INTERACTIONS BETWEEN RNA POLYMERASE AND DNA DURING T4 BACTERIOPHAGE DEVELOPMENT

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ABSTRACT

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An RNA polymerase mutation (rif^R-2) of *Escherichia coli* poorly supports the growth of T4 because T4 is unable to utilize the mutated RNA polymerase for at least one of its essential functions. Mutants of T4 have been isolated which grow better than wild-type T4 on the Rif^R-2. These are missing the function β-glucosyl transferase and can grow better because the incoming parental DNA is partially unglucosylated. Only the β-, not the α-, glucosyl transferase is involved, suggesting a special role for at least some of the hydroxy-methyl cytosines normally glucosylated with a β linkage. This phenomenon is independent of restriction. Only the state of glucosylation of the parental, not the progeny, DNA matters for this phenomenon.

Rif^R-2 has been shown to have a direct effect on both DNA synthesis and late gene expression. The effects of rif^R-2 are partially suppressed by β-glucosyl

transferaseless mutants (βgt^-) of T4, and β-glucosylation is inhibitory both early and late in infection.

Rif^R-2 also has an effect on early gene expression, causing disproportionate synthesis of some early T4 proteins. However, β-glucosylation has no effect on this early gene expression defect, although we have shown that β-glucosylation inhibits an early function required for phage production. Rif^R-2 also causes defective host nucleoid unfolding and a delay in host DNA degradation. β-glucosylation is inhibitory for host DNA degradation, suggesting that the transcription of β-glucosylated hydroxymethyl cytosines in T4 DNA is somehow involved in this step. These data further support the model proposed earlier that RNA polymerase must alter the structure or association of T4 parental DNA early in infection for DNA replication and late transcription to occur.

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INTRODUCTION

Dr. Snyder's laboratory is studying the roles of host RNA polymerase in T4 development, with emphasis on the interactions between T4-coded proteins and RNA polymerase. The approach taken has been to use RNA polymerase mutations (rif^R-2) of *E. coli* which inhibit T4 growth. Characterization of Rif^R-2 infections has shown that RNA polymerase is directly required for T4 DNA synthesis, as well as for viral transcription.

The first article in this dissertation is a published manuscript describing the isolation, mapping, and partial characterization of T4 mutants which grow better than wild type T4 ($T4^+$) on Rif^R-2. These mutants all mapped in the gene for β-glucosyl transferase, the enzyme which glucosylates about 30% of the hydroxymethyl cytosines in T4 DNA, with a β-linkage. It is the unglucosylated state of the T4 DNA, and not the malfunctioning enzyme, which allows β-glucosyl transferaseless mutants to grow better than $T4^+$ on Rif^R-2.

The second article in this dissertation is a manuscript that will be submitted for publication. It describes further effects of the rif^R-2 mutation on T4

development, and compares these effects in $T4^+$ and βgt^- infections. Besides affecting late T4 gene expression and T4 DNA synthesis, the rif^R-2 mutation also causes a delay in host nucleoid disruption and degradation, and affects early T4 gene expression.

The Appendix contains a published manuscript (Snyder and Montgomery, 1974) which describes some effects of rif^R-2 on T4 development, and further describes effects of β-glucosylation. This publication was included because the experiments described in the second article rely heavily upon the data reported therein.

LITERATURE REVIEW

Host Macromolecular Synthesis After T4 Infection

The bacteriophage T4 causes a cessation of all *E. coli* macromolecular synthesis within a few minutes after infection (Monad and Wollman, 1947; Volkin and Astrachen, 1956; Nomura *et al.*, 1960; Hosoda and Levinthal, 1968). T4 could accomplish this by one, or any combination, of the following mechanisms. One or more immediate early T4 proteins could be required for shutoff, or the adsorption and binding of T4 to the host cell wall could cause conformational changes in the membrane which lead to shutoff. Other possibilities are that the T4 protein(s) required for shutoff of macromolecular synthesis is injected with the DNA from parental T4, or that T4 DNA competes with *E. coli* DNA for some critical site in the host.

In fact, two mechanisms for shutoff of both DNA and RNA synthesis have been proposed. The first mechanism requires T4 protein synthesis, since in the presence of various inhibitors (i.e., chloramphenicol, rifampin, and histidine starvation) host DNA and RNA synthesis continue

(Duckworth, 1971; Hayward and Green, 1965; Nomura *et al.*, 1962; Nomura *et al.*, 1966; Terzi, 1967). However, shutoff does occur in the presence of inhibitors at high multiplicities of infection (Nomura *et al.*, 1966), and in *E. coli* infected with T4 phage "ghosts" (T4 protein coats without their DNA) (Duckworth, 1970). Therefore, another mode of shutoff, not requiring T4 protein synthesis, must also be functioning in *E. coli*. Terzi concluded that only this second mode of shutoff functions in T4 infections of *Shigella*, since the shutoff of host macromolecular synthesis is incomplete and is dependent upon the multiplicity of infection (Terzi, 1967). It has been suggested that this second mode is due to conformational changes occurring in the host membrane when T4 adsorb to *E. coli* (Nomura *et al.*, 1966; Duckworth, 1971). This type of shutoff is seen only in "ghost" infections or at high multiplicities of infection in the absence of T4 protein synthesis, because in normal T4 infections the damage to the host membrane is repaired by a T4-directed function(s) (Duckworth, 1971).

Probably the mode of host DNA synthesis shutoff which does not require protein synthesis plays a minor role in normal T4 infections of *E. coli*. However, it does show that a membrane change occurs when T4 adsorb to the cell wall, and it signifies that this change alone is capable of altering internal functions of the cell. This is

reminiscent of the action of colicins on *E. coli*. Colicin E2 does not enter the cell, yet is capable of killing *E. coli* by solubilizing its DNA, even in the presence of chloramphenicol (Swift and Wiberg, 1971). Therefore, it must cause existing host enzymes to degrade the DNA by altering the membrane, and perhaps acts by releasing the host DNA from its membrane-bound site, making it more available to nuclease attack.

E. coli Nucleoids

Besides causing host macromolecular synthesis to cease, T4 infection causes host nuclear disruption, unfolding, and subsequent degradation of the DNA to nucleotides. A review of the properties of the bacterial nuclear structure is presented here so that the significance of the T4 disruption and unfolding can be better appreciated.

Although the DNA of *E. coli* is over 1 mm in length (Cairns, 1963), it is packaged in nuclear bodies about 1 μm in diameter. Cytological studies have shown these bodies to be multilobed and located in the center of the cell (see, for example, Fuhs, 1965; Kellenberger *et al.*, 1958). When cells are gently lysed, DNA can be isolated from *E. coli* as highly folded structures, referred to as nucleoids, which correlate in size to the nuclear bodies seen cytologically (Stonington and Pettijohn, 1971). In addition to DNA, the nucloids contain nascent RNA chains

(about 30% by weight) and protein (about 10% by weight) (Pettijohn *et al.*, 1973), with over 90% of the protein being core RNA polymerase (Worcel *et al.*, 1973).

At least some of the RNA found in the nucleoid is required to hold DNA in the highly folded structure, as shown by the nucleoid unfolding when treated *in vitro* with RNase (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972). *In vivo* treatment of cells with rifampin, a drug which inhibits RNA polymerase activity by binding to the β-subunit (Zillig *et al.*, 1970), yields only unfolded DNA after lysis (Pettijohn and Hecht, 1973; Dworsky and Schaechter, 1973), suggesting that RNA polymerase must be continually functioning to keep DNA in the highly folded structure.

Worce and Burgi (1972) and Pettijohn and Hecht (1973) have proposed a model for nucleoid structure. They suggest that the DNA is folded into 12-80 loops per chromosome, with RNA molecules defining the positions of these folds by their binding to DNA. The bound RNA molecules also prevent rotation between loops, thus separating the DNA into domains of supercoiling.

Nucleoids have been shown to be bound to the membrane of *E. coli* (Dworsky and Schaechter, 1973; Worcel and Burgi, 1974; Delius and Worcel, 1973). This membrane attachment seems to be required for synthesis since replicating DNA is isolated from *E. coli* as membrane-bound

nucleoids whereas nonreplicating DNA is isolated only as membrane-free nucleoids (Worcel and Burgi, 1974). Dworsky and Schaechter (1973) have shown that RNA polymerase is involved in the DNA attachment to the membrane, by decreasing the number of attachment sites (by a factor of 4) with rifampin treatment of the cells. They suggest that the role RNA polymerase plays in stabilizing the folded structure of the nucleoid may be related to the role it plays in binding DNA to the membrane; that the RNA "core" may be associated with the membrane, but can be released without damage to the structure of the nucleoid (Dworsky and Schaechter, 1973).

Host Nuclear Disruption, Unfolding, and Degradation After T4 Infection

The *E. coli* genome is disrupted within the first two or three minutes after infection by T4, and accumulates in clumps along the cell membrane (Kellenberger, 1960; Kellenberger *et al.*, 1959; Luria and Human, 1950; Murray *et al.*, 1950; Snustad *et al.*, 1972). Disruption requires the product of T4 gene D2b, because mutants in that gene no longer cause disruption (Snustad and Conroy, 1974). Nuclear disruption has been found to be nonessential for T4 growth, and is not required for host DNA degradation (Snustad *et al.*, 1974).

Tutas *et al.* (1974) have shown that T4 rapidly convert the folded bacterial genome to a less compact structure

within the first five minutes after infection, and that this process, called nucleoid unfolding, may require an early, or immediate early, T4 protein(s). Nucleoid unfolding is independent of nuclear disruption since mutants defective in nuclear disruption do not prevent unfolding (Snustad *et al.*, 1974). However, no mutants of T4 have been found which affect nucleoid unfolding, so the required T4 protein(s) has not yet been identified (Snustad *et al.*, 1974; Tutas *et al.*, 1974). Recently, we have found that the rif^R-2 mutation of *E. coli* causes a delay in nucleoid unfolding (see body of dissertation), thus being the first genetic system which affects bacterial genome unfolding.

After dirsupting and unfolding the nucleoid, T4 causes subsequent degradation of the host DNA to nucleotides, which can be reincorporated into T4 progeny DNA (Hershey *et al.*, 1953; Koch *et al.*, 1952; Kozloff, 1953; Kozloff and Putnam, 1950; Weed and Cohen, 1951). Degradation seems to occur in two stages. First the host DNA undergoes limited cleavage to fragments with a minimum molecular weight of about 10^6, then these fragments are further degraded to acid soluble pieces of DNA (Kutter and Wiberg, 1968). Bose and Warren (1969) proposed that this two-step process requires at least one exonuclease and two endonucleases that are highly specific and probably coded by T4. T4 endonuclease II is required for

the second stage, since mutants in that gene degrade the host DNA to fragments no smaller than 10^6 (Hercules *et al.*, 1971; Warner *et al.*, 1970). It has been proposed that T4 endonuclease IV is also involved in the second stage of degradation because it degrades DNA to fragments of 150 nucleotides, cleaves adjacent to cytosine residues, and has no effect on denatured T4 DNA, either glucosylated or nonglucosylated (Sadowski and Hurwitz, 1969). Also, some rII deletions which do not make endonuclease IV are defective in host DNA breakdown (Bruner *et al.*, 1973).

No T4 mutants have been found which affect the proposed first step of degradation, so the required T4 protein(s) is not yet known. It seems likely that nuclear unfolding may coincide with the first step in host DNA breakdown by T4, since unfolding also requires an early or pre-early T4 enzyme (Tutas *et al.*, 1974). However, there is no proof of this relationship at the moment.

Modification of Host RNA Polymerase After T4 Infection

Besides causing injury to the cell, T4 modifies much of the cell's macromolecular machinery so that it functions specifically for T4 development. Only the modifications of the host RNA polymerase will be reviewed, since the main emphasis of my research has been to study the roles of host RNA polymerase in T4 development.

Although T4 codes for its own DNA polymerase, it uses the host RNA polymerase for all of its transcription (Haselkorn *et al.*, 1969; di Mauro *et al.*, 1969). Numerous modifications have been found to occur to the host RNA polymerase during the course of T4 development, some of which are essential for transcriptional control. Transcription of T4 genes is a complex process which has been classified into three categories: immediate early (IE), delayed early (DE), and late (Bolle *et al.*, 1968a; Grasso and Buchanan, 1969; Milanesi *et al.*, 1969; Salser *et al.*, 1970). Each one of these steps requires some mechanism of control, and some involve known modifications of RNA polymerase.

No modification of the polymerase by T4 proteins seems to be required for immediate early transcription, since IE genes can be transcribed in the presence of chloramphenicol (Salser *et al.*, 1970). There is evidence that the polymerase uses *E. coli* sigma (σ) factor for transcribing IE genes, because it does not lose the σ factor until two or three minutes after infection (Bautz *et al.*, 1969). It has been proposed that loss of the factor may explain the shutoff of some early genes at two to three minutes after infection (Bautz *et al.*, 1969).

Delayed early transcription has been defined as that occurring between 1.5 and 5 minutes after infection. This requires a T4 protein(s) because delayed early genes

cannot be transcribed in the presence of chloramphenicol (Salser *et al.*, 1970).

The shutoff, as well as the initation, of T4 early messenger RNA occurs in different stages. Some early mRNA are produced throughout infection, while others are turned off at the initiation of DNA synthesis (Hosoda and Levinthal, 1968; Salser *et al.*, 1970) or when host RNA synthesis is shut off (Hosoda and Levinthal, 1968; Matsukage and Minegawa, 1967; Nomura *et al.*, 1966; Salser *et al.*, 1970; Terzi, 1967). With all the transcriptional switches required for early gene expression, only one mutant of T4 has been found to have an effect on early transcription, that effect being to alter the timing of early mRNA synthesis (Mattson *et al.*, 1974).

Late transcription requires continuous DNA synthesis (Bolle *et al.*, 1968b) and at least three viral coded gene products (Bolle *et al.*, 1968b; Notani, 1973; Wu, 1973). The RNA polymerase undergoes additional modifications by the time late transcription begins. Stevens (1972) has shown that four polypeptides are bound to RNA polymerase by five minutes after infection. She has identified two of these polypeptides as the products of genes 33 and 55, both of which are required for late transcription (Bolle *et al.*, 1968b; Notani *et al.*, 1970). The other two polypeptides (mol. wts. 14,000 and 10,000) have not yet been identified with a gene, so their function is unknown.

Recently, there has been evidence, both biochemical (Ratner, 1974) and genetic (Coppo *et al.*, 1974; Snyder and Montgomery, 1974), that the gene 45 product also interacts with RNA polymerase. Since the 45 product is required for late gene expression (Wu, 1973), it is probably involved in modifying the polymerase for late transcription.

Use of Host RNA Polymerase in DNA Synthesis

Recently there has been much evidence supporting the idea that RNA polymerase is required directly for DNA synthesis in a number of systems, including T4 (Brutlag *et al.*, 1971; Buckley *et al.*, 1972; Lark, 1972; Sugino *et al.*, 1972; Sugino and Okazaki, 1973). Buckley *et al.* (1972) have shown the existence of a transitory RNA:DNA copolymer early in T4 infection, and Speyer *et al.* (1972) have found low levels of RNA covalently bound to DNA in viral particles, thus implicating RNA polymerase in T4 DNA synthesis. Recent genetic evidence from our laboratory has shown that T4 DNA synthesis requires RNA polymerase for some function(s) other than gene expression (Snyder and Montgomery,1974). It is of interest in this regard that the gene 45 product which probably binds to RNA polymerase (see above) is required for T4 DNA replication as well as late messenger RNA synthesis (Epstein *et al.*, 1963).

These additional functions of RNA polymerase in T4 development may also require T4 coded control proteins, either to modify the RNA polymerase or the DNA template. The number and complexity of the roles that RNA polymerase play in phage production suggest that there are many T4 functions required for control. Since only a few T4 gene products are known to be required for control of RNA and DNA synthesis, there are probably others which have not yet been identified.

T4 DNA After Infection

RNA polymerase must interact with a DNA molecule for all of its known functions. Therefore, the structure of the DNA, and its modification during T4 development, may play an important role in regulation of transcription or DNA replication. It is known that T4 DNA binds to the host membrane early in infection (Altman and Lerman, 1970; Earhart *et al.*, 1968), and that binding does not require protein or DNA synthesis, but does require RNA synthesis (Earhart *et al.*, 1973). Membrane attachment seems to be required for DNA synthesis (Altman and Lerman, 1972; Earhart *et al.*, 1968; Kozinski and Lin, 1965; Miller and Kozinski, 1970), and is terminated late in infection when phage head formation releases progeny DNA from the membrane (Siegel and Schaechter, 1973). Thus head encapsulation of T4 DNA acts as one control over DNA synthesis. Besides binding to cell membrane, DNA also begins to be

associated with viral-coded proteins by four to five minutes after infection (Miller and Kozinski, 1970).

Structure of T4 DNA

The structure of parental T4 DNA differs from *E. coli* DNA even before T4-directed modification occurs. All T-even bacteriophage contain hydroxymethyl cytosine in place of cytosine in their DNA. These hydroxymethyl cytosine residues are glucosylated, with glucosylation of T2, T4, and T6 being characteristically divided among α-glucose, β-glucose, and α-gentiobiose groups (Kuno and Lehman, 1962; Lehman and Pratt, 1960). The hydroxymethyl cytosines of T4 DNA are all singly glucosylated, with about 70% containing the α-linkage and 30% the β-linkage (Lehman and Pratt, 1960). Glucosylation functions by protecting parental T4 DNA from host restriction, but seems to have no effect on DNA replication, since unglucosylated T4 (T4*) can grow normally in *Shigella* and restriction$^-$ mutants of *E. coli* (Hattman, 1964; Hattman and Fukasawa, 1963; Luria and Human, 1952).

The distribution of α- and β-glucosyl groups reflects the specificity of the α-glucosyl transferase, which is influenced by the nature of neighboring nucleotides (Lunt and Newton, 1965). It has been shown in all T-even phage that α-glucosyl transferase is unable to glucosylate those hydroxymethyl cytosines which are attached to another hydroxymethyl cytosine through their 5'-carbons,

and is limited in its ability to glucosylate those hydroxymethyl cytosines which have a purine nucleotide on either side of it (Pu-H-Pu) (Burton *et al.*, 1963; de Waard *et al.*, 1967; Lunt and Newton, 1965).

In T2 and T6, all of the hydroxymethyl cytosines not α-glucosylated are unglucosylated, while in T4 they are all β-glucosylated. Since α-glucosyl transferases from all T-even phage are similarly restricted in their glucosylation ability, it is possible that those left unglucosylated in T2 and T6, and β-glucosylated in T4, have a special function in phage development.

Pyrimidine clusters have been found in many organisms, both procaryotic and eucaryotic, suggesting by their ubiquity that they may play a specific role in DNA or RNA synthesis (for example, see Burton *et al.*, 1963; Champoux and Hogness, 1972; Szybalski *et al.*, 1966). Szybalski *et al.* (1966) showed that pyrimidine clusters are only found on the transcribing strand of DNA from a variety of organisms, and Champoux and Hogness (1972) showed these types of clusters to be grouped on the late third of λ DNA. Because they are not randomly distributed throughout the DNA molecule, and are found on transcribing strands, it is possible that they are involved in DNA synthesis-directed late messenger RNA transcription. However, no known function has yet been assigned to pyrimidine clusters.

Rif^R-2 Mutation in *E. coli*

Although much is known about the sequential steps in T4 development, details of the molecular mechanisms involved are still few. Our laboratory is studying the roles of host RNA polymerase in T4 development on a molecular basis. Our approach to this problem has been through the host's contribution, by using RNA polymerase mutations of *E. coli* which inhibit T4 growth. The mutation we have most fully characterized is referred to as rif^R-2 because it was obtained as a mutant resistant to rifampicin (Snyder, 1972). Strains harboring the rif^R-2 mutation grow normally, so the mutated RNA polymerase is only defective in T4 development, probably because it is unable to interact correctly with either a T4 coded protein(s) or with T4 DNA.

Although the rif^R-2 mutation is not temperature dependent (i.e., the strain is resistant to rifampicin at all temperatures), the effects it causes on T4 production are all cold sensitive (Snyder and Montgomery, 1974). At 40°, T4 produce normally-sized, clear plaques, while at 27°, the plaques are barely visible. The rif^R-2 mutation causes incomplete shutoff of host transcription (Snyder, 1972), a delay and reduction in rate of T4 DNA synthesis, and defective late gene expression (Snyder and Montgomery, 1974), all of which are also cold sensitive. Therefore, RNA polymerase is required for either gene

expression or some other function in all of these processes. We have shown that RNA polymerase is required for a function other than gene expression in DNA synthesis. This was done by using temperature-shift experiments to show that all the proteins required for replication accumulate at the nonpermissive temperature (Snyder and Montgomery, 1974).

T4 Mutants That Grow on Rif^R-2

We have also isolated and mapped mutants of T4 which grow better than wild type T4 ($T4^+$) on Rif^R-2. These mutations are referred to as gor, for *g*rows *o*n *R*if^R-2. The first gor mutations all mapped in the gene for β-glucosyl transferase (Montgomery and Snyder, 1973). β-glucosyl transferaseless mutants of T4 (βgt^-) partially overcome the rate reduction in DNA synthesis (but not the delay), and the block in late gene expression. We have shown that these effects are not due to the β-glucosyl transferase itself, but are caused by the unglucosylated state of the T4 DNA. This implies that at least some of the hydroxymethyl cytosine residues which are normally β-glucosylated may be important sites of interaction with RNA polymerase for both DNA synthesis and late gene expression (Snyder and Montgomery, 1974).

Two other gor-type mutations have been found and mapped, but they have not yet been further characterized. The second gor-type mutations found map in a new gene

(gor-2) located between genes 55 and αgt (Snyder and Montgomery, 1974). The product of this new gene may be a nonessential protein that interacts with host RNA polymerase during infection and becomes inhibitory on Rif^R-2. However, more experiments are needed to prove this point.

We found that known amber mutants in gene 45 show the gor phenotype when grown on su_2^+ strains of *E. coli* harboring the rif^R-2 mutation, probably because the reduced amount of 45 product produced in a suppressed amber restores some essential balance (Snyder and Montgomery, 1974). As mentioned above, this adds further genetic evidence that the gene 45 product interacts with RNA polymerase during T4 development.

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ARTICLE I

A Negative Effect of β-Glucosylation on T4 Growth in Certain RNA Polymerase Mutants of *Escherichia coli*: Genetic Evidence Implicating Pyrimidine-Rich Sequences of DNA in Transcription

By

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A Negative Effect of β-Glucosylation on T4 Growth in Certain RNA Polymerase Mutants of *Escherichia coli*: Genetic Evidence Implicating Pyrimidine-Rich Sequences of DNA in Transcription[1]

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In a previous publication, an RNA polymerase mutant of *Escherichia coli* was described which poorly supports the growth of the bacteriophage T4. Mutants of T4 have been isolated which grow better than wild-type T4 on the RNA polymerase mutant. These are missing the function β-glucosyl transferase and can grow better because the incoming parental DNA is partially unglucosylated. Only the β-, not the α-, glucosyl transferase is involved, suggesting a special role for at least some of the hydroxymethyl cytosines normally glucosylated with a β linkage. This phenomenon is independent of restriction.

Only the state of glucosylation of the parental, not of the progeny, DNA matters for this phenomenon, suggesting that part of the parental DNA may play a special transcriptional role throughout phage infection. An argument is presented that these experiments are consistent with the idea that pyrimidine-rich sequences are *in vivo* recognition sites for RNA polymerase.

INTRODUCTION

T4 promotes many transcriptional switches during its development. Host RNA synthesis is shut off very early in infection as well as the synthesis of some of the earliest appearing T4 RNAs (Terzi, 1967; Nomura *et al.*, 1966; Matsukage and Minagawa, 1967). Some later appearing RNAs are not made in the presence of chloramphenicol (Brody *et al.*, 1970; Grasso and Buchanan, 1969) which may indicate a separate regulatory mechanism. Also, the synthesis of some of the later appearing RNAs continues throughout infection while the synthesis of some ceases at about the time of onset of the latest appearing messenger RNAs which are in turn made only after T4 DNA replication (Bolle *et al.*, 1968). The program of T4 protein synthesis substantially coincides with the program of RNA synthesis (Hosoda and Levinthal, 1968).

Despite this spate of transcriptional switches, there are only two genes of T4 whose products are known to be involved in transcriptional regulation. These are genes 33 and 55, whose products are partially and completely required, respectively, for the synthesis of T4 late messenger RNA (Bolle *et al.*, 1968). There are a number of possible explanations for the dearth of known regulatory genes. One is that they have remained undiscovered or, if they have been discovered, their role in regulation has remained unnoticed. Another possible explanation is that they are relatively nonessential and thus were not included among the original conditional-lethal assortment (Epstein *et al.*, 1963). Different approaches

[1] This is Journal Article No. 6205 from the Michigan Agricultural Experiment Station.

may lead to the discovery of heretofore undiscovered regulatory genes.

RNA polymerase mutants of the host offer another approach to this problem. The rationale is as follows. T4 uses the host RNA polymerase for all of its transcription (Haselkorn *et al.*, 1969; di Mauro *et al.*, 1969). Presumably, at least some of the transcriptional switches which occur during T4 development are due to the interaction between T4-coded factors and host RNA polymerase. These T4-coded factors could either bind to the polymerase or alter it by some enzymatic mechanism. While mutant RNA polymerases must of necessity function for the growth and replication of the cell, it is possible that some may not properly interact with the responsible virus-coded factors, leading to a defective transcriptional switch and possibly defective growth of the virus. Conversely, T4 mutants may exist which overcome the effect of the mutant RNA polymerase and these may be in genes involved in regulation.

With this rationale in mind, RNA polymerase mutants of *E. coli* were isolated and T4 growth on them was studied. The phenotype of T4 growth on one such mutant has been discussed (Snyder, 1972). The RNA polymerase mutation leads to a significant delay in the production of T4 viral particles.

T4 mutants do exist which grow better than wild-type T4 on the RNA polymerase mutant. They are the subject of this publication.

MATERIALS AND METHODS

Phage strains. T4 R4 (βgt^-)[2] and $R20_a$ (αgt^-)[2] and the double mutant HA 57 (αgt^-, βgt^-) were from the collection of H. Revel (Georgopoulous and Revel, 1971), and were obtained from J. Wiberg. The amber mutants used in the mapping were N122 (gene 42), N81 (gene 41), and E1140 (gene 62) from W. Wood. Plke was obtained from D. Freidman.

[2] βgt^-: β-glucosyl transferaseless; αgt^-: α-glucosyl transferaseless.

Bacterial: The following strains were used as recipients of the Rif^R-2 mutation because of the underlined property.

C600 (λcI857):	am su$^+$ TL$^-$
NF58:	am su$^-$ arg$^-$ met$^-$
K803:	r6$^-$,r2,4$^-$ (permissive for unglucosylated T4) r$^-$m$^-$, met$^-$, gal$^-$, (Wood, W. B., 1966)

The strains NF58-Rif^R-2 and K803-Rif^R-2 were constructed by transduction from C600λ857-Rif^R-2. Met$^+$ recombinants were selected, and in each case about 20% were also rifampicin resistant. One of each of these was chosen for the experiments. The K803 transduction was performed at a low multiplicity to avoid P1 lysogens which are restrictive for unglucosylated T4 (Revel and Georgopoulos, 1969). The UDPG ppase$^-$ mutant used was W4597, obtained from J. Wiberg.

Growth of virus was measured at 30° in M9S medium: 5.54 g Na_2HPO_4 (anhyd.), 3 g KH_2PO_4 (anhyd.), 5 g NaCl, 1 g NH_4Cl, 4 g glucose, 10^{-3} mole $MgSO_4$, and 10 g casamino acids (Difco) per liter. The multiplicity of infection was determined from a careful titer of the virus and the turbidity of the bacterial suspension. The bacteria were at a concentration of 4×10^8 per ml for all experiments. For low multiplicity experiments ($\sim$0.1) it was assumed that every virus infected a cell. CsCl-purified T4 were used for the high multiplicity experiments (>3). Intracellular phage production was measured by disrupting the cells with $CHCl_3$ at the time indicated and plating with an exponentially growing bacterial indicator and a soft agar overlay.

Genetic crosses were performed by absorbing the viruses to be crossed to the permissive bacteria for 5 min at room temperature at an m.o.i. of 3 of each and then diluted 1:100 into tryptone broth (10 g tryptone, 5 g NaCl per liter) at 30° and shaken for 45 min before $CHCl_3$ was added.

RESULTS

Isolation of T4 Mutants Which Grow Better Than Wild-Type T4 on Rif^R-2

T4 mutants which make distinct plaques on Rif^R-2 arise spontaneously with a frequency of about 1 in 10^4. Three such mutants which had arisen independently were isolated by growing three separate lysates on Rif^R-2 starting from wild-type plaques and picking *gor*-type plaques after plating on Rif^R-2. The growth of all three mutants is enhanced on Rif^R-2 to about the same extent and is relatively independent of the multiplicity of infection and the strain of *E. coli* harboring the Rif^R-2 mutation. The growth of one mutant on Rif^R-2 is compared to wild-type T4 growth on Rif^R-2 and on wild-type *E. coli* in Fig. 1. The mutant grows

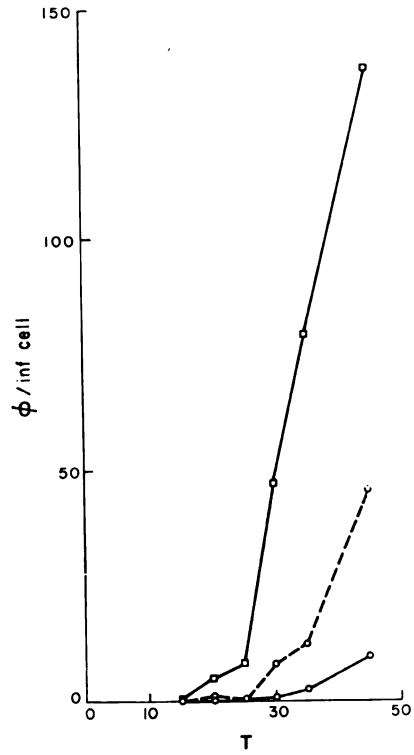

FIG. 1. Growth of a T4 mutant on Rif^R-2: T4$^+$, C600 (□---□); T4$^+$, C600-Rif^R-2 (○ ○); T4 *gor*-1, C600-Rif^R-2 (○- - ○); each at an m.o.i. of 5.

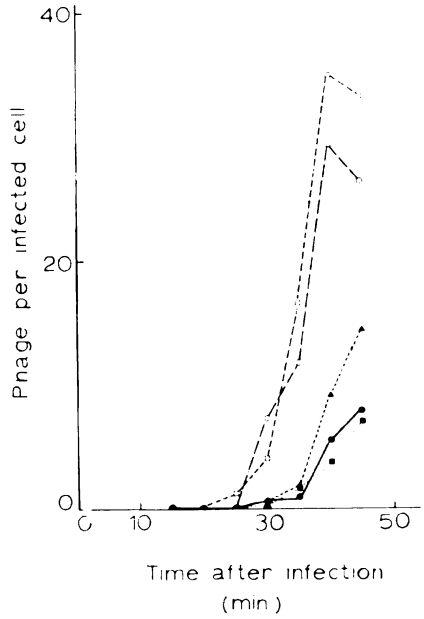

FIG. 2. Wild-type dominance and inability of *gor* mutants to complement. *gor*-1, *gor*-2 mixed (□- - -□); *gor*-1 (○— —○); *gor*-1, T4$^+$ mixed (▲-----▲); T4 (●——●); *gor*-2, T4$^+$ mixed (■···■). All on C600-Rif^R-2 and at a total m.o.i. of 7.

significantly better than the wild-type T4 on Rif^R-2. On wild-type *E. coli*, the mutants grow about as well as wild type T4. (Data not shown.)

These mutants have been called *gor* (*g*row *o*n *R*if^R-2).

The Three Independently Isolated gor Mutants Have Mutations in the Same Cistron

The wild type is dominant to *gor* in mixed infections as shown in Fig. 2. This fact allows a simple test of whether *gor* mutants can complement each other since, if they occur in different cistrons, they should grow like the wild type in mixed infections. None of the three *gor* mutants can complement, so they are all mutant in the same cistron. Data for two are shown in Fig. 2. Mutations to the *gor* phenotype may occur in other cistrons, but there has been no thorough search for them.

TABLE 1

MAP POSITION OF *gor* MUTANTS

Cross:	*gor*, 41⁻, 42⁻ × 62⁻		*gor*, 42⁻, 62⁻ × 41⁻	
	Plaques	PFU/ml	Plaques	PFU/ml
Indicator bacteria				
C600	414	4.14×10^{10}	466	4.66×10^{10}
C600 Rif^R-2	230	2.30×10^{10}	269	2.69×10^{10}
NF58 Rif^R-2	150	1.50×10^{8}	548	5.48×10^{8}
% Recombination	0.36		1.18	
No. of *am*$^+$ which are *gor*	0/20		7/20	

Mapping gor Mutations

Recombination frequencies between known amber mutations and *gor* mutations have been measured by constructing the double mutant, crossing it against wild-type T4, and measuring the frequency of *gor*, *am*$^+$ recombinants by plating on an *am* su$^-$ *E. coli* harboring the rif^R-2 mutation. A low recombination frequency (~4%) was obtained with a mutation in gene 42. To ascertain on which side of the 42 mutation the *gor* mutations lie, the triple mutants *gor*, *am* 41$^-$, *am* 42$^-$ and *gor*, *am* 42$^-$, *am* 62$^-$ were crossed with *am* 62$^-$ and *am* 41$^-$ single mutants, respectively, and the frequency of *gor*, *am*$^+$ recombinant types was measured. If *gor* mutations lie to the left of the *am* 42$^-$ mutation (but to the right of the *am* 41$^-$ mutation), three crossovers are required in the first cross and only one in the second cross. The reverse is true if the mutation lies to the right of the *am* 42$^-$ mutation (but to the left of the *am* 62$^-$ mutation). The results of the crosses are shown in Table 1; *gor*, *am*$^+$ recombinants arose with a frequency of 0.36% and 1.18% in the first and second crosses, respectively, suggesting that the *gor* mutations lie between the *amber* mutations in genes 41 and 42.

Additional evidence for this map position was obtained by picking *am*$^+$ recombinants and testing these to see how many were also *gor*. Fewer would be expected to be *gor* in the first than in the second cross, and this was observed. Thus, the *gor* mutations lie between the *am* 41 and *am* 42$^-$ mutations.

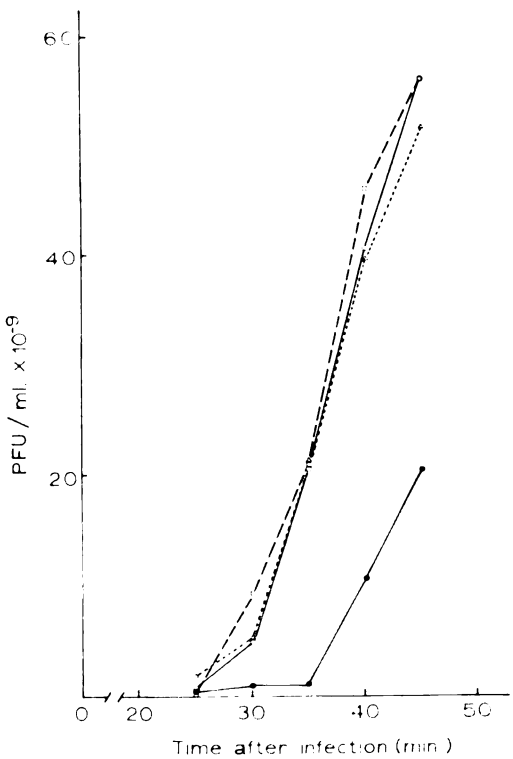

FIG. 3. Identity of *gor* and βgt$^-$. R4 (βgt$^-$) (□ □), *gor*-1 (○ ○), T4$^+$ (● ●), all on C600-Rif^R-2 at a m.o.i. of 7. R4 + *gor*-1 on C600-Rif^R-2 at a m.o.i. of 3.5 of each (△-----△).

They are complemented by both of these mutants (data not shown), so they must lie in a distinct cistron between genes 41 and 42.

The Identity of gor and βgt$^-$

There is one known gene which maps in this region, the gene for the enzyme β-glucosyl transferase (Georgopoulos, 1968). A

TABLE 2

gor ARE βgt⁻

Cross	No. of progeny which do not spot on B r
αgt⁻ × βgt⁻	4/48
αgt⁻ × *gor*-1	5/48
αgt⁻ × *gor*-2	6/48
αgt⁻ × *gor*-3	5/48

βgt⁻ mutant grew like *gor* and did not complement *gor* mutations, as shown in Fig. 3. It is possible that this βgt⁻ mutant is a deletion which includes an adjacent cistron responsible for the gor⁺ phenotype. To test this, all three *gor* mutants were crossed against a mutant with a mutation in the cistron for α-glucosyl transferase and the frequency of the progeny which could grow only on *E. coli* permissive for unglucosylated T4 was measured. Only the double mutant (αgt⁻,βgt⁻) is completely restricted on wild-type *E. coli* and these should arise at a frequency of about 10% if the *gor* mutants are also βgt⁻. The result is shown in Table 2. About 10% of the progeny of each cross are capable of growing only on permissive *E. coli*. Thus, all three *gor* mutants are also βgt⁻ indicating strongly that it is an inability to make an active β-glucosyl transferase which makes them *gor*.

Influence of α-Glucosyltransferase on T4 Growth in Rif^R-2

Since the strain of *E. coli* which was used in the original selection of *gor* mutants is nonpermissive for unglucosylated T4, αgt⁻ mutants and the double mutants αgt⁻,βgt⁻ would not have been detected even if the α-glucosylation is also inhibitory since both these mutants would be at least partially restricted. Accordingly, a Rif^R-2 strain permissive for unglucosylated T4 was constructed and the growth of the double mutant αgt⁻,βgt⁻ was compared to that of βgt⁻. The result is shown in Fig. 4. The double mutant grew no better than the single mutant demonstrating that α-glucosylation is not inhibitory even though

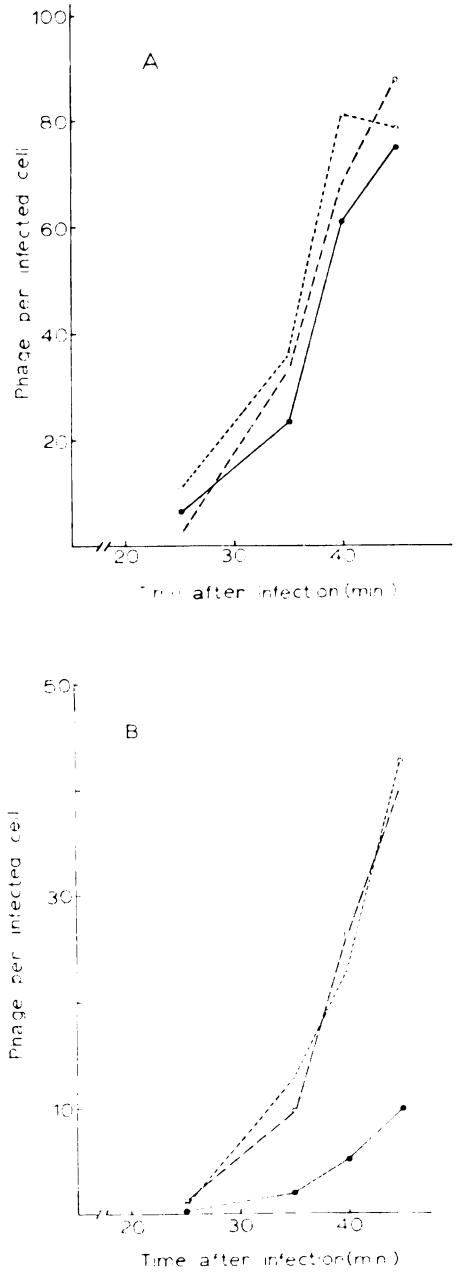

FIG. 4. Growth of the double mutant αgt⁻, βgt⁻ compared to βgt⁻ on Rif^R-2. T4βgt⁻ (○ - - -○); T4βgt⁻, αgt⁻ (□ □); T4⁺ (●— —●). (A): All grown on K803, m.o.i. = 7. (B) All grown on K803-Rif^R-2, m.o.i. = 7.

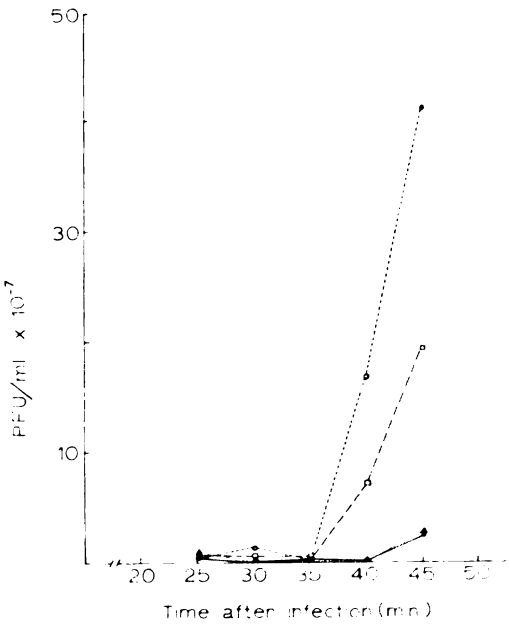

FIG. 5. The phenotype *gor*$^+$ is subject to phenotypic mixing. T4βgt$^-$ (○-----○), T4$^+$ (●—●); βgt$^-$ and T4$^+$ mixed 1:1 before infection (□ - - □); βgt$^-$ and T4$^+$ grown together in the previous infection (△···△). M.o.i. = 0.1 throughout. Growth on K803-Rif^R-2.

80–86% of the hydroxymethyl cytosines are α-glucosylated with the βgt$^-$ mutant (Georgopoulos and Revel, 1971.)

The fact that βgt$^-$ mutants are *gor* even on permissive *E. coli* indicates that this effect is directly due to β-glucosylation of the DNA and has nothing to do with restriction.

Full glucosylation of Only the Parental DNA Is Sufficient to Inhibit Growth on Rif^R-2

If βgt$^-$ mutants are grown with wild-type T4 in mixed infections, the progeny viruses will be fully glucosylated, even though half of them will have the βgt$^-$ genotype. If these viruses are then used to infect cells at low multiplicity one-half of the infected cells will receive a wild-type virus and one-half will receive a βgt$^-$ virus. The ones that receive a βgt$^-$ will exhibit a growth curve like cells infected by the wild type, if only the state of glucosylation of the parental DNA matters for this phenomenon since the progeny DNA will again be only partially glucosylated. If glucosylation of the progeny DNA is inhibitory, we would expect the βgt$^-$ progeny to grow better than wild type. Figure 5 shows the results of such an experiment. The growth is as though every bacterium received a wild-type virus even though approximately 50% of the progeny of the original mixed infection exhibited the βgt$^-$ genotype. (Data not shown.) Thus only the state of glucosylation of the parental DNA matters. Shown for comparison is the growth of wild-type T4 and T4 βgt$^-$, mixed in equal numbers, and used for infection at the same low multiplicity.

T4 Growth on Rif^R-2 Can Be Prevented by β-Glucosylation of Parental DNA after Infection

The above experiment demonstrates that full glucosylation of parental DNA is sufficient to interfere with growth of the virus on Rif^R-2. However, the growth of the virus might also be affected by β-glucosylation of parental DNA after infection either by *de novo* synthesized β-glucosyl transferase or by enzyme conceivably encapsulated in the viral particle.

Unglucosylated T4 can be prepared by growth on uridine-diphosphoglucose-less (UDPG ppase$^-$) mutants of *E. coli* because UDPG serves as the donor of glucose in the glucosylation reaction (Hattman and Fukasawa, 1963). These unglucosylated T4 should grow like βgt$^-$ on nonrestricting *E. coli* harboring the Rif^R-2 mutation if only the state of the incoming parental DNA is important, and they should grow as the wild type if β-glucosylation of parental DNA after infection can inhibit phage growth. The results of one such experiment are shown in Fig. 6. Even unglucosylated wild-type growth is inhibited and the wild type is still dominant to βgt$^-$ in mixed infections. (Data not shown.) In the experiment shown in Fig. 6, T4βgt$^-$* grew less well than T4βgt$^-$ but this is not generally the case. Thus, β-glucosylation of parental DNA after infection can interfere with phage growth on

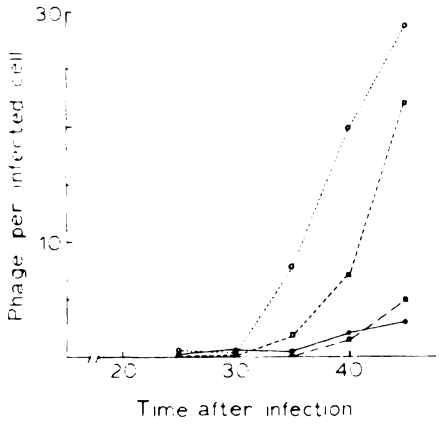

FIG. 6. Unglucosylated wild-type T4 (T4*) grows poorly on Rif^R-2. T4βgt⁻ (○-----○); T4βgt⁻* (□- - -□); T4⁺ (●-- --●); T4* (■— —■) Growth on K803-Rif^R-2, m.o.i. = 7.

Rif^R-2. It should perhaps be mentioned that the UDPG ppase⁻ mutant used in this experiment permits low levels of glucosylation of T4 DNA (Fukasawa and Saito, 1964), and it is difficult to assess what effect this low level of glucosylation may have on the experiment.

DISCUSSION

There is ample evidence to support the contention that the glucosylation of T even phage DNAs performs no necessary function other than protection against restriction since unglucosylated phage can be cultivated in *Shigella* (Luria and Human, 1952) or in *E. coli* mutant in the r6 and r2,4 restriction functions (Revel, 1967). However, the possibility is not excluded that glucosylation may exert a negative effect on phage growth, interfering, in some situations, with reactions involving glucosylated DNA molecules.

This work demonstrates such a negative effect of glucosylation. Partially glucosylated T4 βgt⁻ mutants are able to grow better than wild-type fully glucosylated T4 on certain RNA polymerase mutants of *E. coli*. This phenomenon only depends upon the state of glucosylation of those hydroxymethyl cytosines normally glucosylated by the β-glucosyl transferase (about 30%) and is independent of the ones (about 70%) normally glucosylated with an α-linkage. Thus a special role in phage development is suggested for at least some of the hydroxymethyl cytosines normally glucosylated by the β-glucosyl transferase. Also, only the state of glucosylation of the parental DNA (or part of it) is important since the phenomenon is subject to phenotypic mixing. However, glucosylation of the parental DNA after infection is also sufficient to prevent growth on Rif^R-2, since unglucosylated wild-type T4 (T4*) grow as poorly as the wild type and are still dominant to βgt⁻ in mixed infections.

There are two possible general explanations for the unique role of parental DNA. One is that the parental DNA is the only DNA present during the synthesis of those RNAs which are limiting in the RNA polymerase mutant. According to this explanation the limiting RNAs could only be made after the synthesis of β-glucosyl transferase and before the onset of T4 DNA replication.

The alternative explanation is that part of the parental DNA containing the hydroxymethyl cytosines whose glucosylation is critical for growth on the RNA polymerase mutant preserves its autonomy as parental DNA throughout some, if not all, of the latent period. If this explanation were true, it would not be the first demonstration of a unique role for parental DNA during viral development. Only the parental replicative form of φX174 can direct the synthesis of progeny replicative forms (Denhardt and Sinsheimer, 1965) and λ parental DNA can only find its way into progeny particles vis-à-vis the recombination system (Stahl *et al.*, 1972).

Why does the inhibitory effect of β-glucosylation only become apparent in the RNA polymerase mutant? The effect of the RNA polymerase mutation could be either direct or indirect. Some RNA polymerase mutations are known to affect cellular morphology (Doi *et al.*, 1970) and, in fact, the

Rif^R-2 mutation does affect T4 adsorption and cellular growth of some strains of *E. coli* especially at lower temperatures (L. Snyder, unpublished observations). It is possible that Rif^R-2 is altered in some structural component (e.g., a membrane binding site for DNA) so that fully glucosylated T4 DNA cannot properly interact with it either directly or because hypothetical T4 gene product(s) which are requiring for the binding of fully glucosylated DNA do not properly recognize the altered cellular component. However, we prefer models in which the effect of the RNA polymerase mutation is direct because the enhanced growth of βgt$^-$ mutants in Rif^R-2 is relatively independent of the strain of *E. coli* harboring the Rif^R-2 mutation and because the Rif^R-2 mutation delays a change in RNA polymerase which occurs after T4 infection (Snyder, to be published).

A very specific model which incorporates a direct role for the RNA polymerase mutation immediately suggests itself. The α-glucosyl transferases of T2 and T4 have a sequence specificity since they will not glucosylate approximately 25% of the hydroxymethyl cytosines in their DNA. The hydroxymethyl cytosines which are left unglucosylated have been shown to be preferentially those which are adjacent to other hydroxymethyl cytosines (Lunt and Newton, 1965; de Waard *et al.*, 1967). Pyrimidine-rich regions have been detected in T4 DNA as well as many other DNAs and have been circumstantially implicated in transcription because they occur on the strand of DNA which is used for transcription at a particular locus (Szybalski *et al.*, 1966). The important fact for this model is that the hydroxymethyl cytosines in these pyrimidine-rich sequences would be left largely unglucosylated by the α-enzyme. Assume that they are recognition signals for RNA polymerase. If they are left unglucosylated, they may closely resemble recognition signals of the host so that the host polymerase unmodified by T4 could recognize them and the modification which may not occur in the RNA polymerase mutant would not be required for transcription of the genes which they serve. Ergo, with the βgt$^-$ mutant the need for the RNA polymerase modification is bypassed and the phage grow better. But why then, by this model, can some T4 signals be recognized by the unmodified host polymerase since the necessary modification may require T4 protein synthesis and T4 DNA can be transcribed by unmodified *E. coli* RNA polymerase *in vitro*? The answer may lie in the fact that there are two types of pyrimidine rich sequences in T4 DNA; those which are rich in hydroxymethyl cytosine and those which are rich in thymidine (Guha and Szybalski, 1968). The thymidine-rich regions would always be unglucosylated and could be the recognition signals for the earliest-appearing T4 messenger RNAs whereas the hydroxymethyl cytosine-rich regions would require an RNA polymerase modification for their recognition if they are glucosylated, and they would then be the signals for some of the later-appearing early messenger RNAs.

The pyrimidine-rich sequences on λ DNA have recently been mapped (Champoux and Hogness, 1972) and found to be sometimes internal to transcription units on λ DNA. These authors have suggested that they may serve a "divider" function in transcription, a hypothesis consistent with our data thus far.

Of the T-even coliphages, T2, T4, and T6, only T4 has fully glucosylated DNA (Lehman and Pratt, 1960) giving it additional protection against restriction and therefore presumably broadening its host range. However, our work indicates that T4 pays a price for this increased protection. By covering the remaining 30% or so of the hydroxymethyl cytosines, at least some of which, according to our work, play a very special role in phage development, it interferes with a reaction in which parental T4 DNA molecules must participate, and very possibly additional gene products are required to

overcome this interference. These may be gene products which are required only because T4 DNA is fully glucosylated and which cannot function properly in the RNA polymerase mutant. Thus, although glucosylation of DNA is specific for T-even coliphages of all the known viruses (and therefore is without general significance), and although it apparently plays no role in phage development except for protection against restriction, it may prove very useful as a probe to discover the nature and function of special sequences on DNA.

ACKNOWLEDGMENTS

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ARTICLE II

Bacteriophage T4 Growth on an RNA Polymerase Mutant of *Escherichia coli*: Effect of β-Glucosylation on Early T4 Functions

By

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Manuscript to be submitted for publication

Bacteriophage T4 Growth on an RNA Polymerase Mutant of *Escherichia coli*: Effect of β-Glucosylation on Early T4 Functions

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In previous publications, we have reported that an RNA polymerase mutation, rif^R-2, of *Escherichia coli* poorly supports the growth of T4. Rif^R-2 has a direct effect on both DNA synthesis and late gene expression. The effects of rif^R-2 are partially suppressed by β-glucosyl transferaseless mutants of T4, and β-glucosylation is inhibitory both early and late in infection.

Rif^R-2 also has an effect on early gene expression causing disproportionate synthesis of some early T4 proteins. However, β-glucosylation has no effect on this early gene expression defect, although we have shown that β-glucose inhibits an early function required for phage production. Rif^R-2 also causes defective host nucleoid unfolding, and a delay in host DNA degradation. β-glucosylation is inhibitory for host DNA degradation, suggesting that the transcription of β-glucosylated hydroxymethyl cytosines in T4 DNA is somehow involved in this step. Our new data further support the model proposed earlier that RNA polymerase must alter the structure or association of T4 parental DNA early in infection for DNA replication and late transcription to occur.

INTRODUCTION

E. coli RNA polymerase performs several functions in addition to transcription of *E. coli* messenger, transfer,

and ribosomal RNA. RNA polymerase has been shown to be involved in DNA synthesis (Lark, 1972; Blair *et al.*, 1972; Sugino *et al.*, 1972) and must function to hold DNA in a highly folded state, referred to as a nucleoid (Stonington and Pettijohn, 1971; Dworsky and Schaechter, 1973; Worcel and Burgi, 1972). In addition, Pettijohn and Hecht have shown that a functioning core RNA polymerase seems to be continuously required to maintain the highly folded state of the nucleoids, since cells briefly treated with rifampicin yielded only unfolded genomes upon lysis (Pettijohn and Hecht, 1973).

E. coli RNA polymerase also performs several functions in T4 development. It is required for both early and late transcription (Haselkorn *et al.*, 1969; di Mauro *et al.*, 1969) and for some additional function required for DNA synthesis (Snyder and Montgomery, 1974). Modifications of the host polymerase are required for some of these functions since after infection the proteins associated with RNA polymerase are changed. Two such proteins, the products of genes 55 and 33, are required for late messenger RNA synthesis (Bolle *et al.*, 1968; Notani, 1973) and are known to bind to host RNA polymerase after T4 infection (Stevens, 1972; Horvitz, 1973; Ratner, 1974). In addition, the gene 45 product is required for late messenger synthesis (Wu *et al.*, 1973) and there is recent evidence, both biochemical (Ratner, 1974) and

genetic (Snyder and Montgomery, 1974; Coppo *et al.*, 1974) that it also binds to RNA polymerase. Ratner has found other T4 proteins which are retained by RNA polymerase columns, but it is not known if their binding is essential for some step in phage development (Ratner, 1974).

We have been studying the roles of host RNA polymerase in T4 development, as well as the proteins which interact with the polymerase, by using RNA polymerase mutants of *E. coli* which inhibit T4 growth. The mutation we have characterized is referred to as rif^R-2. The rif^R-2 mutation has a cold-sensitive effect on T4 development; at 40°, T4 production is almost normal, while at 27° T4 development is severely retarded. We have found that the rif^R-2 mutation inhibits both DNA synthesis and late gene expression (Snyder and Montgomery, 1974).

Several mutants of T4 which grow better than $T4^+$ on Rif^R-2 were obtained with the hope of finding T4 genes coding for transcriptional control proteins. The first gor (*g*rows *o*n *r*if^R-2) mutations which were found are in the gene for β-glucosyl transferase, indicating that the glucosylated state of hydroxymethyl cytosines is important in some interactions of T4 DNA with RNA polymerase from Rif^R-2 cells. Two other types of gor mutations have been found in genes which may code for proteins which interact directly with RNA polymerase. Amber mutants in gene 45

were found to grow better than $T4^+$ on Rif^R-2 strains which are su_2^+, and mutations in a new gene (gor-2), located between genes 55 and αgt, cause T4 to grow better than $T4^+$ on Rif^R-2 (Snyder and Montgomery, 1974).

We have found that β-glucosylation inhibits both late gene expression and the rate of DNA synthesis, but seems to have little or no effect on the delay in DNA synthesis (Snyder and Montgomery, 1974). In phenotypic mixing experiments where the parental, but not the progeny, DNA is β-glucosylated, we have shown that there is a function inhibited by β-glucosylation that occurs early in infection (Montgomery and Snyder, 1973; Snyder and Montgomery, 1974). This suggests that the rif^R-2 mutation has an effect on some early T4 function. This paper reports experiments done to determine the effect of rif^R-2 and β-glucosylation on early T4 functions.

MATERIALS AND METHODS

a) Bacterial Strains and Phage

All bacterial strains used were previously described (Montgomery and Snyder, 1973).

T4 *am* A453 (gene 32) was obtained from H. Revel. The β-glucosyl transferase mutant (βgt^-) used was R4, obtained from J. Wiberg. The double mutants T4 *am* N122,R4 (genes 42, βgt) and T4 *am* A453, r1272 (gene 32, deleted for cistrons rII A and B) were constructed in this laboratory.

b) Media and Buffers

M9S, described previously (Snyder and Montgomery, 1974), was used for all experiments except those in which proteins were labeled. The M9S media were modified for protein labeling experiments by replacing the casamino acids with an amino acid mixture. Alanine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine (all the L-isomers) were added to a final concentration of 20 μg/ml. Arginine and methionine were added to a final concentration of 50 μg/ml.

The M9 buffer and tryptone broth and plates were described earlier (Snyder and Montgomery, 1974).

c) Infections and Survivor Determinations

Infections were carried out as described below for all experiments except those shown in Figure 1. The infection and labeling procedures for those experiments are described in the figure legend.

Cells were grown at 40° to an O.D. of 0.4 at 625 mμ, and infected with CsCl purified phage at a m.o.i. of 5 or 10. At two minutes after infection, cells were shifted to 27°, the nonpermissive temperature for T4 growth on Rif^R-2. Cells were infected at the higher temperature because phage adsorb better and are less sensitive to ghost exclusion at 40° than at 27°.

Survivor samples were taken at 3 minutes after infection (1 minute at 27°) by diluting into M9 buffer on ice.

Infections were terminated by pouring cultures over ice and NaN_3 at a final concentration of 10^{-2}M. Only infections producing survivors of less than 4%, and usually less than 2%, were used for the different analyses.

d) SDS-Polyacrylamide Gel Electrophoresis

Cells (2.5 ml) were infected and shifted as described in "c)" ^{14}C-leucine, at a concentration of 1.3 µc/ml and 1 µg/ml, or ^{3}H-leucine, at a concentration of 7.7 µc/ml and 1 µg/ml, was added after infection for the desired times.

Infection was terminated as described in "c)" and the cells were concentrated 10x by centrifuging and resuspending in 0.25 ml of .01M Tris-HCl, pH 8.0; 0.001M EDTA; 1% SDS. 2-mercaptoethanol was added to a final concentration of 2%, the samples were heated in a boiling water bath for 3 minutes, and stored at 4°. Just before electrophoresis, dye solution (20% sucrose, 0.008% Pyronin Y) was added to the sample at a ratio of 1:3. Samples (0.01 ml) were layered onto each gel.

Gels containing 1% SDS and 9% acrylamide were made according to the procedure of Fairbanks *et al.* (1971), with the following modification. Acid-cleaned glass

tubes were coated with dimethyl dichlorosilane (Sigma) and allowed to dry for several hours. Tubes were rinsed with hot water, 95% ETOH, and water, then allowed to dry before pouring gels.

Gels (8.5 cm) were run at 70 volts. Under these conditions, it took approximately 2 hours for the dye front to migrate through the gel. Slices of 1 mm were collected with a Gilson automatic gel slicer. The samples were incubated overnight at 35° with constant shaking in 0.2 ml of 1% SDS to elute proteins from the gel before adding 5.0 ml Aquasol (New England Nuclear) to each vial for counting.

e) Alkaline Sucrose Gradients

Cells were labeled at 40° for 1 hour before infection, with ^{3}H-thymidine at a concentration of 230 µc/ml and 28 µg/ml. Infected cells were lysed according to the procedure of Hercules *et al.* (1971) except that M9S medium was used in place of the GCA (glycerol-casamino acids) media they described.

Alkaline sucrose gradients (5% to 20%) were generated and run as described by Hercules *et al.* (1971). Fractions were collected from the bottom of the tube, TCA precipitated, collected on glass fiber filters (Whatman, GF/A), and counted in a toluene-base scintillation fluid.

f) Nucleoid Disruption

Cells (5 ml) were labeled at 40° for 30 minutes before infection, with ^{3}H-thymidine at a concentration of 30 μc/ml and 1 μg/ml. Infections proceeded and were terminated as described in "c)". In experiments using chloramphenicol, it was added at a final concentration of 150 μg/ml at 5 minutes after infection.

The lysis procedure of Stonington and Pettijohn (1971) was carried out with the modifications of Worcel and Burgi (1972). However, the Sorvall spin of the lysed mixture was omitted, and 0.1 ml was layered directly onto a 10% to 30% sucrose gradient. Gradients were generated and run as described by Worcel and Burgi (1972).

Fractions were collected from the bottom of the tube, TCA precipitated, collected on glass fiber filters (Whatman, GF/A), and counted in a toluene-base scintillation fluid.

RESULTS

Since the rif^{R}-2 mutation has an effect on late protein synthesis, we wished to determine if it also has an effect on early protein synthesis. Initial studies indicated that rif^{R}-2 does not prevent the appearance of at least some early T4 proteins. The synthesis of two early enzymes, β-glucosyl transferase and dihydrofolate reductase, are produced at nearly normal levels after only

slight lags, and all the proteins required for DNA synthesis are present at the early times (Snyder and Montgomery, 1974). However, a more detailed study of the effect of rif^R-2 on early protein synthesis was needed.

The Effect of rif^R-2 and β-Glucosylation on Amino Acid Incorporation

The rate of protein synthesis after $T4^+$ infection on Rif^R-2 and wild-type *E. coli* was measured as the amount of ^{3}H-leucine incorporated during a 2 minute pulse (Figure 1A). The rate of synthesis is not substantially affected by rif^R-2 early in infection, but it is later. By 36 minutes after infection, amino acid incorporation in Rif^R-2 is reduced by about 30% from that in wild-type cells.

The effect of β-glucosylation on early protein synthesis was also determined in the absence of DNA replication. Figure 1B shows the results obtained with continuous labeling. There is no difference between N122 and N122,βgt^- in the amount of radioactive leucine incorporated, indicating that β-glucosylation has no effect on the rate of protein synthesis early in infection in the absence of DNA replication.

The Effect of rif^R-2 on the Synthesis of Individual T4 Proteins

Although the rate of protein synthesis is not affected by the rif^R-2 mutation early in infection, this does

Figure 1. The rate of amino acid incorporation after $T4^+$ infection.

Figure 1A - The rate of T4 protein synthesis after infection of K803 (-O-) and K803-Rif^R-2 (-□-). Cells were infected at a m.o.i. of 5 at 40° and shifted to 27° at 2' after infection by diluting 1/10 into media at 27°. Cells (1 ml) were pulse labeled for 2 minutes with ^{3}H-leucine at 1.3 μc and 1 μg/ml and incorporation stopped with 0.1 ml of 50% TCA. Four milliliters of 5% TCA were added, the precipitate centrifuged and resuspended in 0.3 ml 2% KOH, reprecipitated with 5% TCA, and collected on membrane filters for counting.

Figure 1B - The rate of T4 protein synthesis after infection with T4 *am* N122 (-□-) and T4 *am* N122, βgt$^-$ (-O-). Cells were infected and shifted as in Figure 1A. ^{3}H-leucine was added at 4' after infection at 1.3 μc and 1 μg/ml. Samples (1 ml) were stopped with 0.1 ml 50% TCA. Samples were precipitated and collected as in Figure 1A.

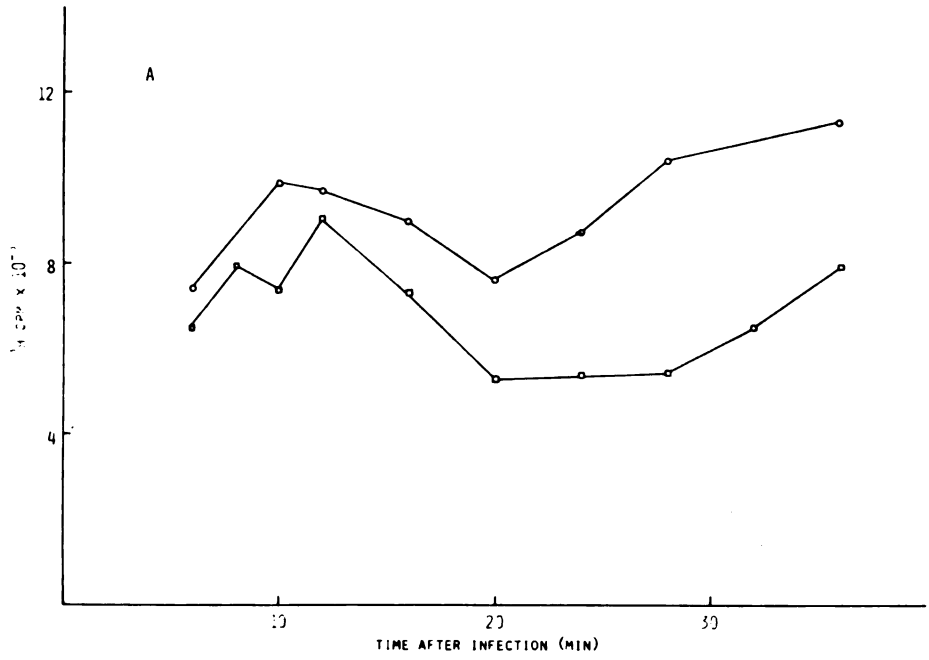

Figure 1A

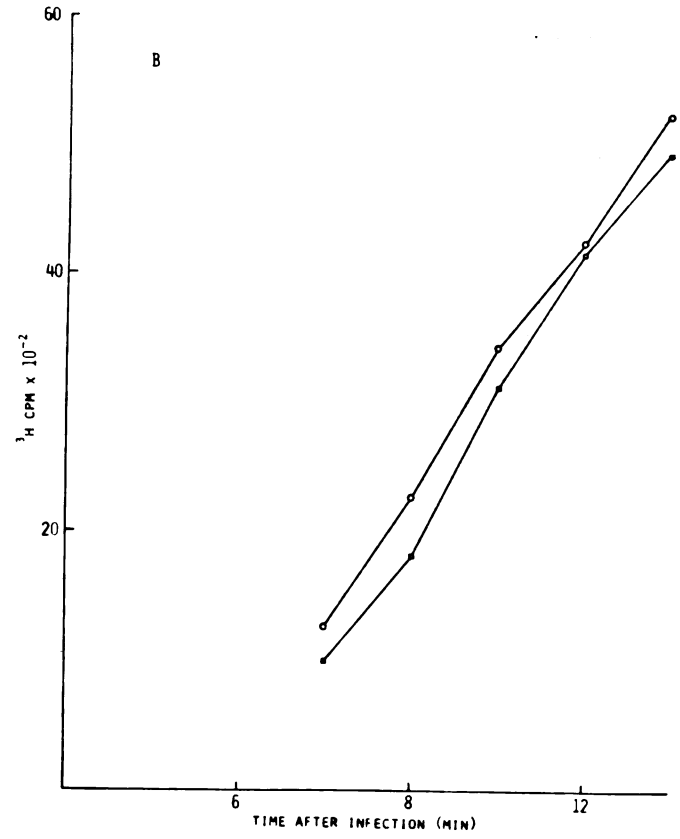

Figure 1B

not necessarily mean that all proteins are being produced at wild-type rates. To determine the distribution of label in various proteins, early proteins were subjected to polyacrylamide gel electrophoresis. To compare the early proteins from two separate infections, proteins were labeled with either ^{14}C-leucine or ^{3}H-leucine and electrophoresed together. Gels were fractionated and counted to determine the amount of radioactivity incorporated into the various peaks during the labeling time. This mixed-label method was chosen because it is more quantitative than slab gel autoradiograms. Small differences in the relative rates of synthesis of different proteins would be difficult to determine from band densities on autoradiograms, but could be detected by the ratio of two different radioactive labels on the same gel.

Since there are probably over 100 early proteins of T4, each peak in our gel patterns undoubtedly represents several proteins of similar size. To determine the degree of resolution in the gels, controls were run comparing different T4 amber mutants missing early gene products. Figures 2A and 2B show the results of both T4 *am* E10 (gene 45) and T4 *am* A453, r1272 (gene 32, deletion in rII A and B) compared to T4 *am* N122 (gene 42). The results are plotted as the percentage of the total

Figure 2. Proteins synthesized from 4 to 12 minutes after infection of NF58 with T4 amber and deletion mutants. The ratio of $^{14}C/^{3}H$ is given below each gel pattern.

Figure 2A - (-□-) T4 *am* N122 (gene 42) infection labeled with ^{14}C-leucine. Survivor rate = 2.15%
(-O-) T4 *am* E10 (gene 45) infection labeled with ^{3}H-leucine. Survivor rate = 3.25%.

Figure 2B - (-□-) T4 *am* N122 (gene 42) infection labeled with ^{14}C-leucine. Survivor rate = 0.52%.
(-O-) T4 A453,r1272 (deletion in RIIA'B) infection labeled with ^{3}H-leucine. Survivor rate = 0.55%.

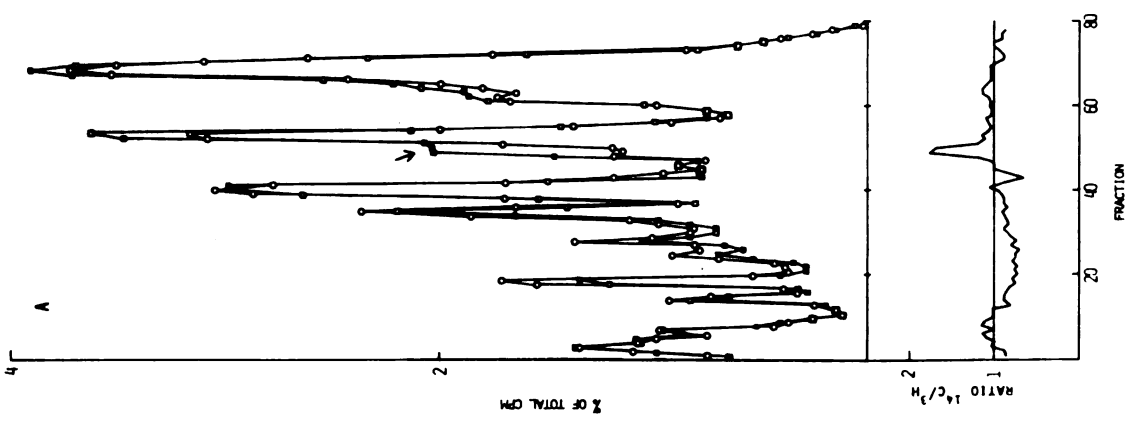

Figure 2A

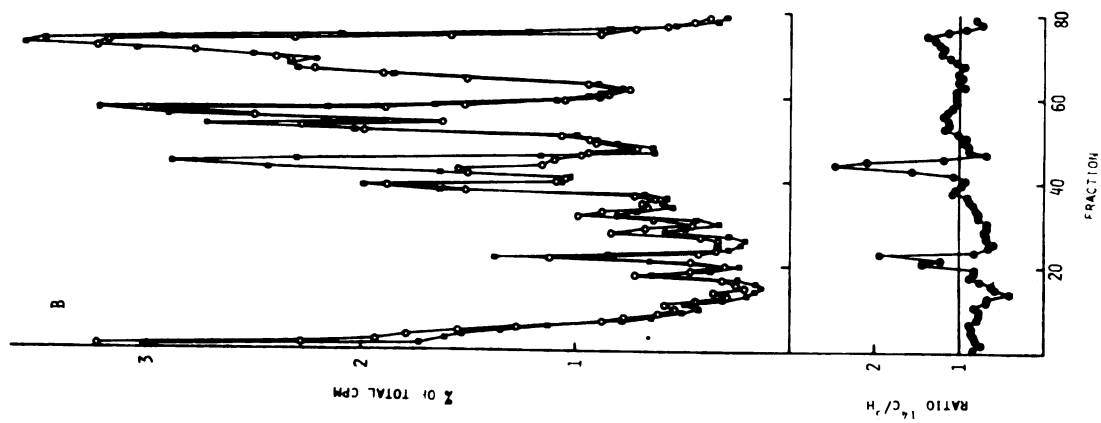

Figure 2B

recovered CPM found in each fraction, with the ratio of the $^{14}C/^{3}H$ per fraction plotted below each graph.

The gene 42 and 45 polypeptides have similar molecular weights and should co-migrate on gels. The resolution of these gels is not good enough to detect the absence of the 42 product in N122 patterns, but it is good enough to detect the absence of the 45 product in T4 *am* E10 (Figure 2A), as a reduced peak shoulder between fractions 48 and 51. This position correlates well to that of the 45 product identified on slab gels (O'Farrell *et al.*, 1973).

In Figure 2B, the large reduction in height of the peak between fractions 41 and 47 in the T4 *am* A453, r1272 infection is due to the absence of both the 32 and rIIB gene products. These two gene products are made in large quantities during the labeling period, and migrate together on slab gels to a position corresponding to the reduced peak on our gels (O'Farrell *et al.*, 1973). The rIIA product is made in smaller amounts and migrates slower than rIIB on slab gels. Its absence is not as evident in these gels, but the rIIA protein migrates to the area between fractions 20 and 24, since there is a variation here in the ratio of the two isotopes. Thus, since we can detect the absence of various gene products, the resolution of these gels is sensitive enough to detect differences in major proteins, and should be able

to detect the more generalized transcriptional defects which might be expected in an RNA polymerase mutant.

The first question we asked was whether the rif^R-2 mutation has an effect on early protein synthesis. Figure 3 shows the gel patterns obtained with T4 proteins labeled from 4 to 12 minutes after infection of both Rif^R-2 and wild-type *E. coli*. Cells were infected with an amber DNA zero mutant to make the two strains comparable in DNA replication. The differences in the gel patterns indicate that the rif^R-2 mutation does have an effect on early transcription.

Because of the overlap of the normalized ^3H and ^{14}C counts seen in our controls, the differences between wild-type and rif^R-2 infections observed in Figure 3 can be considered significant. One of the reduced peaks in the rif^R-2 pattern, that between fractions 48 and 51, may be the gene 45 product, as shown in Figure 2B. Thus, the rif^R-2 mutation may cause the gene 45 product to be synthesized in reduced amounts. This is puzzling considering genetic experiments reported earlier (Snyder and Montgomery, 1974) and which will be mentioned in the discussion.

An experiment similar to that shown in Figure 2 was done on T4 proteins labeled from 2 to 5 minutes after infection to determine if rif^R-2 has an effect on gene expression at earlier times (Figure 4). Here again,

Figure 3. Proteins synthesized from 4 to 12 minutes after infection of NF58 and NF58-Rif^{R}-2 with T4 *am* N122 (gene 42). (-□-) NF58 infection labeled with ^{14}C-leucine. Survivor rate = 1.93% (-O-) NF58-Rif^{R}-2 infection labeled with ^{3}H-leucine. Survivor rate = 2.28%.

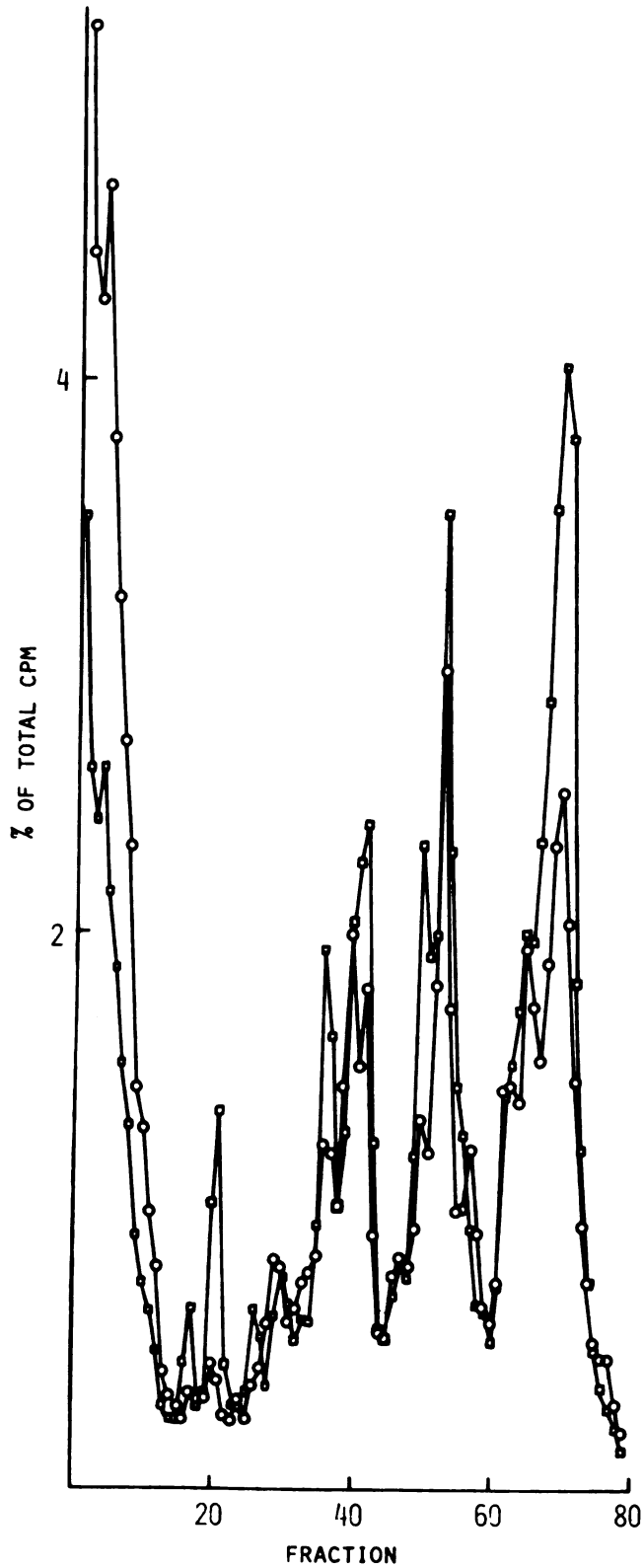

Figure 3

Figure 4. Protein synthesis from 2 to 5 minutes after infection of NF58 and NF58-Rif^{R}-2 with T4 *am* N122. (-□-) NF58 infection labeled with ^{3}H-leucine. Survivor rate <1%. (-O-) NF58-Rif^{R}-2 infection labeled with ^{14}C-leucine. Survivor rate = 1.43%.

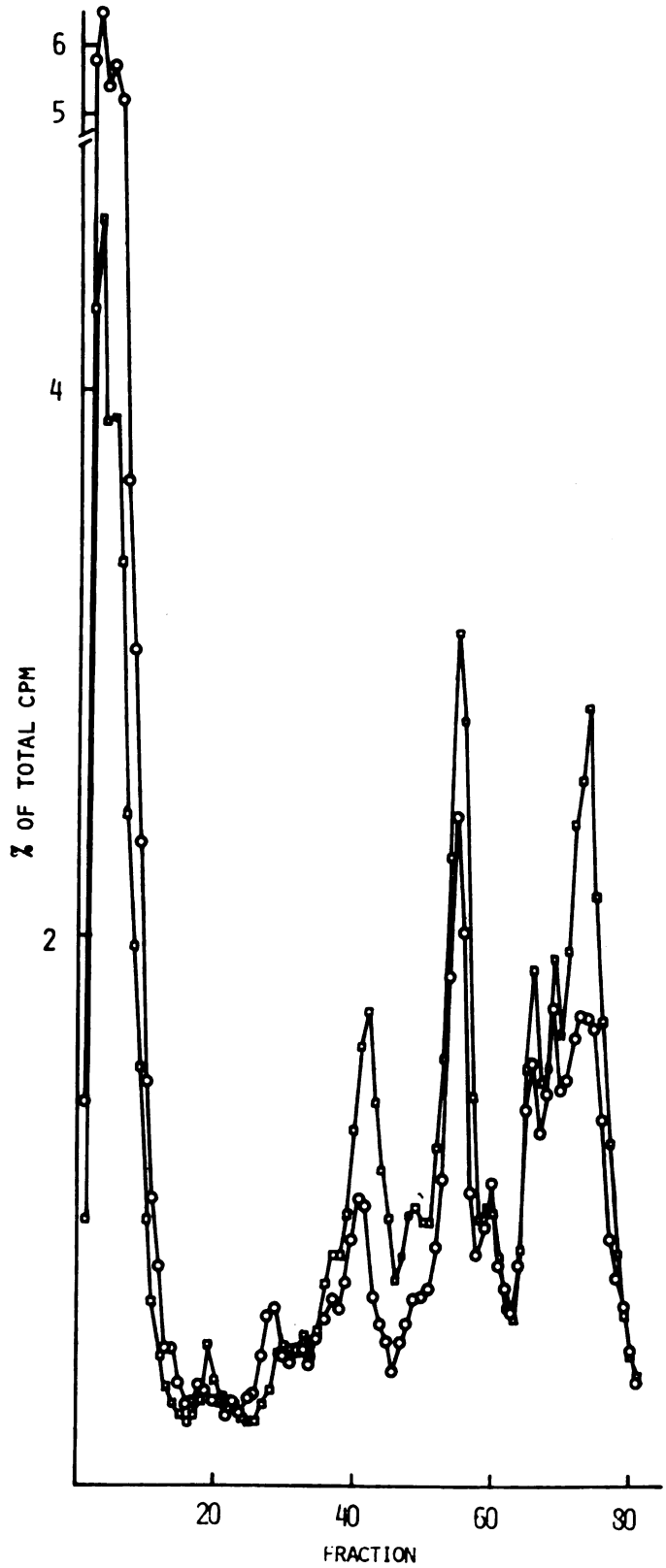

Figure 4

rif^R-2 shows an effect on gene expression, with peak heights lowered and some peaks slightly displaced.

We consistently get more counts remaining at the top of the gels with proteins from rif^R-2 infections. It is possible that some of these large unresolved proteins are *E. coli* proteins, since we have shown earlier that the rif^R-2 mutation causes incomplete shutoff of host mRNA synthesis (Snyder, 1972). However, we have not extended this analysis to determine if host proteins are translated from the residual mRNA. Since our gel results are plotted as the percentage of total recovered counts, the large number of radioactive proteins remaining at the top of the gel will lower the heights of the resolved peaks. Therefore, the differences seen between gel patterns of wild-type and rif^R-2 proteins are probably exaggerated. However, it is very likely that the rif^R-2 mutation has some effect on early transcription, since at least the 45 product seems to be made in reduced amounts.

The Effect of β-Glucosylation on Protein Synthesis

We have reported earlier that the rif^R-2 mutation has an effect on late protein synthesis; amino acid incorporation is reduced about 50% and several proteins [e.g., 34, 37, and 23 (23*) gene products] are synthesized in reduced amounts (Snyder and Montgomery, 1974). Our previous data suggest that βgt$^-$ at least partially

overcomes this late block because phage growth can be inhibited by β-glucosylation late in infection (Snyder and Montgomery, 1974). In order to determine more directly the effect of β-glucosylation on early and late protein synthesis, gene expression after infection of rif^R-2 with βgt^- and $T4^+$ were compared. T4 proteins were labeled 4 to 12, 15 to 20, and 30 to 35 minutes after infection and separated by electrophoresis as described earlier. The gel patterns are shown in Figure 5A, B and C.

Almost identical patterns of early gene expression emerge from Rif^R-2 infected with $T4^+$ and βgt^- (Figure 5A), indicating that early protein synthesis is very similar, and probably identical, in βgt^- and $T4^+$ infections. This is in contrast to the early effect of β-glucosylation on phage production (Snyder and Montgomery, 1974), indicating inhibition of a reaction other than early gene expression.

The differences in the gel patterns of $T4^+$ and βgt^- increase with later times. Figure 5B shows the gel patterns of proteins labeled from 15 to 20 minutes after infection. At this time some differences between βgt^- and $T4^+$ are already becoming apparent. By 30 to 35 minutes after infection (Figure 5C), βgt^- is substantially different from $T4^+$.

Figure 5. Protein synthesis after infection of NF58-Rif^{R}-2 with T4^{+} (-□-) and T4 βgt^{-} (-O-). T4^{+} infections were labeled with ^{14}C-leucine and βgt^{-} infections with ^{3}H-leucine. Survivor rates were less than 3% in all infections.

Figure 5A - Proteins labeled from 4 to 12 minutes after infection.

Figure 5B - Proteins labeled from 15 to 20 minutes after infection.

Figure 5C - Proteins labeled from 30 to 35 minutes after infection.

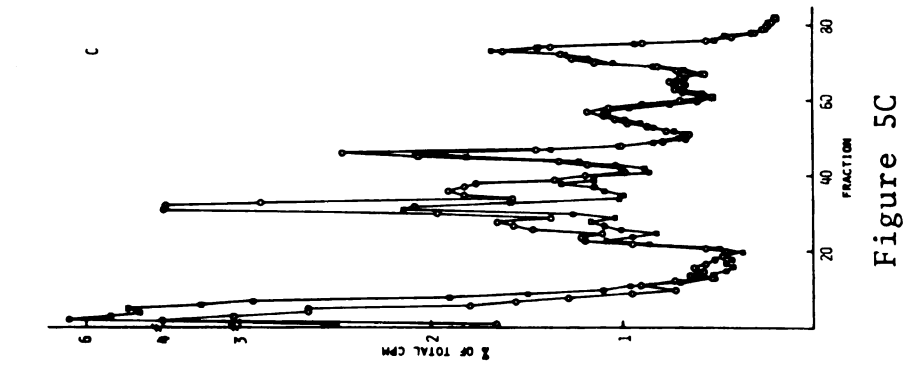

Figure 5C

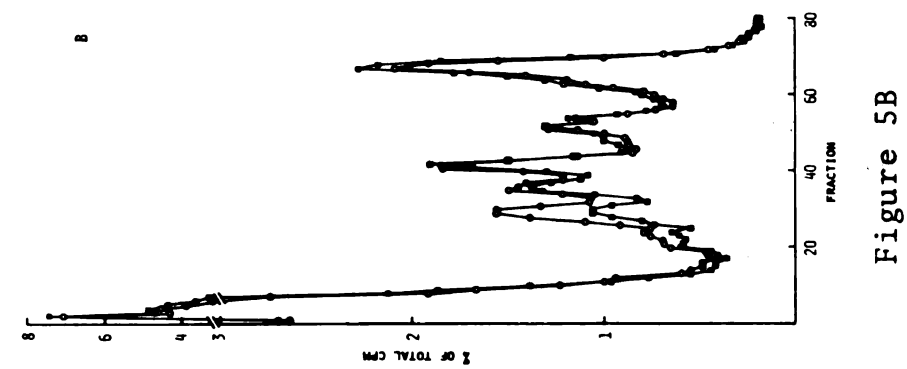

Figure 5B

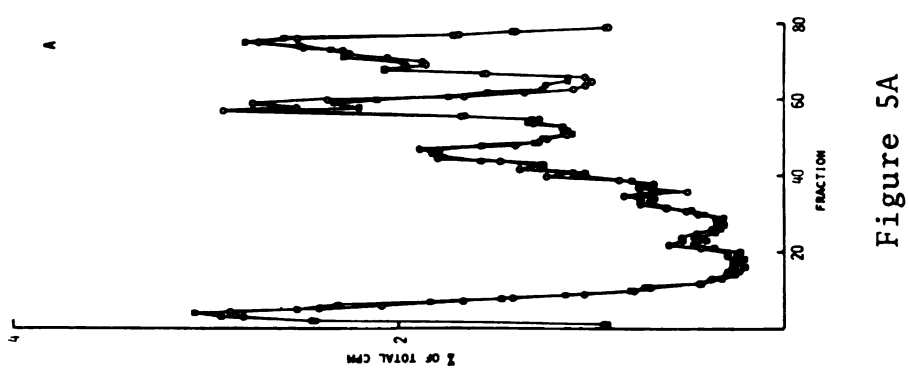

Figure 5A

The increase in differences in βgt^- and $T4^+$ infections of rif^R-2 at later times is further emphasized in Figure 6, where the ratios of $\beta gt^-/T4^+$ (taken from the points in Figures 5A, B and C) were plotted against fraction number. We conclude from these data that β-glucosylation plays at most a minor role in the early protein synthesis block, but becomes more significant during late protein synthesis.

The Effect of rif^R-2 on Degradation of Host DNA

Since protein synthesis does not seem to be the early function inhibited by β-glucosylation, we decided to look at the effect rif^R-2 has on host DNA degradation. The size of DNA fragments after infection was determined by sedimenting prelabeled host DNA through alkaline sucrose gradients. The size of the host DNA was determined at 10 minutes after infection of Rif^R-2 and wild-type *E. coli*. The host DNA from a Rif^R-2 infection has not undergone much degradation by 10 minutes, since it is not much smaller than DNA from uninfected cells (Figure 7). This indicates that host DNA degradation is significantly delayed in Rif^R-2.

The rif^R-2 mutation only delays host DNA degradation, since by 18 minutes after infection DNA from a Rif^R-2 infection is extensively degraded. In fact, our published results indicate that Rif^R-2 infections are

Figure 6. There are greater differences in the relative rates of synthesis of various proteins in $T4^+$ and βgt^- infections of NF58-Rif^R-2 at later times. The ratio of $^3H/^{14}C$ was obtained from the values in Figure 5.

Figure 6A - Protein synthesis from 30 to 35 minutes after infection.

Figure 6B - Protein synthesis from 15-20 minutes after infection.

Figure 6C - Protein synthesis from 4-12 minutes after infection.

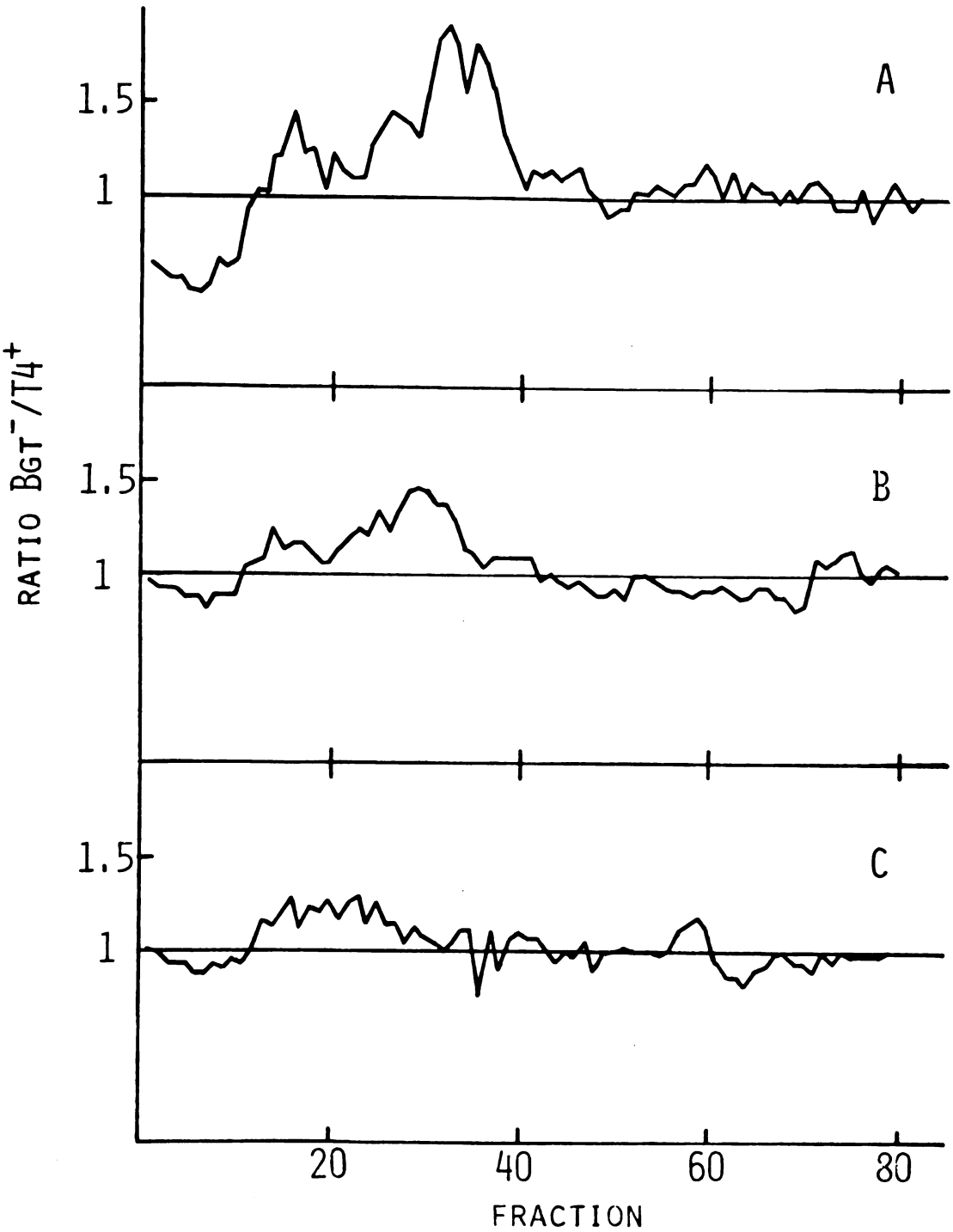

Figure 6

Figure 7. Alkaline sucrose density gradient analysis of *E. coli* DNA from uninfected NF58 (-●-) and from T4 *am* N122 infected NF58 (-□-) and NF58-Rif^{R}-2 (-O-). Infections were terminated at 10 minutes after infection. The position to which marker T4 phage sediment in gradients such as these is indicated by the arrow.

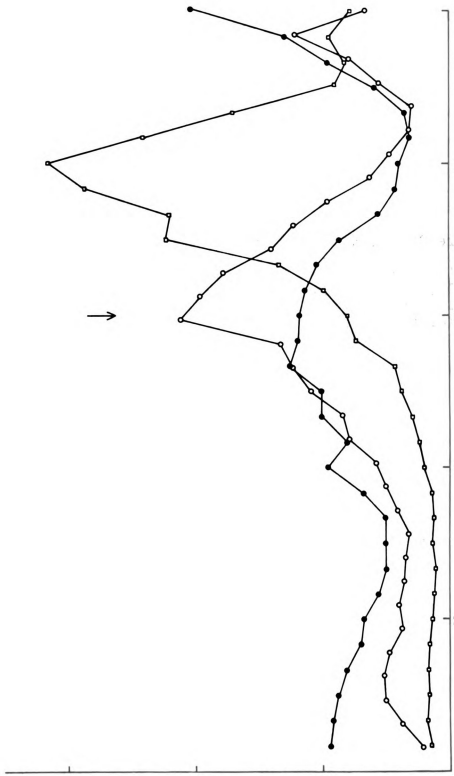

Figure 7

less defective in the degradation of host DNA than infections with T4 denA, which are mutant in the gene for endonuclease II (Hercules *et al.*, 1971).

The Effect of β-Glucosylation on Host DNA Degradation

The extent of host DNA degradation was measured after infection of Rif^R-2 with βgt^- to determine if this early function is inhibited by β-glucosylation.

Prelabeled rif^R-2 cells were infected with βgt^- and $T4^+$, and the size of the host DNA fragments measured at 12 minutes after infection (Figure 8). At this time, DNA from the βgt^- infection is more extensively degraded than DNA from the $T4^+$ infection. The size of the host DNA fragments progressively decreases with time after infection for both βgt^- and $T4^+$, but the fragment size decreases faster after βgt^- infection (data not shown). These data indicate that βgt^- partially overcome the delay in breakdown of host DNA.

The Effect of rif^R-2 on Host Nucleoid Unfolding

It is possible that the first step in host DNA degradation may be coincident with nuclear unfolding. Therefore, we wished to determine if the rif^R-2 mutation also had an effect on host nucleoid unfolding. Our results, shown in Figure 9, indicate that unfolding is delayed in Rif^R-2 infections. At five minutes after infection, host

Figure 8. Alkaline sucrose density gradient analysis of *E. coli* DNA from NF58-Rif^R-2 at 12 minutes after infection. (-●-) NF58 infected with T4 *am* N122 illustrate normal breakdown, (-□-) NF58-Rif^R-2 infected with T4 N122, (-O-) NF58-Rif^R-2 infected with T4 *am* N122,βgt$^-$.

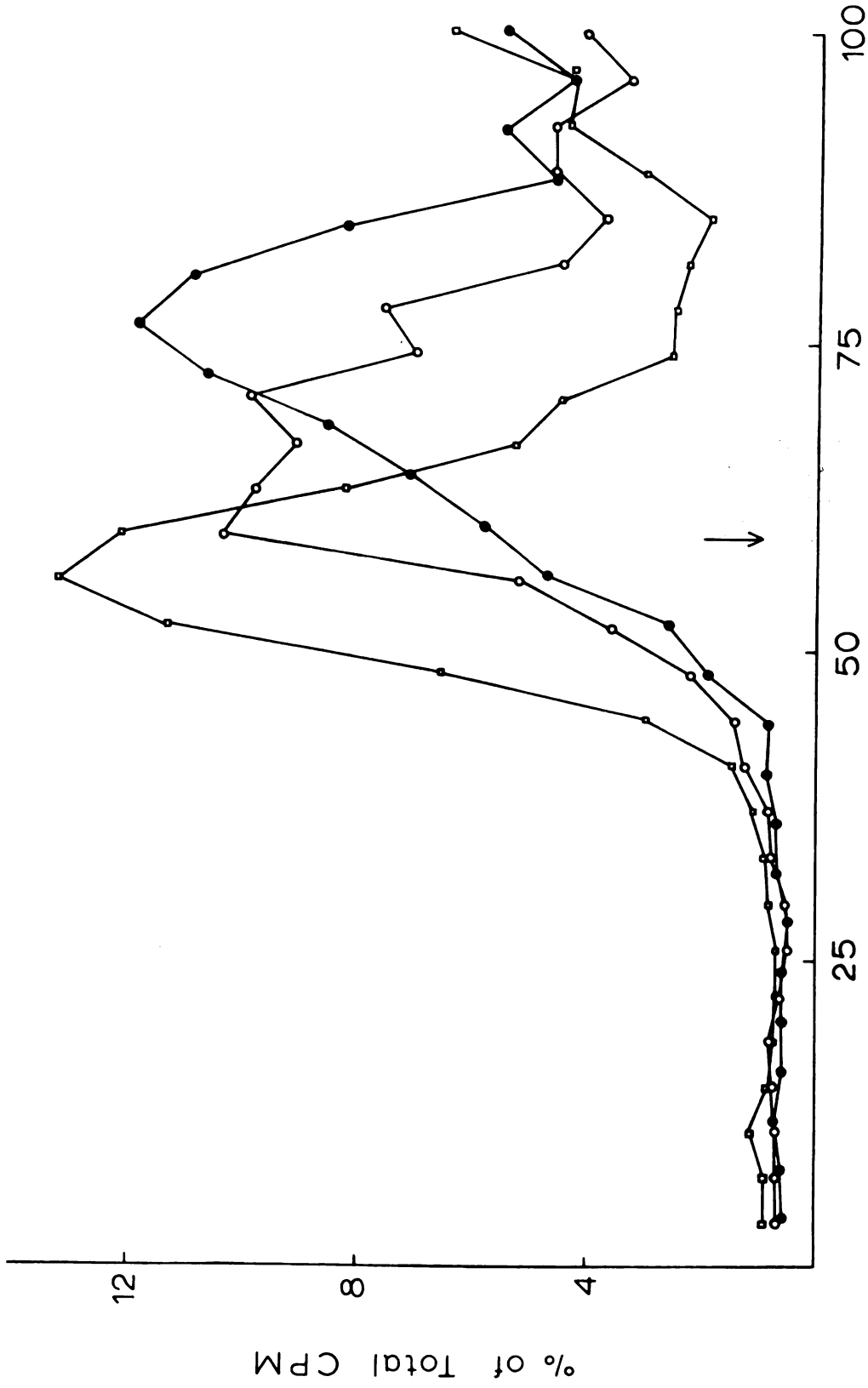

Figure 8

Figure 9. Host nucleoid unfolding after infection of NF58 (-□-) and NF58-Rif^R-2 (-O-) with T4 *am* N122. Cells were gently lysed as described in Materials and Methods and the host nucleoid analyzed on neutral sucrose gradients.

Figure 9A - Host nucleoid unfolding by 5 minutes after infection.

Figure 9B - Host nucleoid unfolding by 10 minutes after infection.

The arrow indicates the position to which marker T4 phage migrate in such gradients.

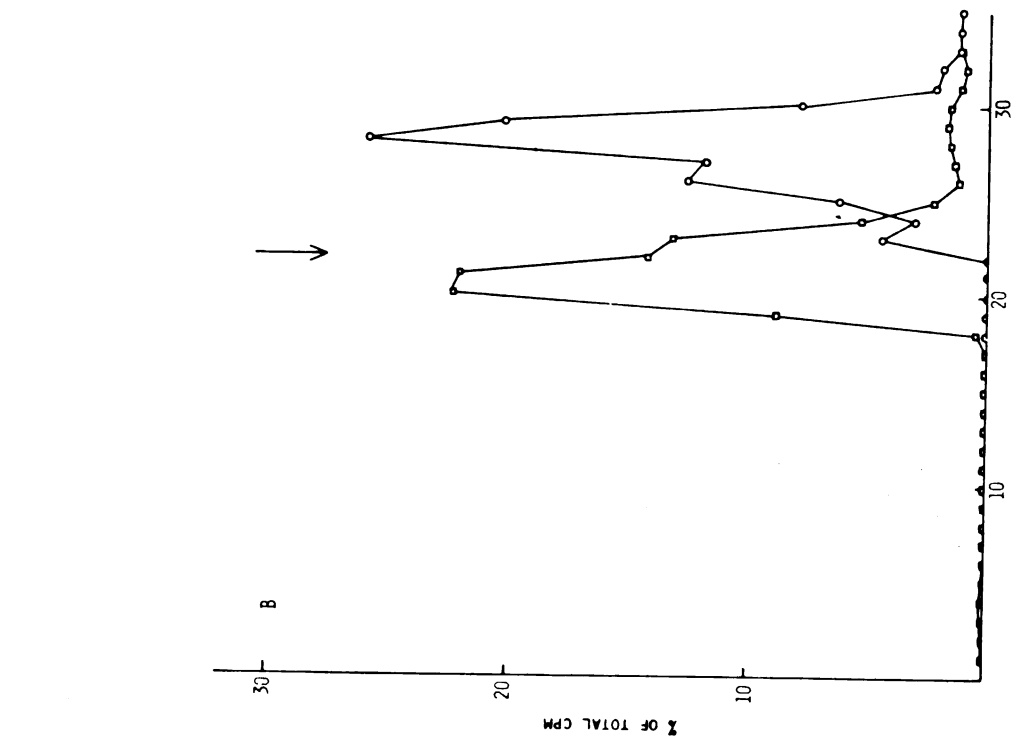

Figure 9B

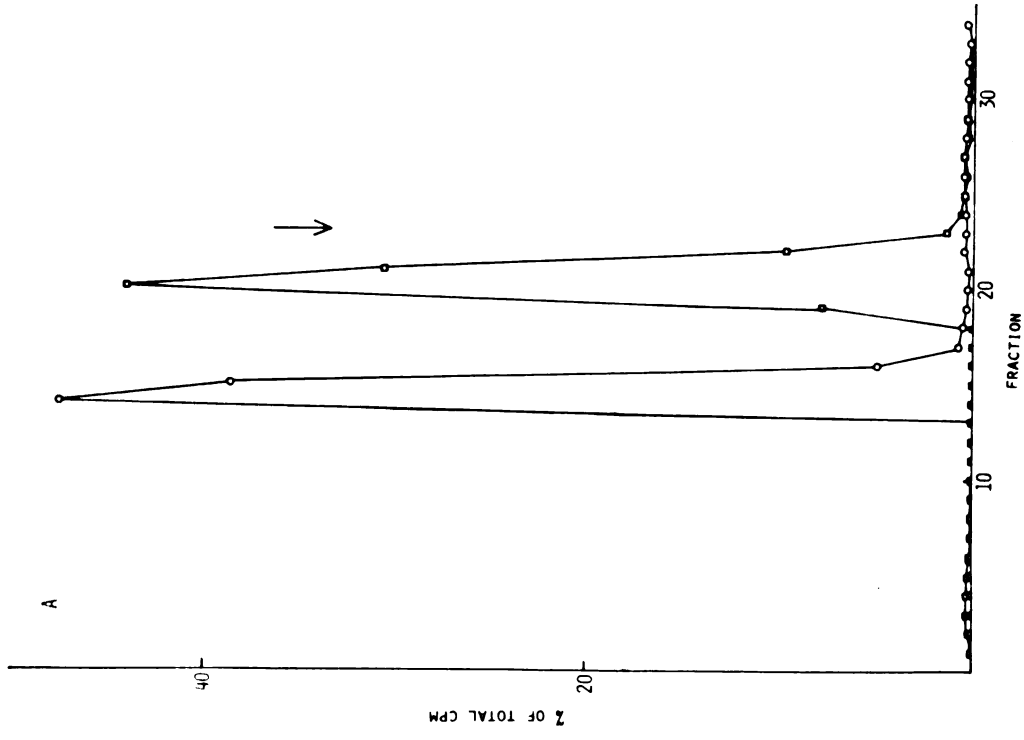

Figure 9A

nucleoids from a Rif^R-2 infection sediment faster than nucleoids from a wild-type *E. coli* infection (Figure 9A). The S value of nucleoids from infected Rif^R-2 cells (about 1800 S) falls within the range of S values obtained from uninfected cells (1600-1800 S) (data not shown), indicating that unfolding has not commenced by 5 minutes in Rif^R-2 infections.

By 10 minutes after infection, Rif^R-2 nucleoids sediment slower than nucleoids from a wild-type infection (Figure 9B), which has not changed from the S value it had at 5 minutes. These data suggest that unfolding of the host nucleoid is considerably delayed by the rif^R-2 mutation. After the delay, the host nucleoid is unfolded to a 500-600 S structure. This very slowly sedimenting structure may correspond to an intermediate form in normal unfolding or may represent an aberrant form found only in Rif^R-2 infections. This point requires further investigation.

Since the first step in nucleoid unfolding does not occur until after 5 minutes in Rif^R-2 infections, we wanted to see if this delay is caused by a delay in synthesis of a protein(s) required for unfolding. To answer this question, chloramphenicol was added 5 minutes after infection to prevent further protein synthesis. Therefore, only those proteins made by 5 minutes will be allowed to function. The infection either proceeded for

Figure 10. Host nucleoid unfolding in Rif^R-2 in the absence of protein synthesis. (-□-) T4 *am* N122 infection of NF58, (-O-) T4 *am* N122 infection of NF58 Rif^R-2. CAM was added at a final concentration of 150 μg/ml at 5 minutes after infection.

Figure 10A - Infections shifted 40°-27° at 2 minutes after infection; shifted 27°-40° at 7 minutes after infection; and terminated at 10 minutes.

Figure 10B - Infections shifted 40°-27° at 2 minutes and allowed to remain at 27° until 20 minutes after infection.

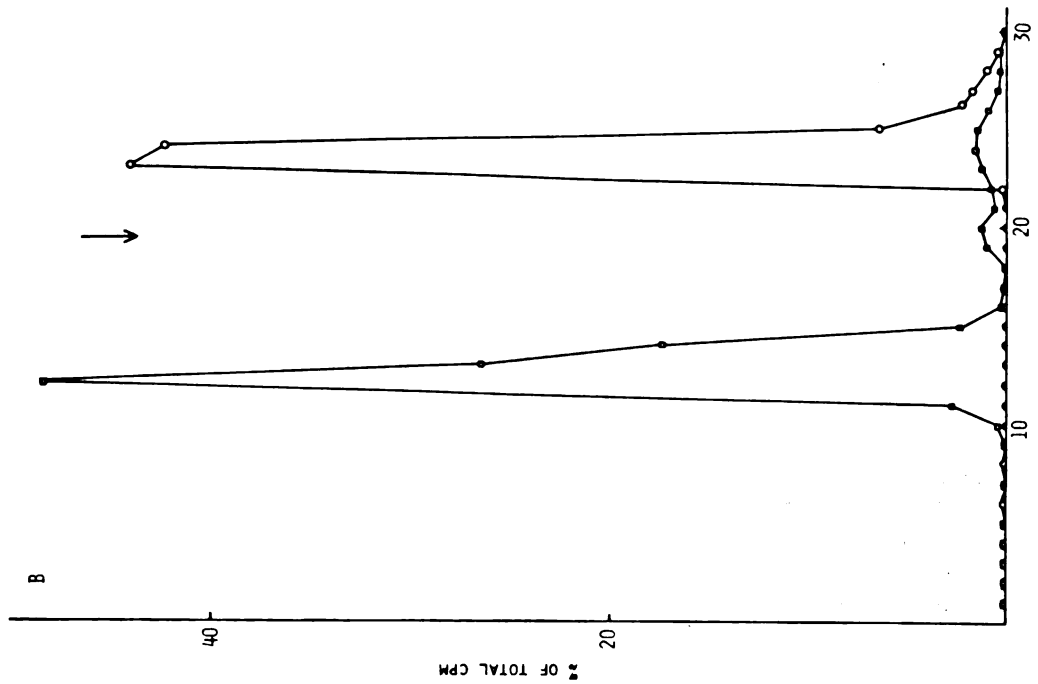

Figure 10B

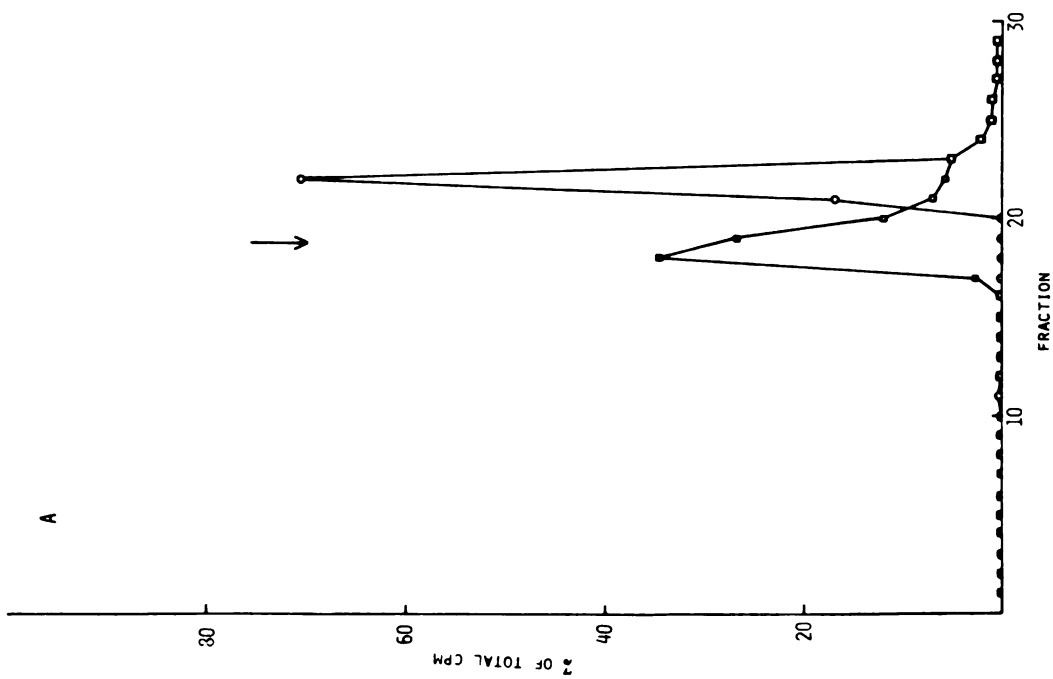

Figure 10A

20 minutes after infection at 27° (Figure 10B), or were shifted to 40° (the permissive temperature for T4 growth on Rif^R-2) at 7 minutes, and proceeded at that temperature until 10 minutes after infection (Figure 10A). Under both sets of conditions, Rif^R-2 nucleoids were unfolded to a 700 S structure. Thus, the delay in host nucleoid unfolding may not be due to a defect in early gene expression.

It is interesting to note that the sedimentation value of the wild-type genome changed in the experiment shown in Figure 10A. When shifted to 40° after adding chloramphenicol at 5 minutes, the wild-type DNA reattains the sedimentation rate of the uninfected form (about 1800 S). Presently, we cannot explain this phenomenon. However, it is possible that the unfolded nucleoid can be restored to the uninfected form by host enzymes if the synthesis of additional T4 proteins is inhibited. We are going to further investigate this possibility.

DISCUSSION

The rif^R-2 mutation reduces the overall rate of late protein synthesis, as well as reducing the synthesis of specific gene products (e.g., 34, 37, and 23 [23*]). We have concluded from the experiments described here that rif^R-2 probably has an effect on early protein synthesis as well. The effect is not a generalized rate reduction since the rate of amino acid incorporation is

not affected, but the rates of synthesis of several proteins are affected, in different ways. For example, dihydrofolate reductase and β-glucosyl transferase are overproduced after a slight lag (Snyder and Montgomery, 1974), while the gene 45 product is probably synthesized in reduced amounts.

It is puzzling to note that the gene 45 product may be synthesized at a reduced rate in both $T4^+$ and βgt^- infections of Rif^R-2. We reported in an earlier publication (Snyder and Montgomery, 1974) that T4 amber mutants in gene 45 grow better than $T4^+$ on su_2^+ strains of *E. coli* harboring the rif^R-2 mutation. We have found that six amber mutants of gene 45 (E10, NG18, E3000-E3003) all show the same phenotype, suggesting that the reduced level of the 45 product synthesized in suppressed amber mutants restores some essential balance. However, we now have reason to believe that the six amber mutants we have tested have the same mutation because, although the different amber mutants recombine normally with temperature-sensitive mutants in gene 45, they are unable to recombine with each other (L. Snyder and J. Hendra, personal communcation). An alternative possibility for the suppressed amber effect is that the glutamine inserted for the amber codon in su_2^+ strains leads to a 45 product which is better able to react with the mutated RNA polymerase. This explanation leads to the prediction that any means of

inserting a glutamine at the amber codon site would also produce a gor phenotype. One of the transitions from the amber codon is a codon for glutamine, and thus one would expect some amber revertants to grow better than $T4^+$ on Rif^R-2. However, no revertants have been found which show this phenotype (Snyder and Montgomery, 1974), suggesting that this is not the correct explanation. Although we have now found that rif^R-2 may cause a reduction in the levels of 45 products, we still favor the explanation that suppressed amber mutants in 45 grow better than $T4^+$ on Rif^R-2 because of even more reduced levels of the 45 product in these infections.

Because rif^R-2 affects the synthesis of only some early proteins and not the rate overall, it is possible that the mutated RNA polymerase is defective in some function required for early transcriptional control. For example, it may be defective in the utilization of some early promotors, or be unable to interact correctly with some T4 protein(s) required for control. Despite evidence that the control of early protein synthesis is at least partially dependent upon T4 coded proteins (Wiberg *et al.*, 1974; Karam and Bowles, 1974; Mattson *et al.*, 1974), no RNA polymerase modifications have been shown to be involved in early regulation. If the effect of rif^R-2 on early protein synthesis is due to the inability of RNA polymerase to interact correctly with

a T4 coded protein(s), a more detailed study of the early defect may identify a specific gene product(s) required for early control.

Our experiments have determined that the rif^R-2 mutation also affects both host nucleoid unfolding and subsequent degradation of the DNA to nucleotides. It has been shown that within 2 to 3 minutes after infection, the host DNA is disrupted from its centrally-located, multi-lobed form and accumulates in numerous clumps along the cell membrane (Kellenberger, 1960; Luria and Human, 1950; Murray *et al.*, 1950). This process is called nuclear disruption. Kellenberger *et al.* (1959) showed that the addition of chloramphenicol at one minute after infection delays nuclear disruption. A T4 mutant has been found which does not cause nuclear disruption (Snustad and Conroy, 1974).

Tutas *et al.* (1974) have shown that T4 infection rapidly converts the folded bacterial genome to a less compact structure. This process, called nucleoid unfolding, can be analyzed biochemically and is distinct from nuclear disruption since mutants defective in nuclear disruption do not prevent unfolding (Snustad *et al.*, 1974). Unfolding may require an early or pre-early T4 protein (Snustad *et al.*, 1972), but no T4 mutant has been found which affects this process. Therefore, the rif^R-2 mutation is the only genetic system known to prevent nucleoid unfolding.

Not only does the rif^R-2 mutation cause a delay in unfolding but the unfolded structure has a lower S value than that of the unfolded genome after wild-type infection. This very slowly sedimenting structure could represent a normal intermediate in the disruption process. If it is a normal intermediate, the process of unfolding may be very complex, with the unfolded forms found after infection not corresponding to the structures proposed by Worcel and Burgi for genomes with these S values (Worcel and Burgi, 1972). This idea was also proposed by Tutas *et al.* (1974) because their unfolded genomes had sedimentation values less than 1000 S but did not have enough nicks to sediment as would a partially nicked genome containing an RNA core. Our unfolded nucleoids after T4 infection have a higher S value than those of Tutas *et al.* (1974). We have no explanation for this, but it may be related to differences in temperature of infection or to the host strains used.

Alternatively, the slow sedimenting structures may represent an aberrant form found only in Rif^R-2 infections. This is an attractive idea for several reasons. First, the slowly sedimenting form obtained in the Rif^R-2 infection corresponds in S value to a completely unfolded form that has lost its RNA "core", as determined by Worcel and Burgi (1972). The S value of the host nucleoid after a wild-type infection corresponds to that

of a partially relaxed form (Worcel and Burgi, 1972); one that still has the RNA "core", but already contains several single-stranded nicks per chromosome. Perhaps loss of the RNA "core" may occur even more quickly after Rif^R-2 infection than after infection of the wild-type.

The defect in nucleoid unfolding could be the cause of the delay in host DNA breakdown in Rif^R-2 infections if normal unfolding is a prerequisite for normal degradation. Since we have shown that the absence of β-glucosylation shortens the delay in degradation, this model predicts that βgt^- mutants would also partially overcome the defect in nucleoid unfolding. However, the effect of the absence of β-glucosylation on nucleoid unfolding has not yet been tested.

In an earlier publication (Snyder, 1972) it was reported that the rif^R-2 mutation causes incomplete shutoff of host transcription. Therefore, the host genome retains a functioning RNA polymerase after infection of rif^R-2. Since a functioning RNA polymerase is required to hold the nucleoid in a highly folded form (Dworsky and Schaechter, 1973; Stonington and Pettijohn, 1971; Worcel and Burgi, 1972) the two defects (incomplete shutoff of host transcription and delay in nucleoid unfolding) may be related. It is possible that the delay in unfolding allows transcription to continue or,

inversely, that the residual host transcription delays the unfolding of the nucleoid.

The effects of rif^R-2 during T4 development show striking similarities to the effects of mutants in genes 52, 60, 39, and 58-61, which also cause a delay in T4 DNA synthesis (Yegian *et al.*, 1971; Epstein *et al.*, 1963), and a defect in late gene expression (Yegian *et al.*, 1971). Recently, DNA-delay (DD) mutants were found to be cold sensitive in phage production as well as T4 synthesis (Mufti and Bernstein, 1974), as is Rif^R-2. Also, at least some DD-mutants (those in gene 52) are slightly defective in host DNA breakdown (Naot and Shalitin, 1973) as is Rif^R-2.

It is possible that DD-mutants affect a membrane function (Yegian *et al.*, 1971; Mufti and Bernstein, 1974). If so, Rif^R-2 may also be altered in a membrane function either directly, if RNA polymerase is involved in attaching DNA to the membrane, or indirectly, if Rif^R-2 for some reason has altered membranes. One difference, however, between DD mutants and Rif^R-2 is that none of the DD mutants is suppressed by βgt^- mutants (Snyder, unpublished observations). There is indication that at least some of the DNA delay gene products interact in their function (Mufti and Bernstein, 1974) so that it may be necessary to inactivate more than one DNA delay gene product before the suppression by βgt^- mutants

becomes apparent. Or, RNA polymerase may act at a different step in the same process but at the only step, the need for which can be partially alleviated by the absence of β-glucosylation.

We have concluded that β-glucosylation has an effect on late protein synthesis but not early protein synthesis in Rif^R-2. Our gel results indicate that the relative rates of synthesis of various proteins by βgt^- are quite different from wild-type T4 at late times, but at earlier times they are very similar, and probably identical. In an earlier publication (Snyder and Montgomery, 1974), we have shown that β-glucosylation inhibits a function which occurs between 2 and 10 minutes after infection at 40°, indicating that RNA polymerase interacts early with the parental T4 DNA at sites which are normally β-glucosylated. The function performed seems to be irreversible and first must be done to parental DNA and then to at least some progeny DNA (Snyder and Montgomery, 1974). Thus, our new data indicating that there is no effect of β-glucosylation on early gene expression give further support to the model proposed earlier (Snyder and Montgomery, 1974) that RNA polymerase must alter the structure or association of T4 parental DNA early in infection for DNA replication and late transcription to occur. Possibilities for this type of interaction have been proposed elsewhere (Snyder and Montgomery, 1974).

The fact that β-glucosylation does not affect early gene expression means that the inhibition by β-glucosylation is not general for all reactions involving RNA polymerase (since early genes presumably have some β-glucose) but is due to β-glucose in certain specific regions. Another simple experiment we have done supports the same conclusion. The experiment is as follows. An amber mutant in βgt (amβgt 10) is partially gor on amber-suppressing strains of *E. coli*. The gor phenotype is due to incomplete β-glucosylation because of inefficient suppression of the amber mutation since DNA from the viruses grown on such strains can accept β-glucose *in vitro* (L. Snyder, unpublished observations). If the incomplete β-glucosylated single mutant is combined with an αgt^- mutation much bigger plaques are formed. Presumably in the double mutant, that β-glucosylation which occurs is more widely distributed on the genome and is then less inhibitory. Thus, β-glucosylation of only some of the hydroxymethyl cytosines affects T4 growth on Rif^R-2.

In any case, since βgt^- mutants of T4 partially suppress the effect of rif^R-2 on DNA breakdown, but do not seem to affect early gene expression, RNA polymerase would seem to be otherwise involved in a step required for host DNA degradation. Somehow the transcription of β-glucosylated hydroxymethyl cytosines in T4 DNA is

involved in this step. Perhaps T4 DNA competes for some essential cellular component (e.g., membrane binding sites) with host DNA and the transcription of certain specific regions of T4 DNA is required for this competition. The subsequent release of host DNA due to this competition may be an essential step in host DNA degradation. The interaction between T4 DNA and the essential cellular component could be required for T4 DNA replication and late gene expression but not for early gene expression.

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APPENDIX

APPENDIX

Inhibition of T4 Growth by an RNA Polymerase Mutation of *Escherichia coli*: Physiological and Genetic Analysis of the Effects During Phage Development

By

Loren R. Snyder and Donna L. Montgomery

Virology 62, 184-196 (1974)

APPENDIX

The following publication describes physiological and genetic studies on the effects of rif^R-2 on T4 development, and further describes effects of the β-glucosylation inhibition. The experiments reported in Article II of this dissertation depend upon the information reported in this publication. Therefore, the paper is included as additional information which should be read to fully understand the significance of Article II.

VIROLOGY 62, 184-196 (1974)

Inhibition of T4 Growth by an RNA Polymerase Mutation of *Escherichia coli*: Physiological and Genetic Analysis of the Effects During Phage Development[1]

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We have previously reported that an RNA polymerase mutation, rif^{R}2, of *Escherichia coli* retards T4 bacteriophage development. The effect of rif^{R}2 on phage production is partially suppressed in β-glucosyl transferaseless mutants of T4 and β-glucosylation of the parental DNA alone is sufficient to inhibit phage growth.

Both physiological studies and the map positions of other suppressing T4 mutations (in genes 45 and maybe 55) support the conclusion that there are two effects of rif^{R}2 during phage development: a direct effect on T4 DNA replication and a direct effect on late gene expression. Both of these defects seem in part to be due to a failure of the mutant RNA polymerase to perform a reaction(s) which physically alters or changes the association of parental and then, probably, some progeny DNA. This reaction is required early for T4 DNA replication and throughout infection for late gene expression.

INTRODUCTION

The host RNA polymerase enzyme performs a number of functions required for the development of the coliphage T4. It is required for both early and late messenger RNA synthesis (Haselkorn *et al.*; di Mauro *et al.*, 1969) and, as we report here, probably for at least one other function required for T4 DNA replication. However, the host RNA polymerase does not remain unchanged after viral infection being altered both in its subunits (Goff and Weber, 1970; Travers, 1970; Schachner and Zillig, 1971) and in the protein with which it is associated (Travers, 1969; Stevens, 1972; 1973; Horvitz, 1973; Snyder, 1973). While the physiological significance of some of the reported changes is unknown, some are obviously related to transcriptional regulation. For example, the products of genes 55 and 33, both of which are required for late messenger RNA synthesis (Bolle *et al.*, 1968; Notani, 1973) bind to the host RNA polymerase as newly synthesized T4 peptides (Stevens, 1972; Horvitz, 1973; Ratner, personal communication).

Since at least some of the modifications of RNA polymerase require the interaction between viral coded gene products and *Escherichia coli* RNA polymerase, it is possible that some mutations in RNA polymerase may interfere with one or more of the interactions thereby interfering with phage growth. We have reported the existence of RNA polymerase mutants of *E. coli* which grow almost normally but in which T4 production is severely retarded (Snyder, 1972). The mutation, rif^{R}2, which was extensively studied has two apparent effects during T4 development causing delays in the synthesis of T4 DNA and in the appearance of some late gene products.

In another publication (Montgomery and Snyder, 1973), we reported the isolation of gor (*grow on rif^{R}2*) mutations of T4 which partially suppress the effect of rif^{R}2 on T4 growth. The first such mutants to be isolated lacked a functional β-glucosyl transferase and could grow because their

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DNA is partially unglucosylated.

Here we report the results of a continued analysis of the effects of the RNA polymerase mutation, rif^{R}2, on T4 phage development including a further analysis of the inhibition by β-glucosylation and the isolation of other gor mutations which map elsewhere than in the gene for β-glucosyl transferase.

MATERIALS AND METHODS

(a) *Phage and Bacterial Strains*

The glucosyl transferase mutations used were R4, R20a, and amβgt10 which are βgt$^-$, αgt$^-$, and βgt$^-$, respectively. They have been described in the literature (Georgopoulos and Revel, 1971). R4 and R20a were obtained from J. Wiberg. The amβgt10 mutant was crossed out of a double mutant amβgt10, αgtam8 obtained from H. Revel. The mutants, amE10, amNG18 and tsCB53 all in gene 45 as well as the mutant tsP36 in gene 43 were obtained from P. Geiduschek's laboratory. amB14 in gene 46 and amE727 in gene 49 came from J. Wiberg. All the other mutants used in the crosses were obtained from H. Revel.

The bacterial strains K803 and NF58 as well as the transduced strains K803-rif^{R}2, NF58-rif^{R}2, and C600-rif^{R}2 have been previously described (Montgomery and Snyder, 1973). K803 is permissive for unglucosylated T4 and is am su$_2^+$. C600 is nonpermissive for unglucosylated T4 and is am su$_2^+$. NF58 is am su$^-$. CR63 is am su$_1^+$. B/r is am su$^-$.

(b) *Media and Buffers*

M9S: 5.54 g Na_2HPO_4, 3 g KH_2PO_4, 5 g NaCl, 1 g NH_4Cl, 4 g glucose, 10^{-3} moles $MgSO_4$ and 10 g casamino acids (Difco)/liter H_2O.

Tryptone broth and plates: 10 g tryptone, 5 g NaCl/liter was mixed with 19 g agar or 7.5 g agar to make indicator plates or top agar, respectively.

(c) *Infections and Growth Curves, etc.*

Cells were grown to an O.D. of 0.4 at 625 nm in M9S, and infected at an m.o.i. of 5, unless otherwise indicated, with CsCl purified virus. Intracellular phage were measured by diluting into saline containing chloroform. Temperature shifts were accomplished by diluting 1:100 into medium preincubated at the desired temperature.

Crosses were performed by adding unpurified viruses at an m.o.i. of 5 of each to cells grown as above. After 2 min the infected cells were diluted 1:1000 into tryptone broth at 30° and shaken for 1 hr until $CHCl_3$ was added.

(d) *SDS-Slab Gel Electrophoresis*

The method and apparatus used were those of Studier (1973). The gels were 20% acrylamide and were run at 10 mA. The gels were dried under vacuum on filter paper and autoradiograms were developed for 17 days on Kodak no-screen X-ray film.

(e) *Enzyme Assays*

Dihydrofolate reductase was assayed in crude sonicates by the method of Mathews and Sutherland, 1965, using conditions which favored the phage-induced enzyme. By 12 min after infection at 27°, there were sixfold and eightfold increases in the specific activity of dihydrofolate reductase on K803 and K803-rif^{R}2, respectively. The reaction was dependent on added dihydrofolate.

β-Glucosyl transferase was assayed in crude sonicates by the method of Josse and Kornberg, 1962. The activity measured was not detectable with wild type T4 DNA as substrate and was not present in uninfected cells or in cells infected by T4 βgt$^-$.

(f) *Isolation of gor-2 Type Mutants*

We have found that the vast majority of gor (grow on rif^{R}2) type mutants are βgt$^-$. However, other types of gor mutants exist which presumably have had subtle specific changes in T4 proteins which are malfunctioning as a result of the RNA polymerase mutation. The map position of such mutations is, of course, of considerable interest, but they are rare compared to βgt$^-$ mutants. It was necessary to eliminate βgt$^-$ mutations from the selection procedure to avoid the onerous task of screening large numbers of gor mutants to find ones

which are not βgt^-; and to allow us to test many more viruses per plate of Rif^R2 indicator bacteria. βgt^-, αgt^- double mutants are completely restricted on wild-type *E. coli*. Therefore, by starting with an αgt^- mutant we restricted the selection to βgt^+ mutants provided the αgt^- mutation has not reverted to αgt^+.

Accordingly, to obtain the gor-type mutant mapped in Table 1, $R20_a(\alpha gt^-)$ was mutagenized with NH_2OH by the method of Tessman, 1968. As a measure of mutagenesis, βgt^-, αgt^- mutants were 30× more frequent as measured by plating efficiency on nonrestricting Rif^R2 bacteria (K803-rif^R2) at 27°, and represented 0.1% of the population. When the mutagenized phage were plated on restricting Rif^R2 bacteria (C600-rif^R2), plating efficiency was only about 10^{-5}% and the only plaques observed were very small. These plaques were picked and purified by replating on restricting Rif^R2 bacteria. They were then tested to assure that they were still αgt^-. This was accomplished in two steps. First they were tested to see if they gave the smaller plaques and reduced plating efficiency (~20%) characteristic of αgt^- mutants. The ones which were still αgt^- by plating criteria were further tested by crossing them against βgt^- mutants and testing to make sure the expected number ~12% were βgt^-, αgt^- double mutant recombinants (e.g., completely restricted). Only about 1 in 10 of the gor type mutants obtained under restricting conditions were still αgt^- by these criteria. The αgt^-, gor mutant for which the mapping data are shown in Table 1 has been back crossed 4× against wild type T4 at ratios of 1–5 and made αgt^+ without its map position being altered.

TABLE 1

MAP POSITION OF gor-2 TYPE MUTATIONS

Cross 1:1	Gene	No. from Rif^R2 which are *am*	Approximate recombination frequency (%)
gor-2 × N116	39	6/20	25
gor-2 × E51	56	5/20	25
gor-2 × N122	42	17/60	25
gor-2 × B14	46	10/100	10
gor-2 × BL292	55	1/260	<1
gor-2 × E727	49	5/60	10
gor-2 × B17	23	5/20	25
gor-2 × N134	33	4/20	25

RESULTS

Effect of the rif^R2 Mutation on T4 DNA Replication

T4 DNA synthesis is "cold sensitive" on Rif^R2 occurring with a lag at low temperatures but at about the normal time at higher temperatures (Fig. 1A and B). Observations made with other systems which show an involvement of RNA in DNA replication (Dove *et al.*, 1969; Lark, 1972; Sugino *et al.*, 1972; Brutlag *et al.*, 1971; Blair *et al.*, 1972) led us to attempt to determine whether the defect in DNA rep-

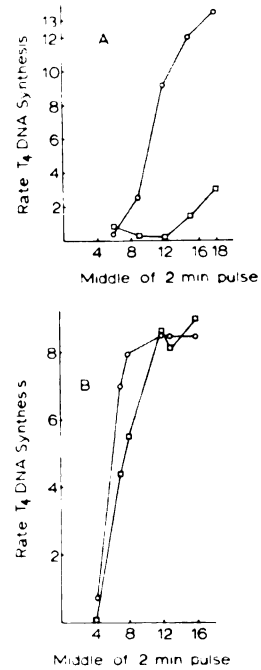

FIG. 1. The rate of T4 DNA replication after infection of K803 (—○—) and K803-rif^R2 (—□—). Cells (0.1 ml) were added to 0.9 ml of [^{3}H]methyl thymidine in M9S at 1 μCi and 1 μg/ml for 2 min followed by 0.12 ml of 50% TCA. Four milliliters of 5% TCA was added and the precipitate was centrifuged, resuspended in 2% KOH, reprecipitated with 5% TCA, and collected on millipore filters for counting. (A) 27° throughout. (B) 40° throughout.

lication was due to a delay in the synthesis of gene product(s) required for replication or to some other malfunction of RNA polymerase in DNA replication.

At least some T4 early gene products appear at about the normal time on Rif^{R}2. The kinetics of appearance of the enzymes, T4-induced dihydrofolate reductase and β-glucosyl transferase, have been measured and neither of these appears with a significant lag. Also, there seems to be no gross effect on the time of appearance of the bulk early messenger RNAs of T4 as determined by hybridization competition experiments (data not shown). However, since a delay in the synthesis of only one gene product required for DNA synthesis would cause a delay in DNA replication on Rif^{R}2, this question should be resolved by other means.

Since T4 DNA synthesis is cold-sensitive on Rif^{R}2, we can do temperature shift experiments and ask how quickly the block in DNA synthesis is imposed after a shift to the nonpermissive temperature. If the block is on gene expression, we would not expect an immediate effect because of the time permitted for translating (and finish synthesizing?) preexisting m-RNA. If, however, the effect is on some other function of RNA polymerase in DNA replication, we might observe a more immediate effect. In fact, the effect of rif^{R}2 on T4 DNA replication is virtually instantaneous, the rate being frozen at the rate achieved at the time of the shift (Fig. 2A-C). If DNA synthesis has achieved its maximum rate before the time of the shift, e.g., 10 min, no effect can be seen (Fig. 2C). We shall discuss the implications of this observation later.

Better evidence that the effect of rif^{R}2 on T4 DNA replication is on some function other than gene expression comes from experiments designed to answer the question whether all of the gene products required for T4 DNA replication are synthesized at the nonpermissive temperature but DNA cannot replicate for some other reason. Cells were infected at the nonpermissive temperature and after DNA synthesis normally has begun on the wild-type cells, but not on Rif^{R}2 cells, chloramphenicol was added to block any further gene expression followed by a shift to 40°. If DNA replication begins on Rif^{R}2, the block is on other than gene expression. In addition, in order to make the Rif^{R}2 cells comparable to the wild-type cells at the nonpermissive temperature, DNA synthesis was blocked by thymine starvation by preventing host DNA breakdown with a den A mutant (Hercules *et al.*, 1971) and *de novo* synthesis of thymine by FUdR. After adding chloramphenicol, furnishing thymidine, and shifting to the permissive temperature, DNA synthesis achieved almost wild-type rates (Fig. 3). If the cells were retained at the nonpermissive temperature, DNA synthesis again began but with a lag on Rif^{R}2 relative to the wild-type (data not shown). Thus the delay in DNA replication does not seem to be due to a defect in gene expression, which also is suggested by the map position of some of the gor-type mutations to be discussed later.

As shown above, the rif^{R}2 mutation

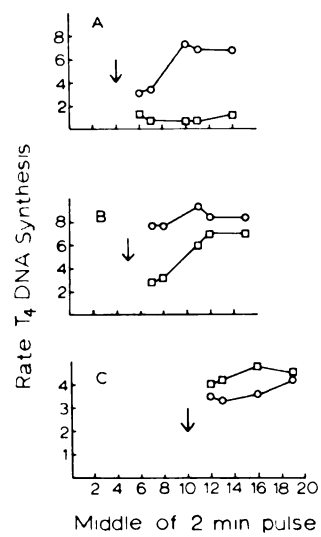

FIG. 2. Same as Fig. 1 except that the cells were infected at 40° and diluted 1:10 into medium at 27° at the time indicated by the arrow. Cells (1 ml) were added to 0.1 ml of [^{3}H]methyl thymidine at 10 μCi and 10 μg/ml. The incorporation was stopped with TCA and the precipitates washed and collected as in Fig. 1 (A) Shift 4 min; (B) Shift 5 min; (C) Shift 10 min (—○—) K803; (—□—) K803-rif^{R}2.

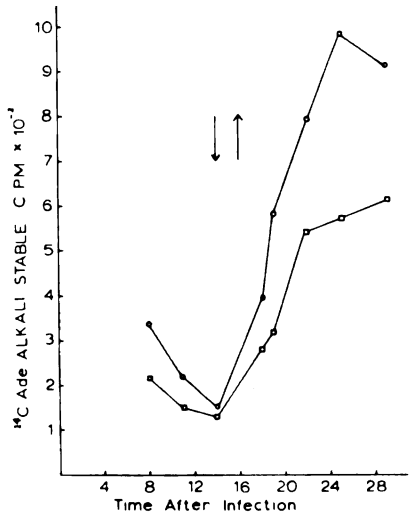

FIG. 3. All of the gene products required for T4 DNA synthesis accumulate in Rif^{R}2 at the nonpermissive temperature. FUdR (5-fluorodeoxyuridine) was added to 20 μg/ml at minus 2 min. T4 den A was added at 0 min and, after 14 min CAM to a final concentration of 150 μg/ml. At 16 min the cells were shifted from 27°-40° with CAM at 150 μg/ml and thymidine at 100 μg/ml. The rate of T4 DNA synthesis was measured as in Figs. 1 and 2 except that [^{14}C] adenine was used as label. Before the time of the shift 0.1-ml aliquots were taken and added to 1 ml of [^{14}C]Ade at 0.2 μCi and 1 μg/ml and FUdR at 20 μg/ml in M9S. After the shift, 1-ml aliquots were taken and added to 0.1 ml of [^{14}C]Ade at 10 × the above concentration. After stopping the incorporation with TCA and centrifuging, the precipitate was resuspended in 0.5 *M* NaOH and incubatd overnight at 37° before reprecipitation with TCA. (—○—) K803; (—□—) K803-rif^{R}2. Arrow down: time of CAM; Arrow up: time of shift into thymidine.

seems to "freeze" DNA replication at the rate achieved at the time of the shift to the nonpermissive temperature. Such a result is expected if rif^{R}2 only affects the formation of new "replication forks" but does not prevent DNA synthesis by "forks" which have already formed at the permissive temperature. However, in conflict with this interpretation are additional experiments showing that the effect on the rate of T4 DNA synthesis is overcome even in the absence of DNA replication *per se*. The experiments were done exactly as those described in Fig. 2 except that the cells were infected with a mutant of T4, tsP36, which codes for a reversibly inactivated DNA polymerase (Riva *et al.*, 1970). DNA synthesis will not begin until the temperature is shifted to 27°. If the shift was made late enough (e.g., 10 min) the effect of rif^{R}2 on DNA synthesis was no longer apparent (Fig. 4). Thus, the function of RNA polymerase in T4 DNA replication which is inhibited by the rif^{R}2 mutation must proceed independently of replication. In the absence of specific knowledge of the nature of this function we shall refer to it as "replication potential" for purposes of further discussion.

Effect of rif^{R}2 on T4 Late Gene Expression

The effect of rif^{R}2 on T4 DNA replication is apparent in thymidine incorporation experiments, but is not the major obstacle to T4 development. As shown above, the effect on T4 DNA synthesis can be "bypassed" by allowing the infection to proceed at the permissive temperature (40°) for sufficient time. DNA synthesis then appears to be normal both in pulse and continuous incorporation experiments and the radioactivity is incorporated into normal length DNA as determined by alkaline sucrose gradient centrifugation (data not shown). However, phage production is still very defective (Fig. 5) indicating a "late block" either in late gene product synthesis or in phage assembly.

Other indications of a late block come from experiments with T4 amber mutants

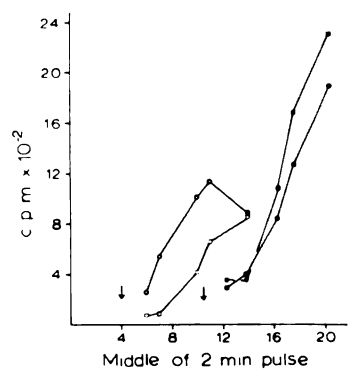

FIG. 4. Effect on DNA replication can be bypassed in the absence of replication. Same as in Fig. 2 except the cells were infected with tsP36 instead of T4. (—○—) K803, (—□—) K803-rif^{R}2. Open figures shift 4 min, closed figures shift 10.5 min. Arrow denotes time of shift.

in genes 46, 47, or 59. Infection by these mutants leads to an early cessation of T4 DNA replication due somehow to the synthesis and function of late gene products (Wu *et al.*, 1972). When Rif^{R}2 was infected with these mutants at the nonpermissive temperature (27°) T4 DNA was replicated but much later than in the wild-type strain (Fig. 6A) suggesting that late gene expression is greatly delayed on Rif^{R}2. If the cells were infected at 40° and the temperature was shifted to 27° late enough so that DNA synthesis is normal but late gene product synthesis has not begun, the arrest of DNA synthesis was delayed (Fig. 6B) indicating that late gene expression occurs more slowly on Rif^{R}2 even if the effect on DNA synthesis is by-passed.

Late gene product synthesis was measured directly by polyacrylamide gel electrophoresis to determine whether the "late block" is on gene product synthesis or on phage assembly. A shift of Rif^{R}2 to the nonpermissive temperature late in infection does not affect the rate of T4 DNA synthesis as discussed above but reduces the rate of amino acid incorporation by about 50% (data not shown). The reduced rate of amino acid incorporation is due in large part to a reduced rate of synthesis of late proteins (Fig. 7). A direct effect of rif^{R}2 on late gene product synthesis is also supported by the map position of some gor-type mutations to be discussed later. At present we do not know if there is an assembly defect as well.

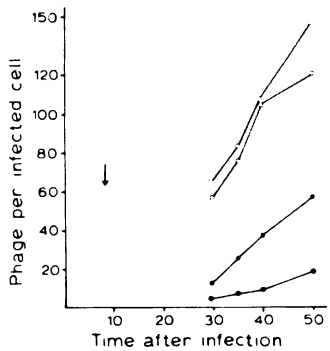

FIG. 5. Phage production after a shift from 40° - 27° at 10 min after infection. (—O—), K803-T4; (—□—), K803-T4 βgt⁻; (—●—), K803-rif^{R}2-T4; (—■—), K803-rif^{R}2-T4 βgt⁻. Arrow denotes time of shift.

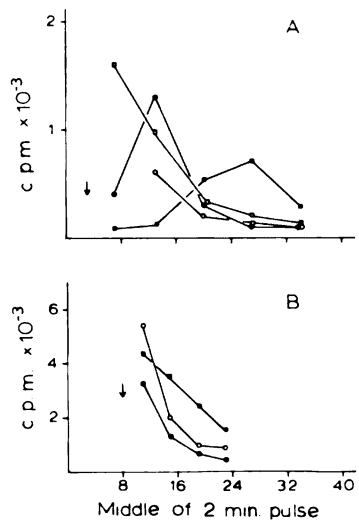

FIG. 6. Rif^{R}2 is blocked in late gene expression. Infection by B14 in gene 46. Rate of T4 DNA synthesis measured as in Fig. 2. (—O—) NF58 40° → 40°; (—●—) NF58 40° → 27°; (—□—) NF58-rif^{R}2 40° → 40°; (—■—) NF8-rif^{R}2 40° → 27°. Arrow denotes time of shift. (A) Shift 3 min; (B) Shift 8 min.

The Effect of β-Glucosylation on T4 Growth in Rif^{R}2

In an earlier publication (Montgomery and Snyder, 1973) we reported that β-glucosyl transferaseless (βgt⁻) mutants grow better than wild-type T4 on Rif^{R}2. In addition, the related coliphages T_2 and T_6, which lack an analogous β-glucosyl transferase, grow better as well (Montgomery and Snyder, unpublished data). Apparently, some hydroxymethyl cytosines which play a special role in phage development are glucosylated with a β linkage. In T_2 and T_6 or in a βgt⁻ mutant of T4 these residues are left unglucosylated thereby avoiding the potential inhibition of a reaction involving RNA polymerase. Similar observations of a suppressive effect of βgt⁻ mutations on T4 growth have been made with RNA polymerase mutants isolated by others (L. Gold, personal communication; R. Horvitz, personal communication; R. Frederick, personal communication).

The inhibition by β-glucosylation seems to be rather specific. While phage production is significantly affected, the lag in

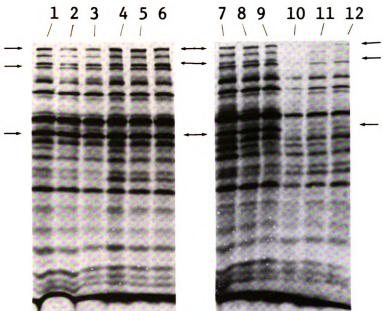

FIG. 7. SDS–polyacrylamide slab gel electrophoresis of T4 proteins synthesized after a shift from 40° to 27° at 10 min. [^{14}C]Leucine was added in 2-min pulses. 1, 2, 3 are K803 shifted 40° → 40° and pulse labeled 13–15 min, 18–20 min, and 23–25 min, respectively. 4, 5, 6 are K803 shifted 40° → 27° with the same pulse times. 7, 8, 9 are K803-rif^{o}2 shifted 40° → 40° with the same pulse times. 10, 11, 12 are K803-rif^{o}2 shifted 40° → 27° with the same pulse times. The proteins migrated from the top to the bottom. The arrows denote some late proteins which were identified by comparison with a parallel infection by a DNA negative mutant of T4 in which late proteins are not made. The arrows point to the products of genes 34, 37, and 23 (23*) from the top down. Some other late bands which can be seen to be significantly affected are due to the products of genes 7, 10, and 18. Presumably, the synthesis of most late proteins is severely inhibited by shifting Rif^{o}2 to the nonpermissive temperature.

onset of T4 DNA replication is not decreased after infection by βgt^{-} mutants although higher rates are achieved later in infection (data not shown). Thus, there may be only one defective function which is inhibited by β glucosylation.

Experiments can be done to determine the time interval during which the inhibited reaction occurs. Since the growth of T4* phage is inhibited on Rif^{o}2 the synthesis of β-glucosyl transferase after infection can inhibit phage development (Montgomery and Snyder, 1973). We expect that the inhibited function is occurring rather late because β-glucosyl transferase is a late appearing early enzyme (Black and Gold, 1971) and β-glucosylation of T4 DNA is completed only late in infection (McNicol and Goldberg, 1973). In fact, the suppressing effect of the absence of β-glucosylation is still apparent even after 10 min at 40° (Fig. 5) indicating that inhibition of phage growth by β-glucosylation can be imposed late in infection.

Other experiments have shown that β-glucosylation of only parental DNA is sufficient to block phage development on Rif^{o}2 (Montgomery and Snyder, 1973). We proposed two possible explanations. One is that part of the parental DNA somehow retains its autonomy throughout infection, the other is that the essential reaction inhibited by β-glucosylation must occur at a time when only parental DNA is present in Rif^{o}2. The latter explanation is given strong support by the following experiments.

The experiments show that the inhibitory effect of parental β-glucosylation can be by-passed early in infection at the

permissive temperature, 40°. We assure that only the parental DNA is β-glucosylated by using amber mutants in the β-glucosyl transferase gene; growing the phage on an amber-suppressing strain of *E. coli*, and following phage production on a non-amber suppressing strain harboring the rif^{R}2 mutation. As shown in Fig. 8, the effect of β-glucosylation of parental DNA is apparent if the cells are shifted to the nonpermissive temperature (27°) early enough after infection, e.g., 2 min, but not if the shift is made somewhat later, e.g., 10 min. Thus the reaction inhibited by β-glucosylation must begin before 10 min after infection at 40° to allow normal phage development.

The fact that parental β-glucosylation is no longer inhibitory late in infection suggests that the function of RNA polymerase which can be inhibited must happen early in infection at a time when only parental DNA is present in Rif^{R}2. Since T4 DNA replication occurs at the normal time at 40° on Rif^{R}2, perhaps β unglucosylated progeny DNA can substitute for β glucosylated parental DNA for the inhibited function if progeny DNA appears soon enough. Accordingly, it would be informative to ask whether T4 DNA replication is required during the early period at 40° in order to bypass the inhibition by β glucosylation of parental DNA. To do the experiment we again took advantage of the properties of the reversible temperature sensitive mutant in gene 43, tsP36. We repeated the experiment shown in Fig. 8, but infected with a double mutant, amβgt, tsP36. DNA synthesis will not occur at the permissive (for the RNA polymerase mutation) temperature (40°) and will only commence after a shift to the nonpermissive temperature. Therefore, progeny DNA will not be present during the period when β-glucosylation of parental DNA is by-passed. As shown in Fig. 9, the effect of parental β-glucosylation was no longer in evidence even in the absence of progeny DNA, so that whatever must happen to parental DNA early in infection and is inhibited by β-glucosylation, has been completed by 10 min after infection at 40°. We cannot say from this experiment if progeny DNA can substitute for parental DNA for the β-glucosylation inhibited function, only that β-glucosylated parental DNA can be used for the inhibited function at 40°. Because the inhibition by β-glucosylation continues late into infection (see Fig. 5), it seems likely that β-glucosylation of progeny DNA is also inhibitory.

Isolation and Mapping of Other gor (Grow on Rif^{R}2) Mutations

If we knew what, if any, T4 gene products are malfunctioning as a result of the RNA polymerase mutation, we could draw upon the wealth of information available

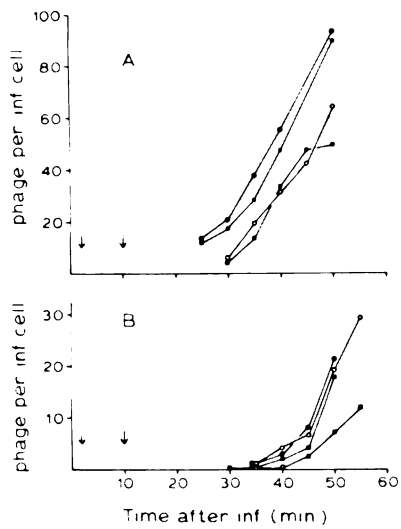

FIG. 8. Inhibition of phage production by parental β-glucosylation can be by-passed early in infection at the permissive temperature. Cells were infected at a m.o.i. of 0.3 with T4amβgt10. (—O—) T4-amβgt10 grown the previous generation on NF58. (—□—) T4 amβgt10 grown on CR63. Open figures shifted 40° → 27° at 2 min after infection. Closed figures shifted 40° → 27° 10 min after infection. (A) NF58; (B) NF58-rif^{R}2. The difference between phage production by amβgt10 grown on the amber suppressing and non-suppressing *E. coli* is less at higher multiplicities of infection suggesting a multiplicity dependent phenomenon. Also, amβgt10 is probably not completely suppressed by either am su$_1^+$ or am su$_2^+$ since it is partially gor on these suppressors even though the β-glucosylation is enough to protect a double mutant amβgt10, αgt$^-$ against restriction. Probably less β-glucosylation is required to protect against restriction than to prevent the gor phenotype.

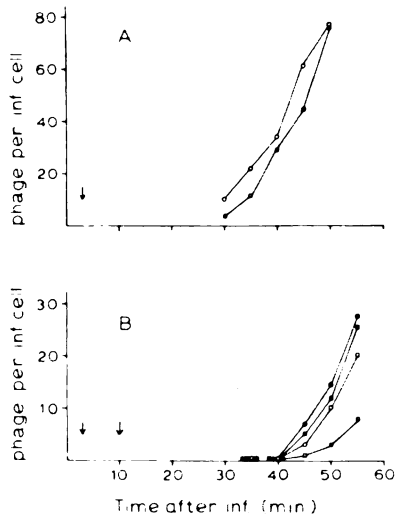

FIG. 9. Inhibition by parental β-glucosylation can be by-passed in the absence of replication. Same as Fig. 8 except cells were infected by a double mutant tsP36, amβgt10. (—O—)tsP36, amβgt10 grown on NF58; (—□—)tsP36, amβgt10 grown on CR63. Open figures shifted 40°-27° at 3 min after infection. Closed figures shifted 40°-27° at 10 min. (A) NF58; (B) NF58-rif^{R}2.

on the function of the various viral gene products to enhance the observations made with the mutant bacteria alone. To find the putative T4 genes directly, we would isolate mutants of T4 which can grow better than the wild-type on Rif^{R}2. Some of these mutants might have a gene product which is altered in such a way that it can function where the wild-type gene product cannot. The responsible mutation should map in the viral gene which is malfunctioning on Rif^{R}2. Since such mutations cause a subtle specific change in a protein, we would expect them to be much rarer than the already isolated βgt$^-$ mutations in which a gene product is simply inactivated. Thus we would expect the vast majority of gor-type mutations to be of the βgt$^-$ variety necessitating a more complex selection procedure such as the one outlined in Materials and Methods. The gor mutants obtained in this way differ from βgt$^-$ mutants in that they vary considerably in their plaque size on Rif^{R}2 suggesting that they are not simply missing a gene product. So far we have three such gor mutants which we call the gor-2 type (βgt$^-$ are the gor-1 type). In all of these, the mutations to the gor phenotype lie in the same region of the map. Some mapping data for one gor-2 mutant is shown in Table 1. The maximum recombination frequency (25%) is seen in crosses with mutations in genes other than in or near 55. For example, crosses with amB14 in gene 46 and am E727 in gene 49 which genes are on either side of gene 55 give recombination frequencies of only about 10%. Crosses with mutations in gene 55, e.g., amBL292 and ts 553 give recombination frequencies of about 1% or less. We have not established unequivocally that gor-2 mutations reside in gene 55, but this is a reasonable map position considering what is known of the function of the product of gene 55 and its role in late messenger transcription.

In the process of mapping the gor-2 type mutations in or very near gene 55, we discovered that an amber mutant, E10, in gene 45 is gor when suppressed by the amber su$_2^+$ suppressor which inserts glutamine. The gor phenotype is due to the suppressed E10 mutation itself since the wild-type recombinants of crosses with amber mutations in genes on either side of gene 45 (genes 44 and 46) are not gor as some of them would be if the gor type mutation were not in or very near gene 45. Also, five revertants of E10 were tested and were found to be no longer gor. In order to establish unequivocally that the suppressed amE10 mutant grows better than its wild-type revertants, amE10 and a revertant were mixed 1 to 1 and plated on K803-rif^{R}2. Twenty plaques were picked and tested to see if they were ambers. This was repeated for five independent revertants. All but one of the 100 plaques chosen were the amber mutant.

The gor phenotype of suppressed amE10 is probably due to an effect of reducing the amount of the gene 45 product in the cell since other suppressed amber mutants in gene 45, e.g., NG18, seem to grow better as well. Apparently, synthesizing less gene 45 product restores a balance of some sort in Rif^{R}2 and enhances phage production. We have not yet tested the other amber suppressors to see if they also give amE10 the gor phenotype.

DISCUSSION

We have concluded from the experiments discussed above that the rif^{R}2 mutation affects at least two events during T4 development. There are direct effects both on T4 DNA replication and on T4 late gene expression. We have also determined the map positions of mutations which permit T4 to grow better on Rif^{R}2. To use λ terminology (Georgopoulos, 1971; Georgopoulos and Herskowitz, 1971), Rif^{R}2 is gro 45 and maybe gro 55.

There is very good evidence that the gene 55 product binds to RNA polymerase (Stevens, 1972; Horvitz, 1973; Ratner, personal communication). The gene 55 product is known to be directly required for late gene expression (Pulitzer, 1970). There is a direct effect of rif^{R}2 on late gene expression and we would expect the responsible gene product to physically interact with RNA polymerase. Thus, it seems very likely that the gene 55 product is malfunctioning on Rif^{R}2 leading to a defect in late gene expression. Conversely, the existence of gor-type mutations which are probably in gene 55 augments our data indicating that Rif^{R}2 is directly blocked in T4 late gene expression.

That there may be other T4 gene products which are malfunctioning on Rif^{R}2 is suggested by the defect in T4 DNA replication and the early inhibition by β-glucosylation. The gene 55 product is not required for T4 DNA replication (Epstein *et al.*, 1963) and is not required early for phage production (Pulitzer, 1970). Accordingly, we may be able to isolate other gor-type mutations. Amber mutations in gene 45, when suppressed, lead to the gor phenotype. Since, in general, reduced amounts of a gene product are made when amber mutations are suppressed it is likely that making less gene 45 product restores a balance of some sort in Rif^{R}2. The gene 45 product is among the T4 gene products retained by RNA polymerase columns made with RNA polymerase from T4 infected cells (Ratner, 1974). The physical data of Rathner, combined with the genetic data presented here, and obtained with Tab D mutants discussed below, makes it seem very likely that the gene 45 product binds to RNA polymerase for its function. Perhaps, on Rif^{R}2, the formation of a complex involving the gene 45 product and RNA polymerase is defective and the presence of reduced amounts of gene 45 product somehow facilitates the formation of the complex. In any case, since the gene 45 product is known to be required for replication, as well as for late messenger synthesis, the existence of gor type mutations in gene 45 strengthens our interpretation that Rif^{R}2 is affected directly in T4 DNA replication and late transcription.

T4 DNA replication is resistant to rifampin. (Rosenthal and Reid, 1973). However, we have presented data that indicate that Rif^{R}2 is directly blocked in T4 DNA replication. This would mean that the rifampin sensitive subunit of RNA polymerase is directly required for T4 DNA replication. Perhaps the RNA polymerase used for replication enters a complex which is impermeable to rifampin; or the synthesis of the RNAs which are required for replication do not require the rifampin sensitive step of transcription; or the role of RNA polymerase in replication, which is defective in Rif^{R}2, does not involve transcription *per se*.

The defects caused by the RNA polymerase mutation involving the gor-2 and 45 products may not be independent. There is, of course, no reason to suspect a relationship between the two defects from the data presented here since the same change in RNA polymerase could cause the malfunctioning of more than one T4 gene product, independently. However, recent data of others also indicate a relationship. They have isolated mutants of *E. coli* which will not support the growth of a particular temperature sensitive T4 mutant in gene 55. The *E. coli* mutants, called Tab D mutants, are probably in a subunit of RNA polymerase (A. Coppo *et al.*, 1974). Some T4 mutations which allow T4 growth on Tab D mutants map in gene 45 (J. Pulitzer, personal communication). The similarity between Rif^{R}2 and the Tab D mutants in that T4 growth on both types of mutants is affected by mutations in both genes 55 and 45 suggests that there may be an interac-

tion between the products of these two genes.

Because we do not know if the only defects on Rif^{R}2 are caused by the products of genes 45 and 55 we cannot say for certain if the function of one (or both) of them is involved in the inhibition by β-glucosylation. However, the gene 45 product is certainly suspect. The inhibition by β-glucosylation occurs early and the absence of the gene 45 product depresses the rate of early RNA synthesis (Mathews, 1968) and/or causes an early expansion of ribonucleotide pools (Mathews, 1972). Furthermore, neither on Rif^{R}2 nor in gene 45 minus infections does the synthesis of any early gene products seem to be prevented (O'Farrell and Gold, 1973; Wu *et al.*, 1973). Also, the need for a functional gene 45 product continues late into infection (Scotti, 1969) as does the inhibition of β-glucosylation. In fact, the product of gene 45 is required for late messenger synthesis even in the absence of replication (Wu *et al.*, 1973). The gene 45 product is also required for DNA replication and the fact that both the DNA replication block and the inhibition by β-glucosylation of only parental DNA are bypassed during the same time interval suggests that there may be a relationship between the two effects. It is not clear what this relationship could be since the delay in T4 DNA replication is independent of β-glucosylation. However, it is possible that there are two defects in replication caused by the same gene product only one of which can be suppressed by the absence of β-glucosylation. In any case, it should be possible to unequivocally establish whether the functioning of the product of gene 45 or some other gene is potentially inhibitable by β-glucosylation if we can quantitatively account for all of the defects in T4 development on Rif^{R}2 and then determine which gor-type suppressions are additive and which ones are not.

When cells are allowed a period after infection of about 10 min at 40°, with or without replication, there is no longer an effect of β-glucosylation of parental DNA on phage production. This could be explained if there are some early gene products which can only be made early and whose synthesis at 27° in Rif^{R}2 is inhibited by β-glucosylation. We have not rigorously excluded this possibility even though our data indicate that at least most early gene products, including all those required for T4 DNA replication (see Fig. 3), are made on Rif^{R}2. However, we would like to propose an alternative explanation. We propose that there is normally a change in the structure or association of parental DNA early in infection which is completed within ten minutes at 40°. The process, involving RNA polymerase, which produces the change is cold sensitive on Rif^{R}2. If the change does not occur normally to parental DNA, and presumably, to at least some progeny DNA, replication and late transcription do not occur properly. The effect on late transcription is somehow suppressed by the absence of β-glucosylation. It is probably worth reviewing the known functions of RNA polymerase to see if one of them could be involved.

One known function for an RNA polymerizing enzyme is to make "primers" for DNA replication. RNA is found covalently attached to the short intermediates of *E. coli* DNA replication (Sugino et al., 1972) although it is not clear if these are synthesized by the characterized RNA polymerase enzyme. Also, there is some evidence that the "primer" RNAs are synthesized at the same time as the DNA fragment (Sugino and Okazaki, 1973) so we might not expect them to accumulate in the absence of replication as does "replication potential" and the ability to by-pass β-glucosylation of parental DNA.

DNA polymerase is also required for the initiation of λ (Dove *et al.*, 1969), *E. coli* (Lark, 1972) and M13 (Brutlag *et al.*, 1971) DNA replication. It is not clear if these are "primer" functions or some other requirement for RNA in replication. It is also not clear if these RNAs can accumulate in the absence of replication although it is known that they must be synthesized anew for each round of *E. coli* DNA replication (Lark, 1972). If such RNAs exist for T4, and if their synthesis is prevented by the rif^{R}2 mutation, then they are able to accumulate

in the absence of replication but must be synthesized throughout infection for normal phage production.

One reaction involving RNA polymerase which may or may not be independent of the role of RNA in initiation of replication is the synthesis of RNAs which hold DNA to membrane in highly folded configurations often called "nucleoids" (Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Gierer, 1973; Dworsky and Schaechter, 1973). At present, there are no published reports of viral nucleoids held together by RNA although there have been many reports showing that viral DNAs enter fast sedimenting complexes after infection. However, if nucleoids are required for some aspect of replication and/or transcription, viral DNA may enter such states as well. But T4 nucleoid formation by RNA polymerase is only one possibility for a function of RNA polymerase which could be inhibited by β-glucosylation. Perhaps there are other, as yet undiscovered, roles for RNA polymerase in viral development.

As we have argued before (Montgomery and Snyder, 1973), an inhibition by only β, and not α, glucosylation suggests an involvement of hydroxymethyl cytosine rich regions on DNA because such regions are heavily β-glucosylated. Whatever the inhibited reaction is finally discovered to be, it is obvious that using host RNA polymerase mutants to study viral regulatory mechanisms offers a different perspective from using only viral mutants and should lead to the discovery of fundamental molecular mechanisms which could not easily be discovered in other ways.

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Note added in proof. Recent data suggest that gor-2 mutations are not in gene 55 but to the left of 55 and to the right of 2gt. Their nature is still under investigation.

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