
8
6
4
2
5
10
15
20
25
Fraction Number
S →

Figure 4

DISCUSSION

Like the shutoff of T_4 true late transcription from cytosine T_4 DNA and some host transcription, the shutoff of λ transcription by T_4 during the late period of the λ lytic cycle requires the T_4 alc gene function. Since alc mutants are defective in preventing at least some transcription from T_4, E. coli, and λ DNA templates, there must be something similar about the three types of transcription which is affected by the alc gene product. It is clear from the data that the alc gene product neither blocks λ DNA synthesis nor directly removes supercoils from closed circular supercoiled λ DNA molecules. Therefore, the molecular basis for the alc-mediated shutoff of some types of transcription remains undiscovered. Several plausible mechanisms for alc function remain. To give two possible mechanisms, the alc gene product could act directly on some promoters, provided they contain cytosine, and prevent their utilization. Alternatively, the alc function could act on some other DNA structure such as the "cores" of nucleoids and thereby indirectly remove supercoiling which, in turn, may be required for the utilization of some types of promoters. Both of these explanations have the alc function blocking the initiation, but not the elongation of λ late in RNA synthesis which may be untenable because of the following considerations. All λ late transcription is thought to begin at a promoter to the right of _Q_ and proceed through the late region requiring around 10 minutes to transcribe the entire region (Green et al., 1970). However, the _alc_ function has shut off λ late transcription within 4.5 min. of T_4 superinfection and, since a finite interval is required for the transcription and translation of the _alc_ gene, the time for _alc_ to function is actually less than this. Therefore, λ late transcription may be shut off more quickly than might be expected if only the initiation of transcription is blocked suggesting that the _alc_ function can block the elongation and not just the initiation of the synthesis of mRNA.

Because T_4 inhibits early period λ transcription independently of _alc_ function, it was not possible to determine if the _alc_ product also effects λ early period transcription. We suspect that _alc_ does not effect λ early period transcription because the shutoff of early period λ transcription was less complete than the _alc_-mediated shut off of T_4 and λ late transcription. Regardless, the results indicate that another T_4 mechanism can inhibit λ early transcription. This mechanism may be merely a manifestation of an inherent property of T_4 DNA. For example, T_4 early promoter sequences may have a greater affinity for the RNA polymerase of the host than λ early promoter sequences. Experiments _in vitro_ demonstrating that T_4 is a more active template for transcription than λ DNA, even with RNA polymerase from uninfected bacteria (Mailhammer et al., 1975), provide some support for this mechanism. Alternatively, a T_4 induced function may be responsible for the non-alc-mediated inhibition of λ early RNA synthesis. For example, the bacteriophage T_4 induces several changes in the host RNA polymerase which include both adenylation (Horvitz, 1974) and addition of polypeptides to the polymerase (Stevens, 1972; Horvitz, 1973). Either the adenylations or the added polypeptides could be responsible for the non-alc inhibition of early λ transcription. Preliminary evidence indicates that the adenylations of the polymerase are not responsible because λ early RNA synthesis is blocked after infection by an alt^-, mod^- mutant infection (R.E.P. unpublished observations). But, since an alc-mediated shutoff could be superimposed on a shutoff due to adenylation of the RNA polymerase, it may be necessary to construct a T_4 containing mutations which make it alt^- mod^- and alc mutant to investigate the effects of the adenylations on λ transcription. With respect to the addition of T_4 induced polypeptides to the host RNA polymerase, it has been reported that there are four, of which two are the products of known T_4 genes 33 and 55 (Stevens, 1972; Horvitz, 1973). The other two are good candidates for the shutoff of λ early transcription. In this connection, we have reported that an alc mutant affects the binding of one of the T_4 induced polypeptides to the host RNA polymerase (Sirotkin et al., 1978), probably the 15,000 M.W. polypeptide of Stevens (1972). This is not a general property of alc mutants and many of them show normal amounts of this polypeptide on the RNA polymerase. Because all of our attempts to isolate deletion mutants or nonsense mutants of the alc gene have failed, we think alc may be an essential (i.e. indispensible) gene for T_4 development. If alc is an essential gene, then all alc mutations may be only partially inactivating. If this is the case, then all alc mutants may have the RNA polymerase binding polypeptide, but it may be lost in some alc mutants from the RNA polymerase during purification. We also think that T_4 alc mutants may accumulate second site mutations perhaps because they are selected with cytosine in their DNA and without the phage induced dCTPase. Perhaps a second site mutation, in an alc mutant, was in the gene for the 15,000 dalton polypeptide of Stevens (1972). More experiments are needed to clarify this issue.

Out data suggest that a T_4 mechanism inhibits λ late period DNA synthesis and, even more dramatically, λ early period DNA synthesis (REP, unpublished observation). It has been reported that the T_4 ndd gene product inhibits host DNA synthesis (Snustad et al., 1976a) and may have a similar effect on λ DNA synthesis. However, the parent of the alc mutant we used has a deletion which includes ndd, so this gene product could not have been responsible in our case. It should be pointed out that the shutoff we saw could have been an indirect effect of shutting off λ translation. For example, the O gene product of λ is an unstable protein (Wyatt and Inokuchi, 1974) and stopping its synthesis will result in a cessation of λ DNA synthesis (Klinekert and Klein, 1978).

We have presented direct evidence for a T_4 mechanism that prevents translation of λ late RNA molecules. We think this is the most dramatic demonstration to date of a T_4 induced mechanism which prevents translation of non-T_4 mRNA molecules. The failure to translate the λ late RNA was not due to its size because high molecular weight λ RNA was synthesized, but still not translated. Other mechanisms can be proposed. For example, the shutoff of host and λ protein synthesis could be due to one of the several T_4 induced ribosome binding proteins which are synthesized during a T_4 infection (Dube and Rudland, 1972; Smith and Haselkorn, 1969). The *regA* protein, which is involved in translation regulation of early T_4 proteins (Karam et al., 1974; Trimble et al., 1976; Wiberg et al., 1973), might also affect the shutoff of translation. However, the *regA* gene product may not be synthesized early enough to be responsible for the translation shutoff we observe (J. Wiberg, personal communication). Whatever the mechanism, we predict that T_4 mRNA has some property which distinguishes it from host and λ mRNA. A potential candidate would be an unusual sequence at the 5' end of T_4 mRNA which is involved in the initiation of protein synthesis. We have begun to try to isolate mutations which prevent the shutoff λ late protein synthesis after a T_4 *alc* mutant superinfection.

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APPENDIX

APPENDIX

The Shutoff of Lambda Monomeric Circle Replication

Lambda DNA replicates as monomeric circles during the first ten minutes of development at 37°C and then replicates as concatamers. Because monomeric circle replication differs from concatameric DNA replication, T_4 may effect the two forms of Lambda DNA replication differently. To examine the affects of T_4 on monomeric circle replication, an experiment analogous to that used to investigate the affect of T_4 on Lambda concatameric replication was performed. The results of the experiments are presented in Table 2. Also presented, for comparative purposes, are the results of the experiment showing that the alc gene product did not block λ concatameric DNA replication. It was clear from the data that both monomeric circle DNA replication and some concatameric DNA replication were shut off during T_4 superinfection. But, monomeric circle replication was more sensitive to a T_4 shutoff mechanism than concatameric DNA replication.

There are several plausible explanations for the different sensitivities of Lambda monomeric circle DNA and Lambda concatameric DNA replication to T_4 superinfection. One explanation, is that the difference may be simply due to the difference in the number of

TABLE 1.--Lambda synthesis after infection by T_4. Heat induced cells (DG 75λcl857susS7/DG75) in M9S supplemented with 5 μg./ml. thymidine and 10 μg./ml. tryptophan were infected with T_4 and pulse labelled with 10 μCi./ml. methyl-H^3 thymidine as described. Pulse labelled DNA from 5 ml. aliquots was extracted and hybridized to 20 ug. of λ DNA per filter.

	T_4	Time After Induction	Input c.p.m.	Filter + λ DNA c.p.m.	Filter - λ DNA c.p.m.	% Input Hybridized
A	None	Ininduced	1092	23	22	.09
B	None	Early	8238	262	38	2.7
C	Alc^+	Early	1164	30	24	.5
D	Alc^-	Early	1586	33	23	.6
B	None	Late	8958	2085	86	22.3
C	Alc^+	Late	3042	551	45	16.6
D	Alc^-	Late	4373	436	31	9.3

replicating genomes at the time of T_4 superinfection. Alternatively, the difference could be due to the shutoff of Lambda protein synthesis. Because both monomeric circle replication and concatameric DNA replication require different unstable Lambda gene products, the shutoff of Lambda protein synthesis may affect Lambda monomeric circle replication more severely than Lambda concatameric replication. Another possibility is that T_4 induces several functions that prevent DNA synthesis. Perhaps, monomeric circle replication was more sensitive to a shutoff function than concatameric DNA replication.

The Shutoff of Lambda Late Transcription After alt⁻ mod⁻ Mutant T_4 Infection

T_4 blocks Lambda late transcription during superinfection. At least one T_4 gene involved in the shutoff of late transcription has been identified as the T_4 alc gene. Recently, the T_4 induced adenylation of the host RNA polymerase has been linked to the shutoff host RNA synthesis and may be involved in the shutoff of Lambda late transcription. This can be studied with alt^- mod^- mutant T_4 because this strain of T_4 fails to adenylate the host RNA polymerase during infection (see above).

An experiment analogous to those investigating the affect of alc gene on Lambda late period transcription was performed with alt^- mod^- mutant T_4. The results of this experiment are presented in Figure 5 and, for comparative purposes, the experiment on the alc-mediated shutoff of λ late period transcription is also presented.

Figure 1.--Hybridization of λ RNA synthesized after T_4 infection to λ DNA. A heat induced lysogen (S655/594) in M9S supplemented with 10 ug./ml. tryptophan was infected with T_4 as described earlier. The RNA was pulse labelled with 10 uCi./ml. 5-H^3 uridine, extracted, and hybridized to 25 ug./ml. of Lambda DNA. Each point represents the amount of RNA hybridized to DNA minus the amount of RNA hybridized without DNA. The specific activities of the RNAs were Uninfected late RNA at 6.6 c.p.m./ng., *alc*$^+$ infected RNA 2.9 c.p.m./ng., *alc* mutant RNA at 3.4 c.p.m./ng., and *alt*$^-$ *mod*$^-$ RNA at 1.5 c.p.m./ng. Uninfected RNA is represented by (o———o), alc$^+$ RNA by (o———o), *alc* mutant RNA by (———), and alt$^-$ mod$^-$ RNA by (———).

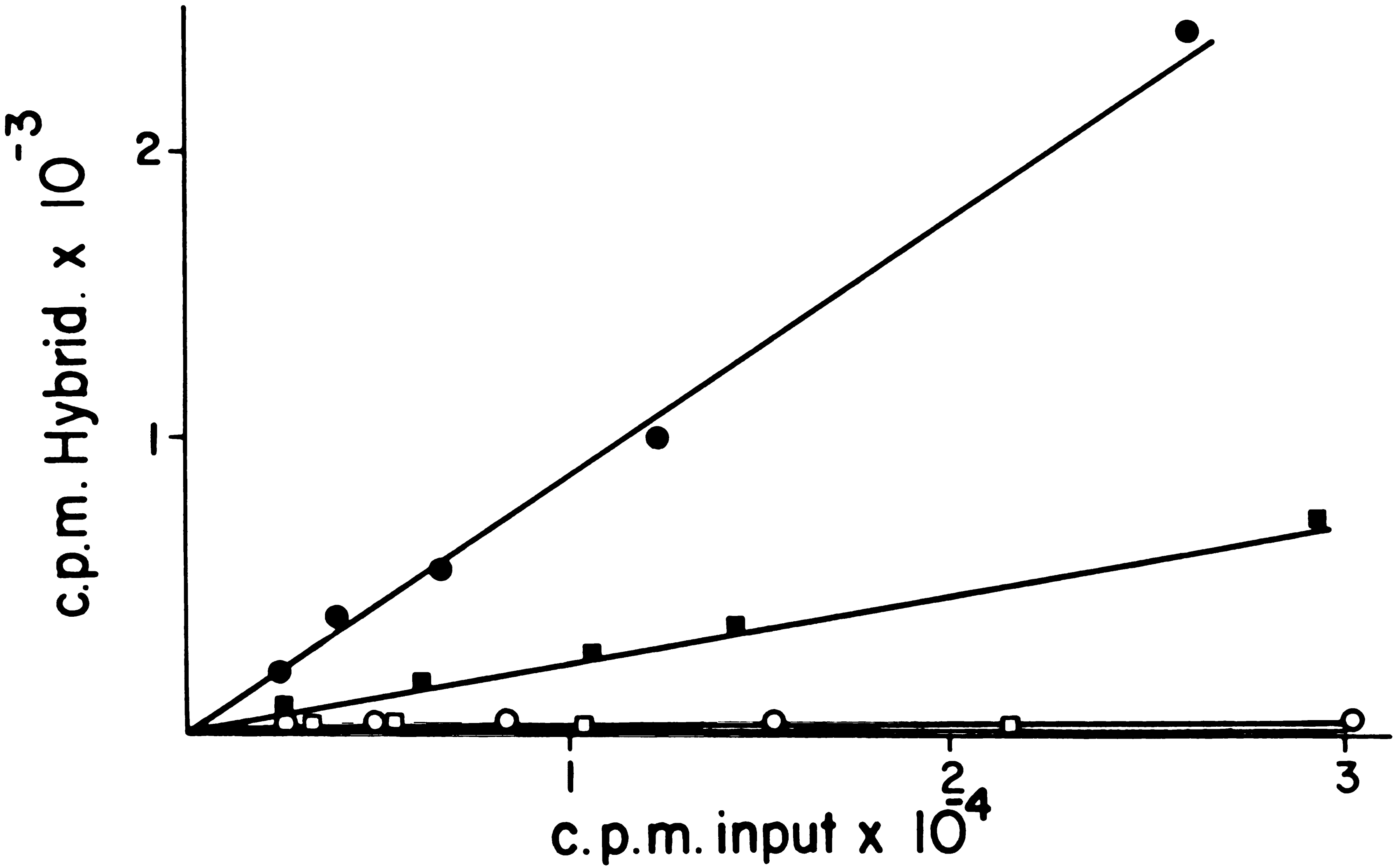

Figure 1

It is clear that the alt⁻ mod⁻ mutant T_4 shut off late RNA synthesis. Therefore, the T_4 induced adenylation of the host RNA polymerase is not involved in the shutoff of Lambda late period transcription.

There are a number of explanations for the failure to detect an effect of the T_4 induced adenylations on the shutoff of late period transcription. First, the T_4 induced adenylation of the RNA polymerase may not be related to the shutoff of Lambda late transcription. On the other hand, adenylation of the RNA polymerase may be involved in the shutoff of Lambda late period transcription, but the alc-mediated transcriptional shutoff may be superimposed on it. To study this alternative, a triple mutant containing alt⁻ mod⁻ and alc mutants may be necessary to determine if the adenylation of the RNA polymerase is responsible for some of the shutoff of Lambda transcription.