ANTI-LATE RNA AND TRANSCRIPTIONAL CONTROL IN T4 BACTERIOPHAGE INFECTED ESCHERICHIA COLI

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ABSTRACT

ANTI-LATE RNA AND TRANSCRIPTIONAL CONTROL IN T4 BACTERIOPHAGE INFECTED *ESCHERICHIA COLI*

By

Robert J. Frederick

The regulation of transcription in T4 infected bacteria is both complex and diverse. I have examined some of the transcriptional events involved in the development of T4 using two separate approaches. The first takes advantage of the antibiotic rifampicin. The second involves the characterization of a unique RNA species called anti-late RNA.

At a concentration of 100 μg/ml of rifampicin, only partial inhibition of ^{3}H-uridine incorporation occurs in T4 infected *E. coli* K803. The residual transcription activity in the presence of the antibiotic, or rifampicin refractory synthesis, is dependent upon the time the antibiotic is added. It does not occur in *E. coli* B strains at the same rifampicin concentration or in the K12 strains at higher concentrations. The refractory synthesis is at least partially specific for certain RNA transcripts; that is, all of them are not reduced proportinately. In addition, some preliminary results suggest that the effect is also dependent upon the template being transcribed.

The second and major portion of this dissertation is devoted to the characterization of anti-late RNA in T4 infected *E. coli*. The anti-late transcripts are made early during infection but are complementary to late RNA. They constitute only 2% of the T4 specific RNA but are transcribed on over 80% of l-strand (early strand) in the late region of the genome. Anti-late RNAs sediment in a broad peak on neutral sucrose gradients at about 20 to 22 S. They are made in the late region on the l-strand. They are refractory to rifampicin added at 1 minute after infection.

Furthermore, the examination of several T4 and *E. coli* mutants demonstrates that anti-late RNA synthesis is subject to the same control mechanisms as delayed early transcription. In cases where the delayed early expression is altered at the transcriptional level, the synthesis of anti-late RNA is also altered. In one instance where a translational control mechanism has been proposed, anti-late transcripts were made normally. This suggests that quantitation of anti-late RNAs may serve as a criterion for distinguishing between translational and transcriptional regulation.

In the discussion it is postulated that anti-late transcription is subject to strong polarity effects once initiation has occurred. When the polarity constraints are removed, synthesis occurs along the l-strand in the late region making the complementary RNA.

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By

Robert J. Frederick

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DEDICATION

To Mark and Kathy, who unknowingly gave up many
things for their father, and very especially
to Barbara, who endured and persevered
ab initio ad finem

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INTRODUCTION

The transfer of genetic information from a deoxyribonucleic acid (DNA) genome into functional ribonucleic acid (RNA) and protein molecules involves many and varied control processes. Inherent in these is the capacity to regulate growth, maintain physiological order, and preserve the temporal sequence of development. The problems to be examined and discussed in this dissertation are directed toward a better understanding of DNA dependent RNA synthesis (transcription) and its regulation using bacteriophage T4 infection of *Escherichia coli* as a model system.

The study of T4 as a transcriptional model offers many advantages. It is a complex virus with a DNA coding capacity of 150 to 200 genes, approximately 60% of which have been identified. Therefore, a great deal is already known about the genetics of this phage. In addition, it has been well characterized biochemically. More important to this work, however, is T4's diversity of transcriptional regulation. The T4 phage maintain a strict transcription sequence that is regulated on at least two levels, the DNA template and the RNA polymerase. The complexity and diversity of T4 regulation, coupled with the ease of handling are why I chose to work with this system.

The first part of this dissertation describes a change in the sensitivity of the RNA polymerase to rifampicin after infection.

This antibiotic blocks initiation of RNA synthesis by binding to the β subunit of the enzyme. Evidence is presented that transcription is partially refractory to rifampicin at certain times after infection. This can be demonstrated in K12 strains but not B strains of *E. coli*. It does not appear to be due to induced or intrinsic permeability differences, but is dependent upon late gene expression and the state of the template being transcribed.

The second part of this dissertation concerns the characterization of anti-late RNA regulation in T4 infected bacteria. Anti-late RNA is a species of T4 transcripts which is made on the anti-sense strand of the late genes. It is made early during the infection on an extensive length of the late region but constitutes only 2% of the early T4 RNA. They are the size of late mRNA, are made from initiations within the late region and are controlled in the same way as delayed early mRNA transcription. The data are discussed in terms of T4 regulation of early gene expression and transcriptional control mechanisms in general.

LITERATURE REVIEW

The T4 Genome

The T4 genome is a terminally redundant, 130 x 10^6 dalton double stranded DNA molecule. It is physically and genetically separable into four regions corresponding to early (2) and late (2) gene expression or alternatively pre- and postreplicative transcription units (Wood, 1974; Geiduschek et al., 1968). In general, each region has a distinct polarity; that is, transcription occurs only on one strand and, therefore, in one direction. Guha et al. (1971) and Notani (1973) have shown that 98% or more of early mRNA is made on the DNA l-strand and more than 80% of the late mRNA on the r-strand.

The T4 DNA contains hydroxymethylcytosine (HMC) instead of cytosine (Wyatt and Cohen, 1952). This unique base serves several functions for the virus. It has recently been determined, for example, that when cytosine replaces HMC in progeny DNA, the late genes are not transcribed (Wu and Geiduschek, 1975; Kutter et al., 1975). Szybalski et al. (1966) have shown that the HMC residues can exist in clusters. There is some evidence that these clusters have some regulatory role (Montgomery and Snyder, 1973; Snyder and Montgomery, 1974).

Not only does T4 use hydroxymethylcytosine instead of cytosine, but this base is also glucosylated after replication. Lehman

and Pratt (1960) showed that all of the HMC residues are singly glucosylated, 70% with an α-linkage and 30% with a β-linkage. This pattern of glucosylation is characteristic of T4, the other T-even phage differing in the amount and type of glucosylation present (Mathews, 1971; Revel and Luria, 1970). It has been suggested that glucosylation has evolved as a protective mechanism against host restriction nucleases (Revel and Luria, 1970), thereby increasing the host range of the phage.

T4 and Transcriptional Regulation

Shutoff of Host Transcription

Shortly after infection, T4 causes the cessation of all *E. coli* macromolecular syntheses (for a review, see Mathews, 1971). Despite a great deal of study, the mechanisms involved have not been clearly elucidated. In one report, for example, two mechanisms were proposed (Nomura et al., 1966). The first did not require T4 protein synthesis and seemed to be active at the membrane level by means of a colicinogenic (El and K) type reaction. The second required a T4 gene product but just how this caused the arrest of *E. coli* mRNA synthesis was not determined.

T4 Induced Modifications of the Host RNA Polymerase

T4 bacteriophage have a very complex regulatory system for gene expression. Unlike other bacteriophage such as T7 and T3, T4 uses the host RNA polymerase throughout infection (Haselkorn et al., 1969; Mizuno and Nitta, 1969; di Mauro et al., 1969). The *E. coli* polymerase is a large molecule composed of four subunits designated

α, β, β' and δ in the ratio of 2:1:1:1 (Burgess, 1971). During the course of infection, T4 causes many changes in the enzyme, some of which have been shown to be involved in transcriptional regulation.

Snyder (1973) and Pitale and Jayaraman (1975) demonstrated a change in the sedimentation profile and template specificity of RNA polymerase after T4 infection. Schachner et al. (1971) reported a change in the electrophoretic mobility of the β subunit. Goff and Weber (1970) and Seifert et al. (1971) showed that adenosine and phosphorus are covalently linked to the alpha subunit after infection. Goff (1974) later reported that this was accomplished by the enzymatic attachment of adenosinediphosphoribose residues. He also showed that the adenylation occurred by two distinct mechanisms, one requiring T4 gene expression (modification) and one which did not (alteration). Recently, Horvitz (1974b) presented evidence that alteration and modification are nonessential for phage production. Rohrer et al. (1975) have successfully isolated the alteration enzyme and characterized it as a 70,000 dalton NAD^+:protein ADP-ribosyltransferase. It is carried in the phage heads and is injected with the DNA (Horvitz, 1974a; Rohrer et al., 1975).

Not only are these structural modifications of cellular RNA polymerase induced, but phage specific activity directed to the enzyme also occurs. For example, both T4 induced sigma (Travers, 1970) and anti-sigma (Bogdanova et al., 1970; Khesin et al., 1972; Stevens, 1974) factors have been reported. Furthermore, several phage specific polypeptides have been shown to have an affinity for the polymerase (Ratner, 1974). Some of these bind so tightly they can be isolated with the enzyme (Stevens, 1972; Horvitz, 1973).

Stevens (1972) tentatively identified two of these as the T4 gene 55 and gene 33 products. Subsequently, Horvitz (1973) proved that the latter was indeed the T4 gene 33 product. In his study of RNA polymerase binding proteins, Ratner (1974) used affinity chromatography to show that the gene 45 product bound to the RNA polymerase isolated from infected *E. coli*. The association of the gene 45 polypeptide was also implicated *in vivo* by Snyder and Montgomery (1974) and Coppo et al. (1975a,b). All of the above genes are required for late gene expression of T4 (Bolle et al., 1968b; Notani, 1973; Wu and Geiduschek, 1975). Very recently, Snyder (personal communication) has obtained evidence that another of the tightly bound polypeptides originally described by Stevens (1972) is responsible for the inability of the phage to make late mRNA on cytosine containing DNA.

Regulation of Early or Prereplicative Transcription

Early gene expression encompasses the transcriptional events occurring before the start of DNA replication. The early transcripts have been subdivided into three classes: immediate early (IE), delayed early (DE) and quasi-late. The nomenclature derives essentially from the time they appear after infection and the length of time they are synthesized. A diagram of representative transcriptional units as discussed by O'Farrell and Gold (1973) is shown in Figure 1.

IE gene products can be detected by SDS polyacrylamide gel electrophoresis approximately one minute after infection, while DE gene products are not made until two or three minutes after infection

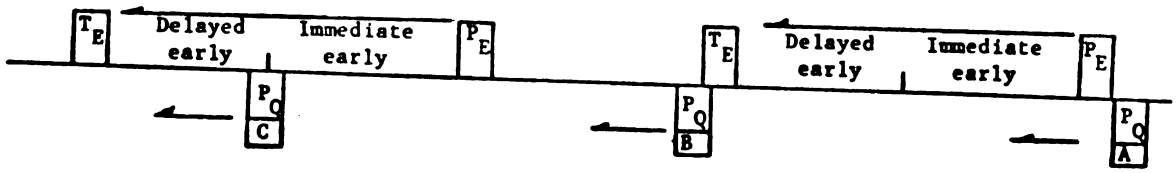

Figure 1. Topological model of prereplicative transcription units as proposed by O'Farrell and Gold (1973a). Abbreviations: (P_E) immediate early promoters; (T_E) termination site; (P_Q) quasi-late promoters; A immediate early; B quasi-late; C delayed early.

(O'Farrell and Gold, 1973a). Immediate early transcripts are made in the presence of chloramphenicol, but delayed early are not (Grasso and Buchanan, 1969; Salser et al., 1970; Lembach and Buchanan, 1970; Peterson et al., 1972). This led to the conclusion that delayed early transcription required a T4 protein. On the other hand, amino acid analogs did not have any effect on DE transcription (Black and Gold, 1971; Lembach and Buchanan, 1970). Furthermore, other methods to disrupt protein synthesis such as amino acid starvation (Witmer et al., 1975; Baros and Witmer, 1975) or depletion of potassium in a cell which cannot concentrate or retain the ion did not inhibit DE transcription (Morse and Cohen, 1975). It was assumed, therefore, that chloramphenicol was affecting transcription by some other manner than inhibiting protein synthesis. In 1969, Morse demonstrated that this antibiotic exerted a polar effect on the transcription of the trp operon. If, then, the DE genes were read from IE promoters, chloramphenicol could have been causing an analogous polar effect on early T4 transcription.

In fact, there has been considerable evidence, *in vivo* and *in vitro*, that many if not most prereplicative genes are transcribed as polycistronic messages (Brody et al., 1970; Stahl et al., 1970; Milanesi et al., 1969, 1970; Black and Gold, 1971; Witmer, 1971; O'Farrell and Gold, 1973a,b; Hercules and Sauerbier, 1974). Milanesi et al. (1970), for example, showed that only IE transcripts were made *in vitro* on a sheared DNA template. That is, DE regions could be physically separated from their promoters. In *in vivo* and *in vitro* experiments, O'Farrell and Gold (1973a,b) showed that DE

gene products could be made when transcription initiations were blocked with rifampicin at one minute after infection.

Several authors (Schmidt et al., 1970; Brody and Geiduschek, 1970; Milanesi et al., 1970; Brody et al., 1970; Schachner et al., 1971) proposed that a termination site existed between the promoter proximal (IE) and promoter distal (DE) genes. According to this hypothesis a termination factor or intrinsic termination site would have to be somehow suppressed by an anti-terminator to allow read through to the DE genes. Roberts (1969) reported one such termination factor, rho, in *E. coli* which prevented transcription of bacteriophage. There have been conflicting reports of the effect of rho on T4 transcription. Jayaraman (1972), Travers (1970) and Richardson (1970) all reported a positive rho effect (i.e., termination) on the *in vitro* transcription products. O'Farrell and Gold (1973b), however, did not see any effect of the termination factor in a T4 transcription-translation system. This may have been due to the high salt concentration which is necessary for translation inhibiting the binding of rho (Richardson, 1970; Schafer and Zillig, 1973b).

Recently, Ratner (1976) and Richardson et al. (1975) have established that rho is coded for by the suA gene. Mutations to suA have been shown to suppress the polarity effect of chloramphenicol on the trp operon (Morse, 1970, 1971). However, chloramphenicol inhibition of delayed early T4 transcription was not affected in suA hosts (Young, 1975; Baros and Witmer, 1975; Witmer et al., 1975). This suggests that rho may not be involved in termination of immediate early transcripts *in vivo*. Of course, some other termination factor

(e.g., kappa, Schafer and Zillig, 1973) or intrinsic termination sites could be involved. In any event, if a termination step is part of the prereplicative regulatory events, then one might expect there also to be some anti-termination factor, perhaps analogous to the N gene product of lambda bacteriophage (Roberts, 1970). There is no direct evidence for this type of mechanism in T4.

An alternative to the termination factor model has been suggested by Black and Gold (1971) and O'Farrell and Gold (1973a,b). They propose a passive control due to the topography of the prereplicative transcription units. The promoter proximal genes (IE) are transcribed first, followed by the promoter distal genes (DE) from pre-early promoters (P_E). The time DE are synthesized thereby would depend upon the length of the IE transcripts, that is how far the polymerase had to move before reaching the DE regions. This hypothesis is also supported by Baros and Witmer (1975).

The third class of prereplicative genes is the quasi-late. These are made from newly recognized promoters as early as two minutes after infection (O'Farrell and Gold, 1973a). This was concluded from experiments using rifampicin to block new RNA polymerase initiations. When the antibiotic was added at one minute after infection, the quasi-late gene products were either reduced in quantity or not made. Those made in reduced amounts were made from both P_E type promoters, i.e., promoter distal genes, and from P_Q (quasi-late promoters) located between the IE and DE regions. Those not made at all in the presence of rifampicin were made only from P_Q promoters. Additional evidence that some early

genes have more than one promoter has been reported by Hercules and Sauerbier (1974).

The study of prereplicative gene expression has been slowed somewhat by the lack of mutants affecting the early regulatory events. In 1974, Mattson et al. isolated and characterized a T4 mutant which made the P_E to P_Q switch inefficiently at high temperatures. In addition, M. Nelson and L. Gold (unpublished observations) isolated an rIIB revertant that does not make the P_E to P_Q switch at all low temperature. Such mutants should help in the examination of prereplicative controls in T4.

Late Gene Expression

Our understanding of T4 late transcription has lagged behind that of early transcription. One reason for this has been the lack of a well defined *in vitro* system for making late transcripts. Only crude systems could be made to transcribe late regions (Snyder and Geiduschek, 1968; Maor and Shalitin, 1974). Despite a general lack of *in vitro* data, many interesting events involved in late transcription have been reported.

Late gene transcription has been shown to require modifications in the host polymerase, processing of the DNA and simultaneous replication, and HMC instead of cytosine in the template. RNA polymerase modifications have already been discussed (see above). The coupling of replication to late transcription was shown by Riva et al. (1970a). Stopping DNA synthesis in temperature shift experiments also resulted in the cessation of late transcription. This was not an absolute requirement, however. In another report, Riva

et al. (1970b) demonstrated that the replication dependence could be bypassed by mutations in the T4 DNA ligase gene which resulted in nicked DNA. This led to the hypothesis that "competent" DNA was necessary for late transcription (Riva et al., 1970b). Subsequently, Wu and Geiduschek (1973, 1975) have shown that some late transcription does occur in the absence of replication but at a much reduced level.

A more complete block of late transcription occurs when cytosine replaces HMC in the DNA. Kutter (1975) has shown that normal amounts of DNA are made in mutants which introduce cytosine into progeny DNA and in which certain T4 nucleases are not present. In these infections, less late protein synthesis occurs than when replication is inhibited. As mentioned earlier, there is new evidence that one of the T4 gene products which binds to the polymerase is responsible for the block to late transcription on cytosine containing DNA (Snyder, to be published).

There is also some suggestion that glucosylation may have some role in late transcription. Riva et al. (1970a), for example, reported a more dramatic cessation of late gene expression after blocking replication when the progeny DNAs were unglucosylated. In addition, Montgomery and Snyder (1973) demonstrated that a block of T4 late transcription caused by a mutation to the host polymerase was suppressed by mutations in the T4 β glucosyl transferase gene (βgt). Interestingly, the βgt^- mutations did not suppress other defects in the T4 reproduction process in the host mutant (Snyder and Montgomery, 1974; Montgomery, PhD Dissertation, MSU, 1975). Beta glucosylation occurs predominantly on adjacent HMC residues

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(diWaard et al., 1967). Since HMC can occur in clusters (Szybalski et al., 1966) and these would be primarily β glucosylated, it was suggested that these regions may have some significance in the regulation of late transcription (Montgomery and Snyder, 1973).

As mentioned before, the glucosylated state of the DNA protects it from host nucleases (Revel and Luria, 1970). This is observed as a 100,000-fold difference in plating efficiencies between the wild type T4 and unglucosylated T4 (Revel and Luria, 1970; Cox and Conway, 1973). If the glucosylated state did evolve under what appears to be strong selective pressure, it seems reasonable to think that there are phage specific mechanisms to facilitate the transcription of glucosylated DNA. This may be indicated in the decreased burst size of unglucosylated phage when grown on a non-restricting host (Georgopoulos and Revel, 1971) and the data of Montgomery and Snyder (1973) mentioned above.

Anti-Late RNA

The accepted view that transcription is strictly asymmetric has recently come under some question. There is now increasing evidence that symmetrical RNA is made in a number of biological systems. These include the animal viruses: SV40 (Aloni, 1972, 1973), vaccinia (Colby and Duesberg, 1969), Adenovirus 2 (Zimmer and Raskas, 1976), herpes simplex (Zeev and Beker, 1975), and polyoma (Aloni and Locker, 1973); HeLa cell mitochondria (Aloni and Attardi, 1971, 1972; Young and Attardi, 1975; Murphy et al., 1975); and the bacterial viruses λ (Bøvre and Szybalski, 1969), the *Pseudomonas* phage, gh-1 (James Jolly, PhD Dissertation, MSU, 1976), and T4 (Jurale et al., 1970; Geiduschek and Grau, 1970; Notani, 1973).

In 1970, Jurale et al. reported the isolation of double stranded RNA fragments from T4 infected *Escherichia coli*. These were shown to be phage specific molecules with molecular weights estimated at 8.5×10^4 to 8×10^5. The authors presumed these arose from the overlapping transcription of opposite DNA strands; a proposal that was previously suggested by Bøvre and Szybalski (1969) for the b2 region of lambda phage.

Geiduschek and Grau also published data in 1970 that demonstrated symmetrical RNA synthesis by the T4 phage. Complementary RNA was assayed by using *in vitro* annealing conditions and digesting noncomplementary single strand molecules with RNases. Using this technique, they determined that this RNA was made early and hybridized with late mRNA. They called this complementary species antimessenger RNA and postulated that it was made by termination errors resulting in "read through" from the early to late regions on the l-strand of the DNA.

In related work, Notani (1973) characterized T4 antimessenger RNA further. He found that antimessage transcripts were made between two and six minutes after infection. They are not made in the presence of chloramphenicol. The decay rates of this RNA were comparable to normal mRNA. Antimessenger RNA constituted approximately 2% of the early RNA being transcribed at any one time, but was made from about 81% of the late region. Young (1975) later confirmed this estimate by showing that up to 90% of the l-strand was transcribed early during infection. From his results, Notani (1973) argued against a "read through" type synthesis of antimessenger RNA. If one allowed that T4 transcription proceeds at

the rate of one gene per half minute (Bremer and Yuan, 1968), and remembering that late genes of T4 are clustered, synthesis of the complementary RNA species in the amounts observed would require about 14 minutes by a defective termination mechanism.

In view of this, Notani postulated that these anti-sense transcripts may be the result of mistakes in initiation or a change in initiation specificity of RNA synthesis.

However they are made, it is possible that antimessenger RNA may be functional. Wu et al. (1973) have suggested some possibilities. These included a role in regulating protein synthesis, replication or initiation of RNA synthesis. There is, however, no clue to any functional role for these RNAs at the present time.

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ARTICLE I

RIFAMPICIN REFRACTORY RNA SYNTHESIS IN T4 BACTERIOPHAGE INFECTED *ESCHERICHIA COLI* K12

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Manuscript to be submitted for publication

ABSTRACT

Rifampicin is a potent and specific inhibitor of RNA synthesis. In this report we describe a rifampicin refractory RNA synthesis in T4 infected *E. coli* K12 strains. This occurs at concentrations of the antibiotic that completely inhibit host transcription. It is dependent upon the time rifampicin is added, occurring only after 5 minutes at 37 C. In addition, we present evidence that only some transcription is refractory and that the effect is dependent upon the template being transcribed.

INTRODUCTION

T4 bacteriophage use the *E. coli* RNA polymerase during infection. This was affirmed by the conservation of the phenotype of polymerase mutations, with respect to its rifampicin or streptolydigin sensitivity, throughout the latent period (Haselkorn et al., 1969; di Mauro et al., 1969; Mizuno and Nitta, 1969). All of these studies were performed using *E. coli* B as the host. In attempting to repeat these results using a K12 strain of *E. coli* (K803), we found that at a concentration of 100 μg/ml, rifampicin depressed the rate of ^{3}H-uridine incorporation 70 to 80% of that of an infected untreated control. The same concentration, however, resulted in a greater than 97% inhibition in the uninfected bacteria. In this investigation, we have partially characterized the effect of rifampicin on T4 infected *E. coli* K803. We present evidence that suggests a differential sensitivity to the antibiotic at intermediate concentrations. In addition, we postulate that it is

dependent upon the state of the enzyme as well as the template being transcribed.

MATERIALS AND METHODS

Bacteria and bacteriophage: *Escherichia coli* K803 ($r_K m_K^-$, rgl^-) and NF58 ($\underline{am}su^-$) have been described (Montgomery and Snyder, 1973). *Escherichia coli* B_E was obtained from L. Gold.

$T4^+$ is a laboratory wild type strain. All other T4 strains have been previously referenced (Montgomery and Snyder, 1973; Snyder and Montgomery, 1974). All of the phage were purified on 3 ml CsCl step gradients at 35,000 rpm in a Spinco SW50 Rotor. The phage band was collected and dialyzed against sequential changes of 2, 1, and 0.5 M NaCl and finally M9 buffer.

Media and chemicals: M9S and tryptone are the same as described previously (Snyder and Montgomery, 1974). M9AA is M9 medium supplemented with 20 µg/ml each of alanine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, arginine and methionine.

^{14}C-leucine and rifampicin were purchased from Schwarz-Mann Company. Stock solutions of the antibiotic were prepared by dissolving it first in methanol (10 mg/0.1 ml) and then diluting with continuous mixing in water or M9S to give a final concentration of 1 mg/ml. Uridine-5-^3H and thymidine-methyl-^3H were purchased from New England Nuclear.

Preparation of cultures: The bacteria for each experiment were transferred to fresh medium (1:50) from overnight cultures stored at 4 C. Absorbance at 625 nm was measured on a Spectronic 20 spectrometer. An A_{625} of 0.4 corresponded to 4 x 10^8 bacteria/ml. The bacteria were infected at a multiplicity of infection (MOI) of 5. Bacterial survivors were determined at 2 or 3 minutes after infection by diluting them with 0.85% saline and plating them on tryptone agar. In all experiments reported here, survivors were less than 2%. Phage production was measured as previously described (Snyder and Montgomery, 1974).

Measuring the rate of RNA and DNA synthesis: The procedure for measuring the rate of ^{3}H-thymidine incorporation was previously described (Snyder and Montgomery, 1974). The rate of RNA synthesis was measured in the same fashion using 1 µCi and 1 µg/ml of 5-^{3}H-uridine. Trichloroacetic acid (TCA) precipitates were collected on Gelman cellulose triacetate filters, dried and counted in a Packard ambient temperature scintillation counter using a toluene base fluor.

Alkali stability of ^{3}H-uridine labeled TCA precipitates: TCA precipitates of cultures labeled in the presence of rifampicin were resuspended in 0.5 M KOH. One half was reprecipitated immediately and the other stored overnight at 37 C. After this incubation, the sample was precipitated with 10 volumes of 5% TCA. The precipitates were collected and counted as described above.

SDS-slab gel electrophoresis: To label T4 proteins, the bacteria were grown in M9AA. Samples (2 ml) of the infected cells were transferred to test tubes containing 2 μCi/ml of ^{14}C-leucine (312 mCi/mMole). Samples were prepared and electrophoresed on a 12.5% polyacrylamide gel according to Studier (1973). The gel was dried under vacuum and developed on Kodak no screen X-ray film.

Hybridization competition assays: ^{3}H-RNA was labeled, extracted and assayed as described by Bolle et al. (1968a). All competitions were done at DNA excess. The T4 DNA was extracted from CsCl purified virus according to the procedure of Mandell and Hershey (1960).

RESULTS

Rifampicin refractory ^{3}H-uridine incorporation: When *E. coli* K803 is treated with 100 μg/ml of rifampicin, the rate of ^{3}H-uridine incorporation decreases to less than 4% of untreated bacteria within 10 minutes. However, when these same bacteria are infected with T4^{+} and subsequently treated with the same concentration of antibiotic, uridine incorporation drops to between 20 and 40% of an untreated, infected control (Figure 1). We shall refer to this persistent incorporation in the presence of the antibiotic as rifampicin refractory activity.

As seen in Figure 1, the refractory synthesis is dependent upon the time of addition of rifampicin. In addition, it is concentration dependent. Since doubling the amount of antibiotic added at 5 minutes resulted in a 90% decrease of uridine incorporation (data not shown), this tends to exclude the possibility of some

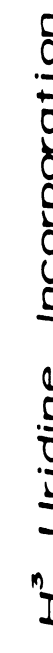

Figure 1. Rifampicin refractory RNA synthesis in T4 infected *E. coli* K803. The rate of ^{3}H-uridine incorporation was measured for 0.1 ml samples of a K803 culture infected at time 0. Rifampicin at a final concentration of 100 µg/ml was added at 2 min (O), 5 min (□), and 10 min (●) after infection. Results are reported as the percent of uridine incorporated in an infected, untreated control during the same 2 min interval.

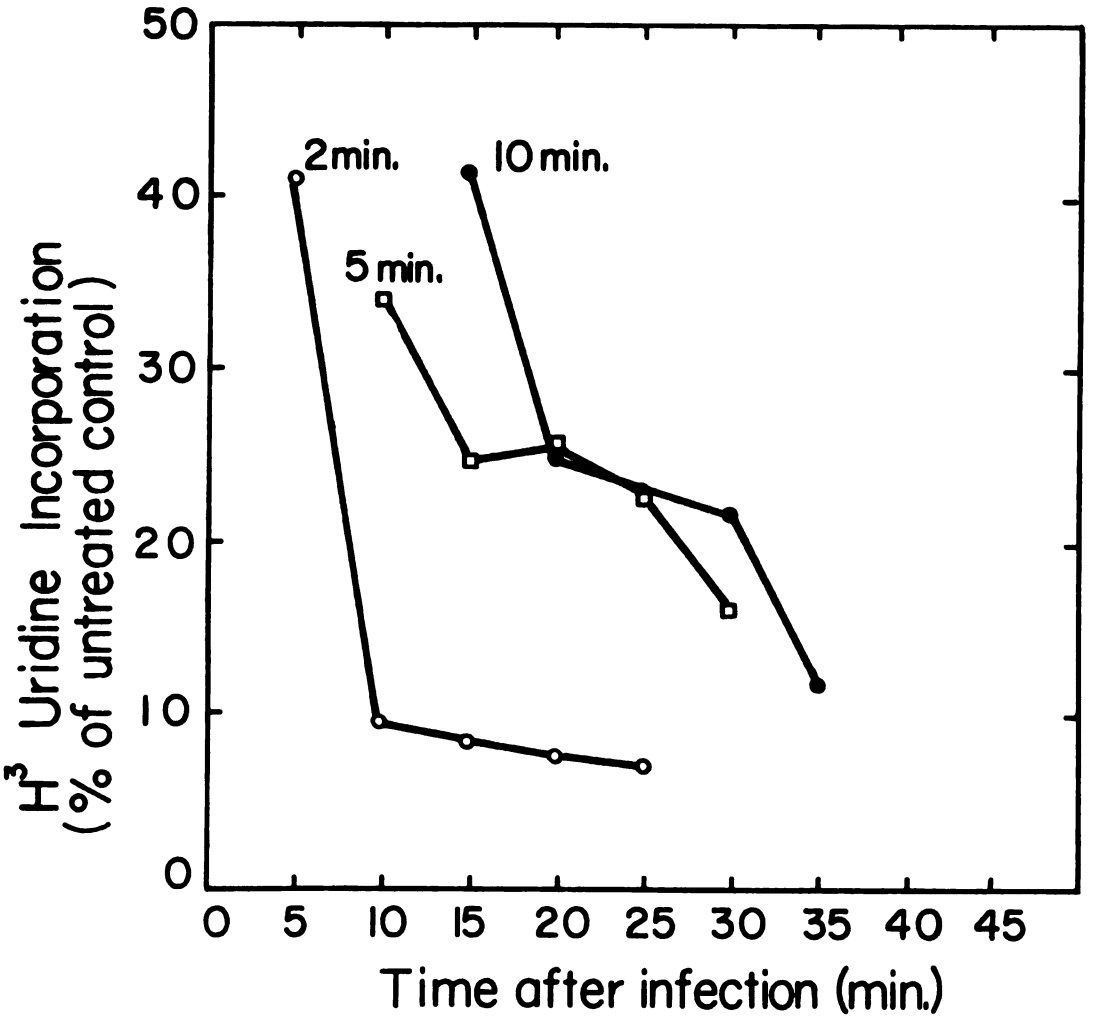
50
40
30
20
10
0
2min.
10 min.
5 min.
H^3 Uridine Incorporation
(% of untreated control)
0 5 10 15 20 25 30 35 40 45
Time after infection (min.)

Figure 1

mechanism for uridine incorporation other than the host polymerase which may be resistant to rifampicin. To insure that we were measuring RNA synthesis, we determined the alkali sensitivity of acid-insoluble counts (see Methods). The alkali treated samples lost 96 to 97% of the TCA precipitable radioactivity.

Since previous studies done with B strains of *E. coli* had not shown any rifampicin refractory synthesis (Haselkorn et al., 1969; di Mauro et al., 1969), we tried to duplicate our results in T4 infected *E. coli* B_E. In this case, RNA synthesis was inhibited to the same extent (approximately 90%) independent of the time of addition of rifampicin (data not shown). The difference between K12 and B strains is also reflected in the phage production and synthesis of T4 gene products when rifampicin was added at 5 minutes after infection at 37 C (Figures 2 and 3). On B_E, phage production was less than 1% of that in the untreated control. Similar data were also reported by Rosenthal and Reid (1973). On K803, however, phage production reached 25% of the control.

Similarly, late gene product synthesis in rifampicin treated B_E is greatly reduced within 15 minutes after the addition of the antibiotic (Figure 3). In addition, the continued synthesis of early gene products is also indicated (see arrows). This effect of rifampicin was also seen by O'Farrell and Gold (1973). The continued early gene expression was attributed to the lack of new mRNA transcripts which could successfully compete with old transcripts for translation.

In contrast to the rifampicin effect on B_E, on K803 all of the normally occurring late gene products were made. The overall amounts

Figure 2. The effect of rifampicin on phage production in *E. coli* K803 and B_E. The bacteria were grown and infected in M9AA medium at 37 C. Rifampicin, 100 µg/ml, was added at 5 minutes after infection. (□) K803 (control), (○) B_E (control), (■) K803 + rifampicin, (●) B_E + rifampicin.

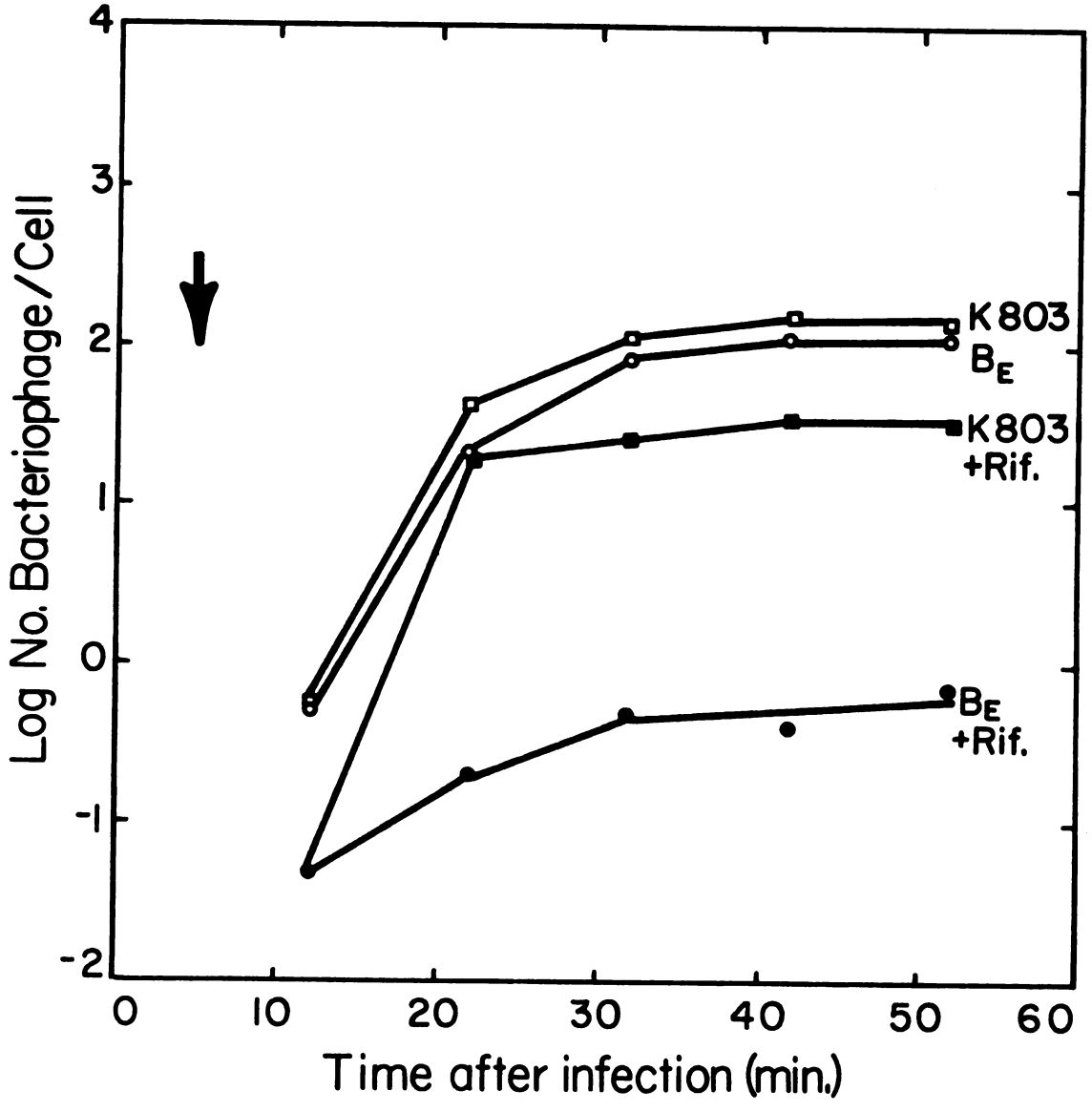
Log No. Bacteriophage/Cell
4
3
2
1
0
-1
-2
0
10
20
30
40
50
60
Time after infection (min.)
K803
B_E
K803
+Rif.
B_E
+Rif.

Figure 2

Figure 3. SDS polyacrylamide electrophoresis of T4 proteins made in the presence of rifampicin. Samples (2 ml) from the infections shown in Figure 2 were labeled with ^{14}C-leucine (2 μCi, 312 mCi/mMole) from 20 to 25 minutes after infection. Samples were treated identically and equal volumes (0.02 ml) containing 2.6 to 6.2 x 10^3 TCA precipitable CPM were analyzed.

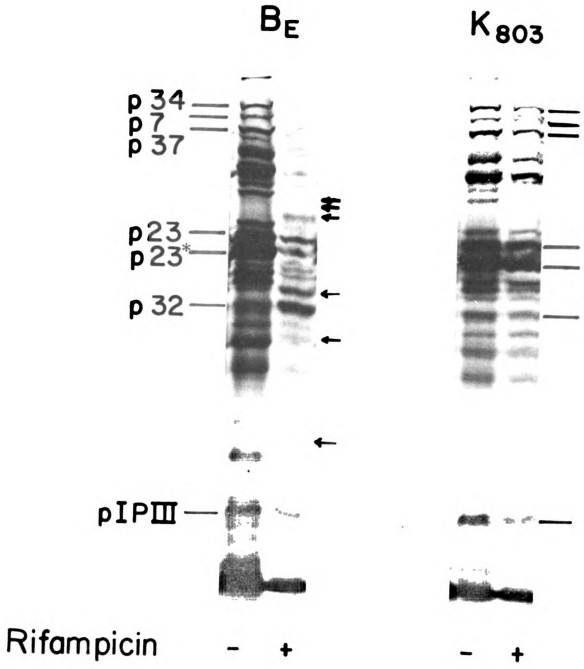

Figure 3

appeared to be less, but the gel pattern was almost identical to the untreated control. This implied that late mRNA transcripts were being initiated in the presence of rifampicin since late synthesis had not yet begun at 5 minutes after infection.

Rifampicin refractory RNA synthesis does not occur in the absence of late gene expression: The implications from the time dependence and differences in late gene expression prompted a test to see if the rifampicin refractory synthesis could occur in the absence of late gene expression. To do this, an amsu$^-$ K12 strain (NF58) was infected with various T4 amber mutants which do not make late gene products on nonsuppressing hosts and the effect of rifampicin on uridine incorporation followed. A comparison of wild type and amN81 (gene 41) is shown in Figure 4. Rifampicin refractoy synthesis did not occur in T4$^+$ infected NF58 until 10 minutes after infection. This was expected since this strain grows slightly less well than K803 and the latent periods are protracted accordingly. The decrease in refractory synthesis at 30 to 35 minutes for the 15 minute curve was due to lysis of the culture. In the amN81 infection, ^{3}H-uridine incorporation was inhibited to the same extent independent of when the antibiotic was added to the culture. These results were repeated with amE10 (gene 45), amN122 (gene 42) and the double mutant amBL292,N134 (genes 55 and 33). In all cases, when late gene expression was prevented, there was no rifampicin refractory activity seen.

Figure 4. The absence of rifampicin refractory RNA synthesis in the absence of late gene expression. The experiments were performed as described in the legend of Figure 1. The times indicate when rifampicin was added. A. $T4^{+}$ infected NF58 (*am* su^{-}). B. T4 *am* N81 (gene 41) infected NF58.

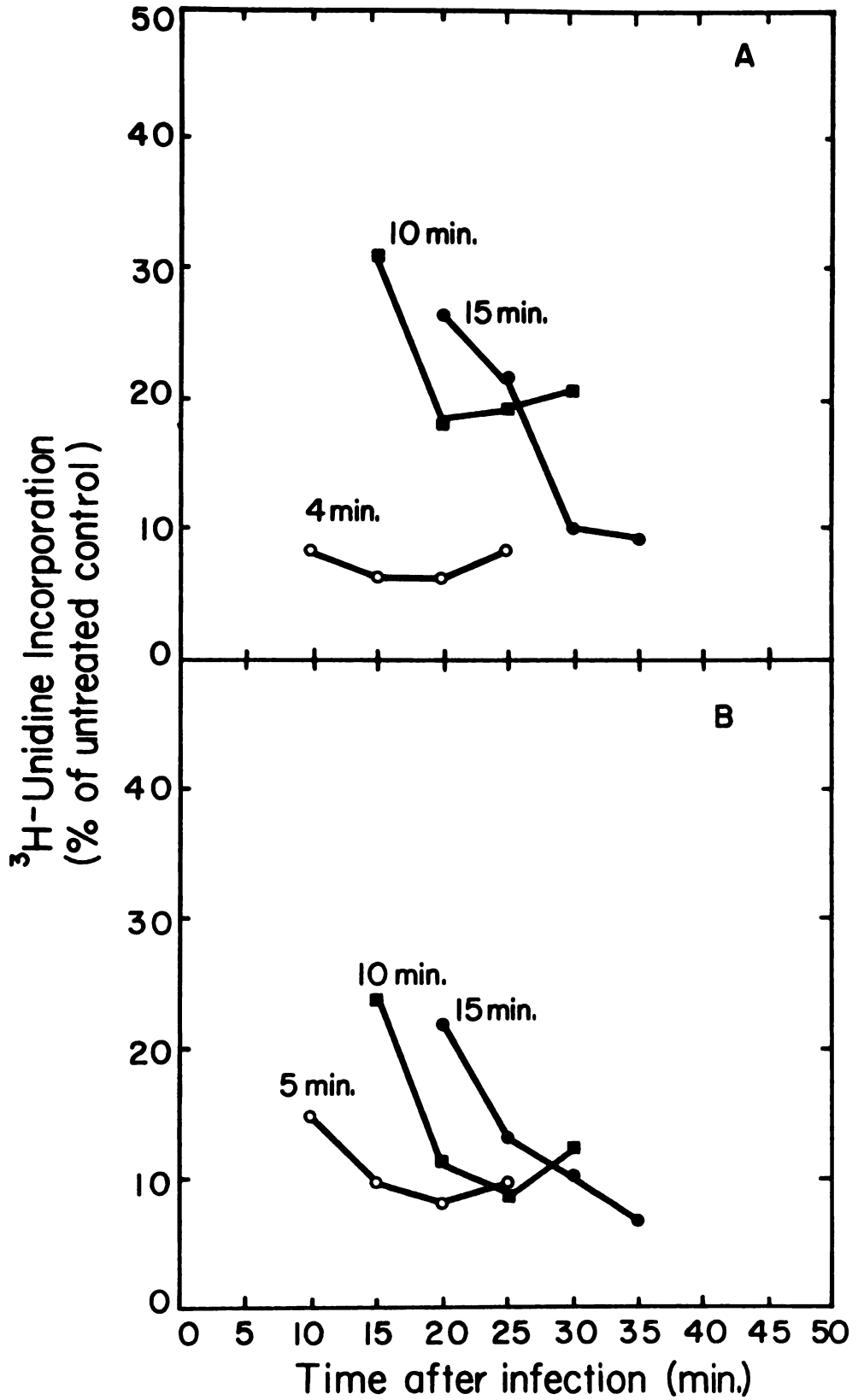
A
B
10 min.
15 min.
4 min.
5 min.
^{3}H-Unidine Incorporation
(% of untreated control)
Time after infection (min.)
0
10
20
30
40
50
0 5 10 15 20 25 30 35 40 45 50

Figure 4

Hybridization competition of T4 RNA made on K803 in the presence of rifampicin: An analysis of the rifampicin refractory transcripts was done using hybridization competition techniques. The RNA was labeled in 2 minute pulses at 5, 10 and 15 minutes after the addition of the antibiotic. The specific activities of the RNA preparations from the treated cultures was 44.5, 26.1, and 27.8% of the equivalent untreated 10-12, 15-17 and 20-22 minute RNAs, respectively. Each RNA sample was competed with 5 and 20 minute RNA from $T4^+$ infected K803. The results are shown in Figure 5. In all cases, 20 minute RNA competed the RNA made in the presence or absence of the antibiotic equally well. On the other hand, 5 minute RNA competed the transcripts made in the presence of rifampicin less well than those made in the absence of the antibiotic. Thus, at least some RNA species may be differentially inhibited by rifampicin.

An effect of DNA glucosylation on rifampicin refractory synthesis: It has been shown that the glucosylation of T4 DNA may play a role in late gene expression (Montgomery and Snyder, 1973). In Table 1 are results from several experiments showing the effect of rifampicin on the production of phage by T4 with different glucosylation patterns. All experiments were done on K803 (rgl^-) to avoid restriction of unglucosylated phage. The βgt^- phage do not have β-glucosyl transferase and as a consequence the DNA is only α-glucosylated (approximately 80% of the hydroxymethylcytosine residues) (Revel and Luria, 1970). Although phage production may be more sensitive at early times (2 minutes) it appears equally as

Figure 5. Hybridization competition of T4 ^{3}H-RNA made in the presence of rifampicin. *Escherichia coli* K803 was grown in M9S and infected with $T4^{+}$ at an MOI of 5. RNA was labeled with ^{3}H-uridine (5 μCi and 5 μg/ml) at 10 to 12 min (A), 15 to 17 min (B), and 20 to 22 min (C) after infection in the presence (●,■) or absence (○,□) of 100 μg/ml of rifampicin added at 5 min after infection. Competition was done in the presence of 10 μg/ml T4 DNA, with 5 (○,●) or 20 (□,■) minute unlabeled RNA from $T4^{+}$ infected K803. Hybridization efficiencies were 28 ± 4%. One hundred percent hybridization equaled: A, 9651 and 4290; B, 5589 and 2028; and C, 3982 and 2654 CPM for the untreated and treated samples, respectively.

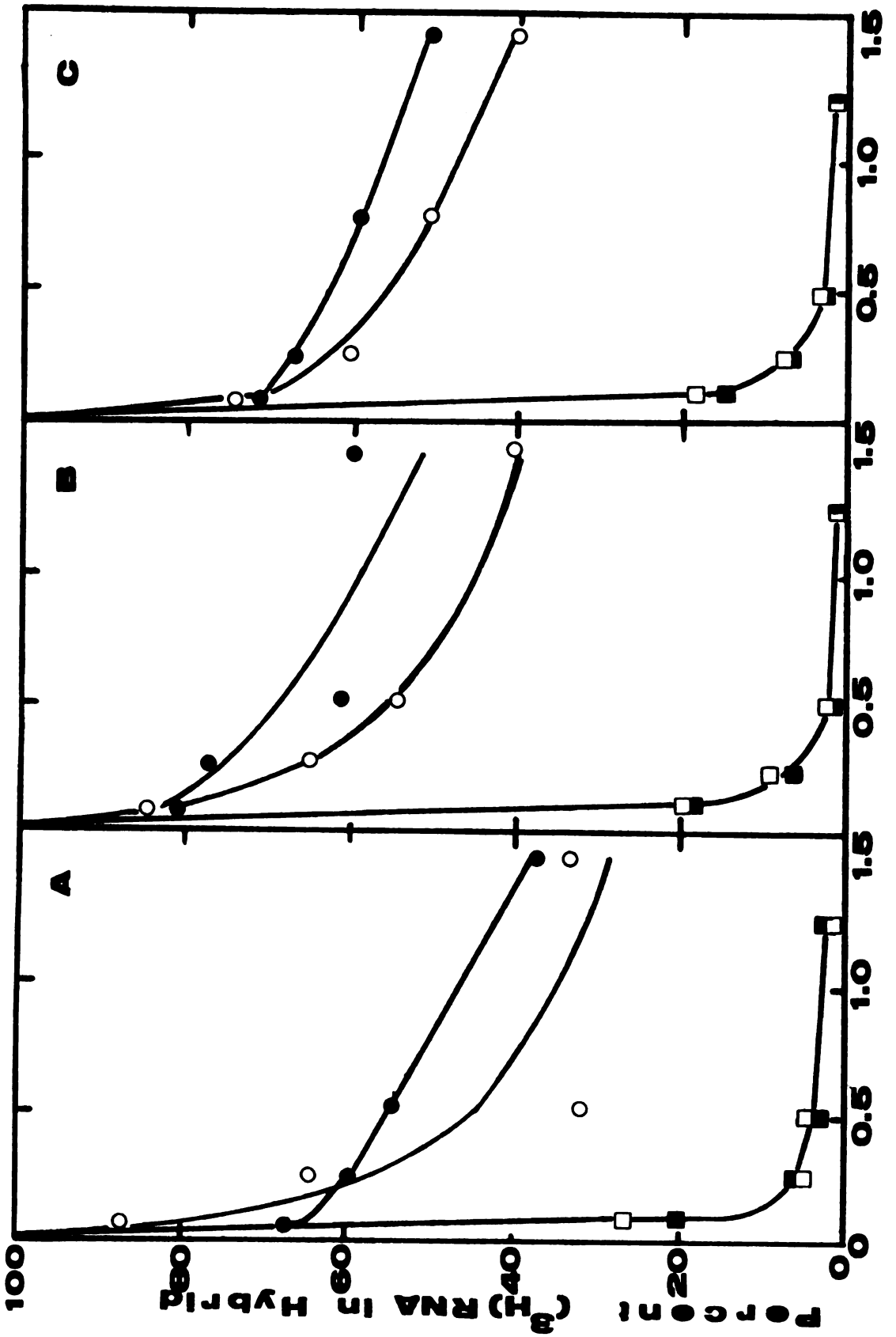
A
B
C
Percent (^{3}H) RNA in Hybrid
Unlabelled RNA (mg/ml)
0
20
40
60
80
100
0.5
1.0
1.5

Figure 5

Table 1. Effect of rifampicin on the phage production by T4 glucosyltransferaseless mutants

Time of Rifampicin Addition (min)	Phage/Cell			
	$T4^+$	βgt^-	αgt^-	$\alpha\beta gt^-$
---	130	110	101	33.5
2	12.5	0.2	---	---
5	27	15	3.8	0.8
10	120	100	25	3.4
15	---	---	30	8.5

refractory at later times. The αgt^- (α-glucosyltransferaseless) phage have all their hydroxymethylcytosine residues fully β-glucosylated. The $\alpha\beta gt^-$ mutants are fully unglucosylated (Revel and Luria, 1970). Both the αgt^- and $\alpha\beta gt^-$ phage are sensitive to rifampicin even at 15 minutes after infection.

The interpretation of these results is complicated by several facts regarding the synthesis of unglucosylated or partially glucosylated phage. For example, even on the nonrestricting hosts, phage production is depressed to varying degrees but is most severe in $\alpha\beta gt^-$ phage infection (Georgopoulos and Revel, 1971). In addition, DNA synthesis is affected in αgt^- phage infection. In experiments not shown here, rifampicin inhibited the rate of ^{3}H-thymidine incorporation when added after DNA synthesis had begun. This would explain the effect shown on phage production. For the $\alpha\beta gt^-$ mutant, however, DNA synthesis is the same as wild type in the presence or absence of rifampicin added at 5 minutes after infection (Figure 6).

Figure 6. The rate of ^{3}H-thymidine incorporation in T4^{+} and T4 HA57 ($\alpha\beta gt^{-}$) infected *E. coli* K803 with and without rifampicin. Samples (10 ml) of the bacteria grown in M9S were infected with the appropriate phage at an MOI of 5. Rifampicin was added at 5 minutes after infection (arrow). (○) T4^{+}; (●) T4^{+} + rifampicin; (□) T4 HA57; (■) T4 HA57 + rifampicin.

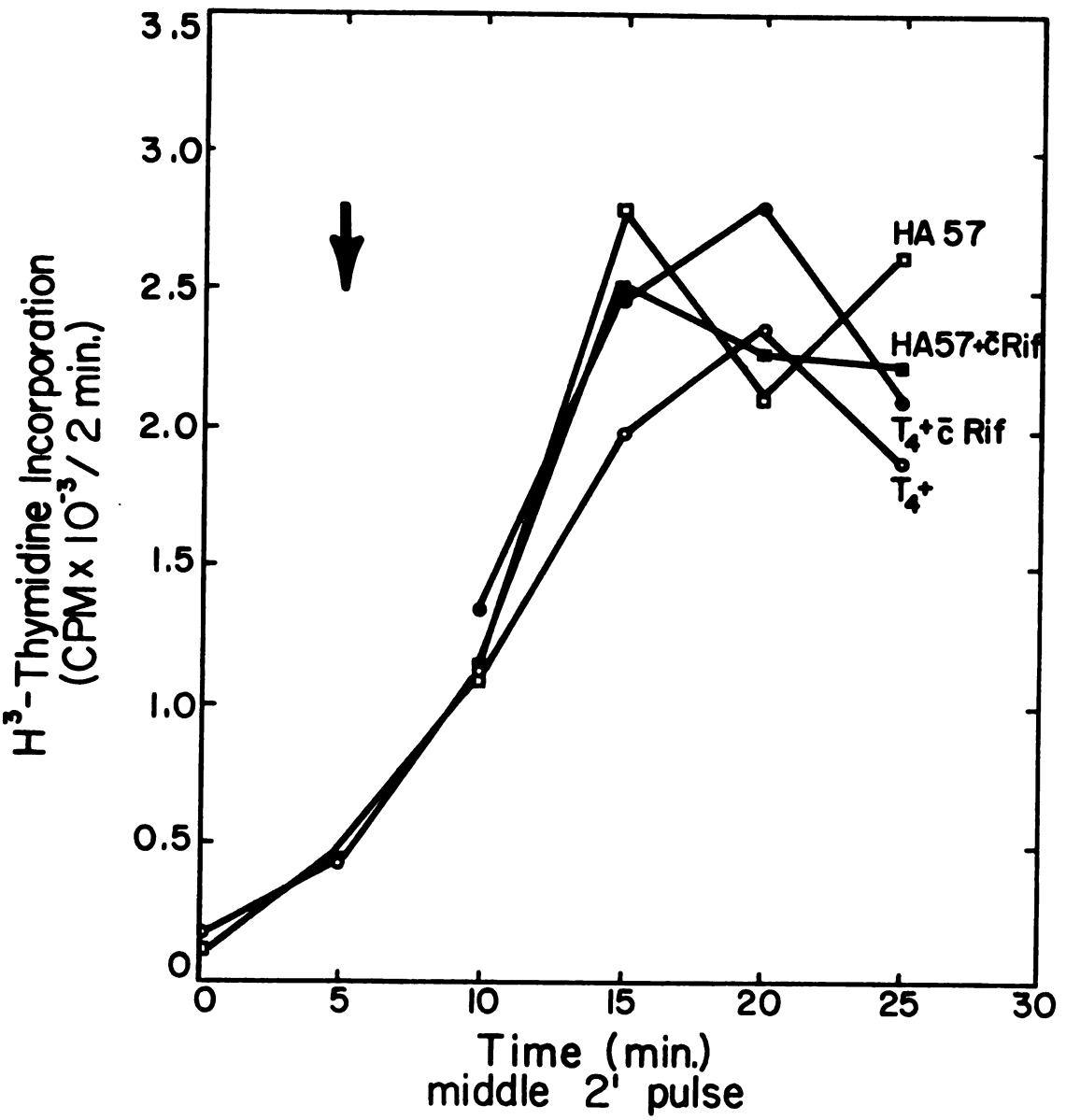
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
H³-Thymidine Incorporation
(CPM x 10⁻³ / 2 min.)
0
5
10
15
20
25
30
Time (min.)
middle 2' pulse
HA 57
HA57+c̄Rif
T4+c̄ Rif
T4+

Figure 6

Furthermore, uridine incorporation appears more sensitive to rifampicin at 10 or 15 minutes after infection than at 5 minutes after infection (data not shown). Therefore, despite the low phage yields, we suggest that the rifampicin refractory activity is affected by the state of the DNA template.

DISCUSSION

We have described a rifampicin refractory RNA synthesis in T4 infected *E. coli* K12 strains. It occurs at an antibiotic concentration of 100 μg/ml, an amount capable of inhibiting RNA synthesis in uninfected bacteria by greater than 97% in 10 minutes. The refractory synthesis is dependent upon the time of addition of the antibiotic. When added before 5 minutes at 37 C to T4 infected *E. coli* K803, inhibition of ^{3}H-uridine incorporation is greater than 90%. After 5 minutes, inhibition is only reduced 70 to 80% and this refractory synthesis is maintained until the cells lyse. On SDS gels, it appears that all the resolvable late gene products are made, while hybridization competition data suggest there is a differential effect on at least some early transcripts.

There are at least two possibilities that come to mind to explain the apparent decrease in sensitivity to rifampicin at late times during infection: a) a T4 induced change in permeability of the cell and b) a change(s) in the RNA polymerase itself. We suggest that our data would argue against a change in the permeability of the cell to rifampicin late in infection. The kinetics of inhibition, for example, are not what would be expected from a decreased peremeability. The initial rate of inhibition immediately after

rifampicin was added was the same whether it was added at 2, 5 or 10 minutes after infection. In addition, the rate of uridine incorporation plateaus. That is, for an interval of 10 to 15 minutes, the rate of RNA synthesis is maintained at a level of about 25% of that of the untreated control (see Figure 1). A second observation that cannot be explained by a permeability change is the differential effect on certain RNAs shown in the hybridization-competition data. The antibiotic must be entering the cell and reacting with the polymerase.

We might postulate, therefore, that there is a change in the K12 polymerase that makes it less sensitive to rifampicin at late times in the T4 infection. This could be a phage induced change in the enzyme or a change in the state of the enzyme. Many phage induced changes in the host RNA polymerase have been demonstrated. These include the association of T4 coded polypeptides with the enzyme (Stevens, 1972; Horvitz, 1973; Ratner, 1974a,b). These polypeptides include the gene 33, 55 and 45 products, all of which are known to be required for late gene expression (Epstein et al., 1963; Bolle et al., 1968b; Notani, 1973). Since rifampicin inhibits by binding to the β subunit of the enzyme (Heil and Zillig, 1970), such changes in the enzyme might make it less sensitive to the antibiotic. A change in the state of the enzyme might be in the formation of (initiation) complexes no longer accessible to the antibiotic. This is suggested by the fact that the refractory synthesis is not seen when late gene expression is blocked or when unglucosylated DNA is used as a template. Furthermore, we do not have any evidence that new initiations are occurring in the presence

of rifampicin; that is, transcription may be from pre-initiated polymerase. The rifampicin refractory state could also be a combination of the events suggested above. This will have to await further analysis.

The findings of Riva et al. (1972) and Romero et al. (1973) may have some bearing on the effect noted here. They were able to demonstrate a differential sensitivity of transcription of certain classes of genes to rifampicin. At sublethal concentrations which had no effect on bacterial syntheses or extrachromosomal DNA replication, the transcription of the latter was prevented. It was also possible to select rifampicin resistant mutants that selectively did not transcribe extrachromosomal genes, yet replication continued and the plasmids or episomes were maintained. A similar effect was noted by Snyder (1972) for a rifampicin resistant mutant of *E. coli* (Rif^R-2) which would not grow T4. The major block to phage growth was the inability to transcribe late genes. Interestingly, when the Rif^R-2 mutation was transduced to *E. coli* B, the inhibition of T4 growth was lost (Snyder, personal observation). We are not sure if this observation and the data presented here are related except that they both reflect differences between K12 and B strains.

In conclusion, we have described a partial inhibition of transcription by rifampicin in T4 infected *E. coli* K12 strains. The refractory synthesis is dependent upon the time of addition and the concentration of the antibiotic used. We suggest that this potentially may provide a useful means for the examination of late gene expression, both at the molecular and genetic level. In

addition, these results portend possible misinterpretations where rifampicin is used exclusively to define temporal transcription sequences.

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ARTICLE II

THE REGULATION OF ANTI-LATE RNA SYNTHESIS IN BACTERIOPHAGE T4: A DELAYED EARLY CONTROL

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SUMMARY

T4 bacteriophage make mutually complementary RNA transcripts. The anti-sense RNA is made in the late region but at early times after infection. Hence it is called anti-late RNA. We have studied some of the physical characteristics and possible regulatory mechanisms involved in the synthesis of these unique early RNA species.

Anti-late RNAs sediment on 5 to 20% sucrose gradients with an average sedimentation coefficient of 20-22 S. They are comparable in size to late mRNA.

We provide evidence that anti-late transcripts are initiated in the late region and they are made *in vivo* on degraded DNA templates. Their synthesis is refractory to rifampicin at one minute after infection.

Anti-late RNA production is examined under several different conditions of altered early gene expression. In all circumstances where the delayed early gene transcription is altered, anti-late synthesis is also altered. From the data presented here, we postulate that anti-late RNA synthesis is controlled by the same mechanism regulating delayed early genes. The data are discussed with respect to the current understanding of T4 gene expression.

INTRODUCTION

Until recently, the transcription of double stranded DNA was thought to be strictly an asymmetric process. That is, for any particular region of the molecule, RNA is made in only one direction from one of the strands (see, for example, Geiduschek et al., 1968, and Davidson, 1972). There are, however, increasing numbers of

reports of symmetrical transcription in many different biological systems. These include the animal viruses SV40 (Aloni, 1972, 1973), Vaccinia (Colby and Duesberg, 1969), Adenovirus 2 (Zimmer and Raskas, 1976), herepes simplex (Zeev and Beker, 1975), and polyoma (Aloni and Locker, 1973); HeLa cell mitochondria (Aloni and Attardi, 1971, 1972; Young and Attardi, 1975; Murphy et al., 1975); and the bacterial viruses λ (Bøvre and Szybalski, 1969), gh-1 (Jolly, PhD Dissertation, Michigan State University, 1976) and T4 (Jurale et al., 1970; Geiduschek and Grau, 1970; Notani, 1973).

Symmetrical RNA synthesis by T4 was evident from the isolation of virus specific double stranded RNA from infected bacteria (Jurale et al., 1970) and the self-annealing properties of RNAs from infected cells (Jurale et al., 1970; Geiduschek and Grau, 1970). In their characterization, Geiduschek and Grau (1970) determined that the symmetric RNA was made early during infection and was complementary to late mRNA. Notani (1973) later demonstrated that this anti-late RNA was made for a short interval, approximately 2 to 6 minutes after infection at 30 C, and was synthesized on 81% of the late region from the early (l) strand. The origin of anti-late RNA, at first attributed to overlapping transcription regions of opposite polarities (Jurale et al., 1970) or mistakes in termination recognition which allowed read through into the late regions from early promoters (Geiduschek and Grau, 1970), was later interpreted by Notani (1973) to be from mistakes in initiations. The reasoning was that, due to the clustering of late genes, it would take substantially longer than the four or five minutes of synthesis for the RNA polymerase to traverse the extensive late region.

Several observations, however, suggest that anti-late RNA may be made in response to or as a consequence of some other mechanism than simply initiation errors. This RNA is not made when chloramphenicol is added at the time of infection. Anti-late RNA is made temporally, starting at 2 minutes and ending at 6 minutes after infection. It is specific for the late regions; i.e., no complementary early RNA is synthesized, at least at early times (Geiduschek and Grau, 1970; Notani, 1973). Consequently, we decided to investigate the synthesis of anti-late RNA to see if it is regulated, if so what controls are involved, and what relationship, if any, it may have with other classes of early RNAs.

In discussing the various pre-replicative mRNA species, we shall use the terms and abbreviations as defined by O'Farrell and Gold (1973a). Immediate early (IE) are made as promoter proximal cistrons from pre-early promoters (P_E). Delayed early (DE) are made as promoter distal cistrons from pre-early promoters and/or from other promoters recognized later in the course of infection (P_Q). Quasi-late are made from P_Q type promoters and continue to be made late in infection.

In this paper we present evidence substantiating the conclusions of Notani (1973) that T4 anti-late RNA is made by initiations on the DNA l-strand (early strand) in the late region. In addition, we show that anti-late transcription is subject to a delayed early type of regulation. The data are discussed in terms of prereplicative transcription and its regulation.

METHODS AND MATERIALS

(a) Phage and Bacteria. The bacteria and phage used are described in Table 1.

(b) Media. M9S and tryptone media were the same as used previously (Snyder and Montgomery, 1974). M9AA is M9 (Bolle et al., 1968) substituted with 20 µg/ml of the following amino acids: alanine, aspartate, cysteine, glutamate, glycine, histidine, isoleucine, lysine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, arginine, and methionine.

(c) Chemicals. ^{3}H-5-Uridine and $^{32}PO_4$ were purchased from Amersham/Searle Co., and ^{3}H-thymidine from New England Nuclear Co. ^{3}H-Uridine triphosphate (^{3}H-UTP), ^{14}C-leucine, and rifampicin came from Schwartz-Mann. Sucrose (grade 1), ribonuclease A (bovine pancreatic, 5X recrystallized), deoxyribonuclease (DN-EP), egg white lysozyme, and calf thymus DNA were purchased from Sigma Chemical Co. Ribonucleotide triphosphates were from P and L Biochemicals. Ribonuclease T_1 (B grade) was purchased from Calbiochem.

(d) Preparation of RNA Polymerase. RNA polymerase was isolated and purified through the DEAE cellulose step and assayed according to Burgess (1969).

(e) Nucleic Acid Extractions. RNA was extracted using the hot phenol method of Bolle et al. (1968). Each preparation was additionally treated with DNase (20 µg/ml, 37 C, 20 minutes) and re-extracted twice with hot phenol. Concentrations were determined spectrophotometrically (Bolle et al., 1968).

Table 1. Bacteria and bacteriophage used

	Strain	Relevant Characteristics	Source or Reference
Bacteria:			
E. coli B	BE	am su^-	L. Gold
K12	HR112	am su^-, rgl^-	H. Revel
	K803	am su^+_{II}, $r_{K-K}m^-_{K-K}$, rgl^-	(Wood, 1966)
	K803 Rif^R-2	K803, rif^r	P1 transductant of K803 (Montgomery and Snyder, 1973)
	DG75	am su^-	(Worcel and Burgi, 1972)
	DG75 Rif^R-2	am su^-, rif^r	P1 transductant of DG75
Bacteriophage:			
T_4	SP62	reg A^-	J. Wiberg
	am N122	gene 42	(Epstein et al., 1963)
	SP62, am N122	reg A^-, gene 42	(constructed in our laboratory)
	am E10	gene 45	(Epstein et al., 1963)
	Ac19rev2	cold sensitive on BE	M. Nelson and L. Gold
	tsG1	mot^-	(Mattson, 1974)
	am E10, tsG1	gene 45, mot^-	(constructed in our laboratory)
	HA57	$\alpha\beta gt^-$	J. Wiberg
	am E10, HA57	gene 45, $\alpha\beta gt^-$	(constructed in our laboratory)

DNA was extracted from CsCl-purified virus according to Mandell and Hershey (1960). Sheared DNA was prepared as described by Brody et al. (1970). The size of the sheared DNA was determined by ultracentrifugation on a 5 to 20% sucrose gradient in 0.1 M NaCl, 0.05 M EDTA. The gradients were centrifuged at 35,000 rpm for 2 hours at 20 C in a Beckman SW 50.1 rotor. The sheared DNA sedimented with a broad peak at approximately 16 S as determined from the location of H-labeled *E. coli* ribosomal RNA on the same ^{3}H gradient. The molecular weight was calculated from the formula of Eigner and Doty (1965) as discussed by Kutter and Wiberg (1969) using this S value or the distance intact T4 DNA sedimented on a companion gradient and assuming a molecular weight of 130×10^{6} daltons. These gave values of 3.16 and 3.04×10^{6} daltons, respectively.

(f) Radioisotopic Labeling. RNA was labeled using appropriate pulses of ^{3}H-uridine as indicated in Results. The bacteria were growtn to an absorbance of 0.4 at 625 nm measured on a Spectronic 20 spectrometer and infected at a multiplicity of infection (MOI) of 5 unless otherwise indicated. ^{3}H-5-Uridine was added to give a final concentration of 50 µCi and 1 µg/ml. The incorporation was stopped by pouring the cell culture over an equal volume of ice and the RNA extracted as indicated above.

T7 viruses were labeled by adding ^{3}H-thymidine (10 µCi and 10 µg/ml) to log phase *E. coli* at a concentration of 4×10^{8}/ml. Fifteen minutes later, T7 phage were added at an MOI of 0.2. The culture lysed spontaneously in approximately 45 minutes. The

labeled viruses were purified on a CsCl step gradient. The final suspension contained 6 x 10^4 CPM/ml.

T4 am E10, HA57 was labeled with ^{32}P according to the procedure of Hattman (1964) except the bacteria were grown in tryptone broth. The CsCl purified viruses had a specific activity of 1.7 x 10^{-5} CPM/plaque forming unit.

Proteins for SDS polyacrylamide electrophoresis were labeled as described in the appropriate legends. The bacteria for each experiment were grown overnight in M9AA and transferred to fresh medium at a 50-fold dilution. Label incorporation was stopped by adding an equal volume of ice to the labeling tubes or pouring the contents of each tube over ice with enough L-leucine to give 100 µg/ml. The samples were centrifuged at 3-5000 x g for 10 minutes. The pellets were resuspended in 0.05 ml sterile water and mixed with an equal volume of 2X concentrated lysing buffer (Studier, 1973). All samples were boiled for 2 minutes before running on 12.5% SDS gels.

(g) SDS Slab Gel Electrophoresis. The procedure was the same as described by Studier (1973). The gels were run at 10 MA per gel for 2 to 3 hours until the marker dye approached the end of the gel. Kodak No Screen X-ray film was exposed to the dried gels for 3 days to 3 weeks. The identification of T4 protein bands in the gels was done using appropriate amber mutants and from published data (O'Farrell and Gold, 1973a,b).

(h) Measurement of Unglucosylated T4 DNA Degradation in a Restrictive Host. A modification of the procedure described by Kutter and Wiberg (1968) was used. Fifteen milliliters of bacteria grown in M9S to 4 x 10^8/ml were infected with ^{32}P labeled T4 am E10, HA57 (genes 45, $\alpha\beta gt^-$) at an MOI of 0.5. At 1, 2, and 4 minutes after infection, 5 ml aliquots were taken, poured over an equal volume of ice, and centrifuged at 8000 rpm for 10 minutes in a Sorvall refrigerated centrifuge. The pellet was washed once in 5 ml of cold M9 buffer to remove unadsorbed phage and centrifuged as above. The resulting pellet was resuspended in 0.2 ml of 0.01 M Tris HCl, pH 7.9, 0.01 M EDTA and 300 µg/ml egg white lysozyme. Labeled T7 phage (0.02 ml) were added and the tubes heated to 65 C for 3 minutes. SDS was added to 0.1% and the incubation continued for 10 minutes. A fraction (0.1 ml) of the lysed samples was put into a scintillation vial, diluted in 0.9 ml water and counted in 5 ml of Aquafluor Scintillation cocktail (New England Nuclear). An equal volume was layered on a 2.8 ml, 5 to 20% sucrose gradient (0.01 M Tris, pH 7.9 and 0.5 M NaCl) over a 0.2 ml shelf of 60% sucrose. The gradients were centrifuged at 38,000 rpm in a Beckman SW 50.1 rotor for 3.5 hours at 20 C. Fractions were collected from the bottom of each tube directly into scintillation vials and treated as above. Samples were counted on a Packard ambient temperature scintillation counter.

(i) Anti-Late RNA Assay. The procedure was the same as previously described (Geiduschek and Grau, 1970). Several different 17 to 20 minute pulse labeled RNA samples were used during the course

of this investigation. The amount of labeled RNA protected from RNAses varied from 8 to 32% depending upon the preparation used. In all cases, the results were consistent and reproducible with all of the assays reported being done at least twice. Pancreatic and T1 RNase were used in concentrations of 10 and 1 μg/ml, respectively.

RESULTS

(a) Size of Anti-Late RNA *in vivo*. An estimation of the *in vivo* size of the anti-late transcripts was made with the thought that the size distribution would give some indication about possible regulatory mechanisms and/or functions of the anti-late RNA. For example, relatively small fragments or a very heterogeneous distribution would be consistent with the suggestion of initiation errors in the late region (Notani, 1973).

The size distribution of anti-late RNA at various times after infection is shown in Figure 1. A zero time control is included to demonstrate the background of the assay since uninfected *E. coli* does not make symmetric RNA (Geiduschek and Grau, 1970; Notani, 1973). Portions of each sample were annealed with labeled 2-4 minute RNA before the gradients were run. Since only background levels of protection were obtained, we concluded there was no T4 DNA in the samples and therefore only complementary RNA is being measured.

On the 5 to 20% sucrose gradients, anti-late RNA sediments as a heterodisperse population in a broad peak. In the 5 minute sample, the bulk of the anti-late RNA (more than 68% of the assayed activity) sediments with a sedimentation coefficient greater than

Figure 1. Sucrose density gradients of anti-late RNA. *Escherichia coli* B_E was grown to 4 x 10^8/ml in M9S at 30 C. The bacteria were infected with $T4^+$ at an MOI of 5. Aliquots (125 ml) were taken at 0, 2, 5, and 8 minutes after infection. The RNA was extracted as described in Materials and Methods. The gradients were prepared and centrifuged as described by Ricard and Salser (1975). Each RNA sample was disaggregated before layering onto a 37.5 ml 5 to 20% sucrose gradient. The samples were centrifuged for 17 hours at 27,000 rpm and 4 C. The gradients had 0.62, 0.68, 1.10 and 1.50 mg of the 0, 2, 5 and 8 minute RNAs, respectively. After centrifugation, 1.2 ml samples were collected from the top using an ISCO Fraction Collector and uv monitor. The fractions were precipitated and the RNAs pelleted as previously described (Ricard and Salser, 1975). The drained pellets were dissolved in 0.1 ml of sterile water. Then 0.3 ml of a solution containing 1 µg of ^3H (17 to 20 minute) RNA and sufficient 10X SSC to give a final concentration of 2X was added to each tube. They were then incubated at 70 C for 3 hours and treated with T1 and pancreatic RNases as described by Geiduschek and Grau (1970). Control tubes without unlabeled RNA and $\pm$ RNases had 231 (average of 3 tubes) and 6399 (average of 3 tubes) TCA precipitable CPM. The results are reported as CPM/tube after background subtraction (-o-) and relative absorbance units at 254 nm (---).

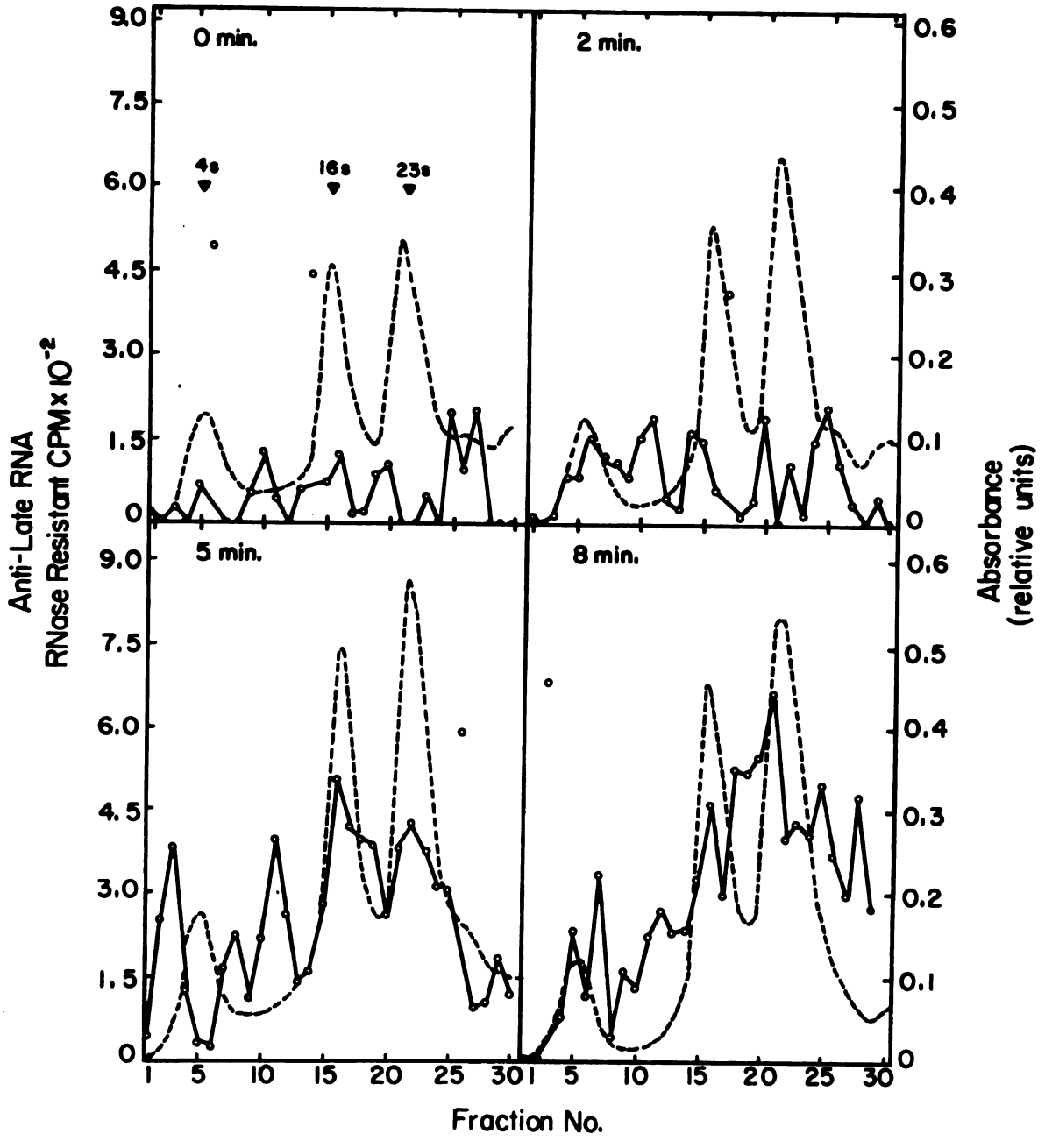
0 min.
2 min.
5 min.
8 min.
4s
16s
23s
Anti-Late RNA
RNase Resistant CPM x 10⁻²
Absorbance
(relative units)
Fraction No.
9.0
7.5
6.0
4.5
3.0
1.5
0
0.6
0.5
0.4
0.3
0.2
0.1
1
5
10
15
20
25
30

Figure 1

16 S and an average value of approximately 20 S. In two separate determinations using independently extracted 5 minute RNA, the same results were found. At 8 minutes, more than 74% of the assayed activity sediments faster than the 16 S ribosomal fraction and has an average S value of 22 S. We did not detect any extremely long transcripts which could be indicative of "read through" on the l-strand. In addition, there was very little anti-late RNA at the top of the gradient, although it is possible that the sensitivity of this assay may be limiting with very small RNA pieces in low concentrations. In general, the size distribution is similar to that observed for late mRNA (Richard and Salser, 1975).

(b) Synthesis of Anti-Late RNA on Less than Unit Length DNA. T4 pre-early transcription can be separated from delayed early transcription *in vitro* on sheared DNA templates (Milanesi et al., 1969, 1970). Considering the tandem nature of delayed early and immediate early cistrons, it would certainly seem reasonable that if anti-late RNA were made by a "run through" from early regions, its synthesis would be limited on a sheared template. In view of this, we developed an *in vivo* system containing degraded T4 DNA and examined the production of anti-late RNA.

The procedure takes advantage of the *E. coli* restriction system for unglucosylated phage DNA. T4 phage normally have fully glucosylated hydroxymethylcytosine residues; 70% with an alpha and 30% with a beta glucosidic bond (Lehmann and Pratt, 1965). Mutants lacking one or both of the respective glucosyltransferase enzymes can be isolated (Georgopoulos and Revel, 1971). If both enzymes are

inactive, such as in T4 HA57, the phage DNA will be unglucosylated and subject to restriction in $\underline{rgl}^+$ bacteria. On *E. coli* DG75 ($\underline{su}^-$, $\underline{rgl}^+$), the unglucosylated DNA is degraded to pieces smaller than 2×10^6 daltons within 2 minutes after infection (Figure 2). The molecular weight was determined by the formula of Eigner and Doty (1965) using 25×10^6 daltons as the molecular weight of T7 DNA. At 4 minutes after infection, nearly 80% of the injected DNA was recovered at the top of the gradient. This is a more extensive and dramatic degradation than noted previously for unglucosylated DNA (Hattmann, 1969; Georgopoulos and Revel, 1971; Dharmalingam and Goldberg, 1976a,b). In these studies, however, degradation was measured as the loss of TCA insoluble radioactivity and there was no correction for unadsorbed viruses. In addition, the phage were grown on $\underline{r^+m^+}$ bacteria. For our study, the phage were grown on K803 $\underline{r_K^-m_K^-}$ and therefore would also be subject to this restriction on DG75 $\underline{r_K^+m_K^+}$.

Early proteins made under conditions where the T4 DNA is degraded are shown in Figure 3. On DG75, only one band (P42) is seen in any significant amounts in a 2 to 5 minute pulse. At later times, other T4 polypeptides are made but in very much reduced amounts.

RNA extracted from DG75 ($\underline{su}^-$, $\underline{rgl}^+$) and HR112 ($\underline{su}^-$, $\underline{rgl}^-$) 5 minutes after infection protected equivalent amounts of 17 to 20 minute ^{3}H-labeled T4 RNA (Figure 4). Pulse labeling experiments indicate that 0.4 to 1.7% of the RNA made early is complementary to late RNA. This is approximately 50 to 100% of control values for the unglucosylated phage in the non-restrictive host (data not

Figure 2. Degradation of unglucosylated T4 DNA in a restricting host, *E. coli* DG75. ^{32}P-labeled DNA was monitored on 5 to 20% sucrose gradients as described in Materials and Methods. The total ^{32}P CPM/gradient were from 101 to 584 (after background subtraction), representing recoveries of 64 to 100%. The percentages indicated are the percent of the total CPM in the top 6 or 7 fractions. Sedimentation was from right to left. ^{3}H-T7 DNA was run on each gradient as a marker.

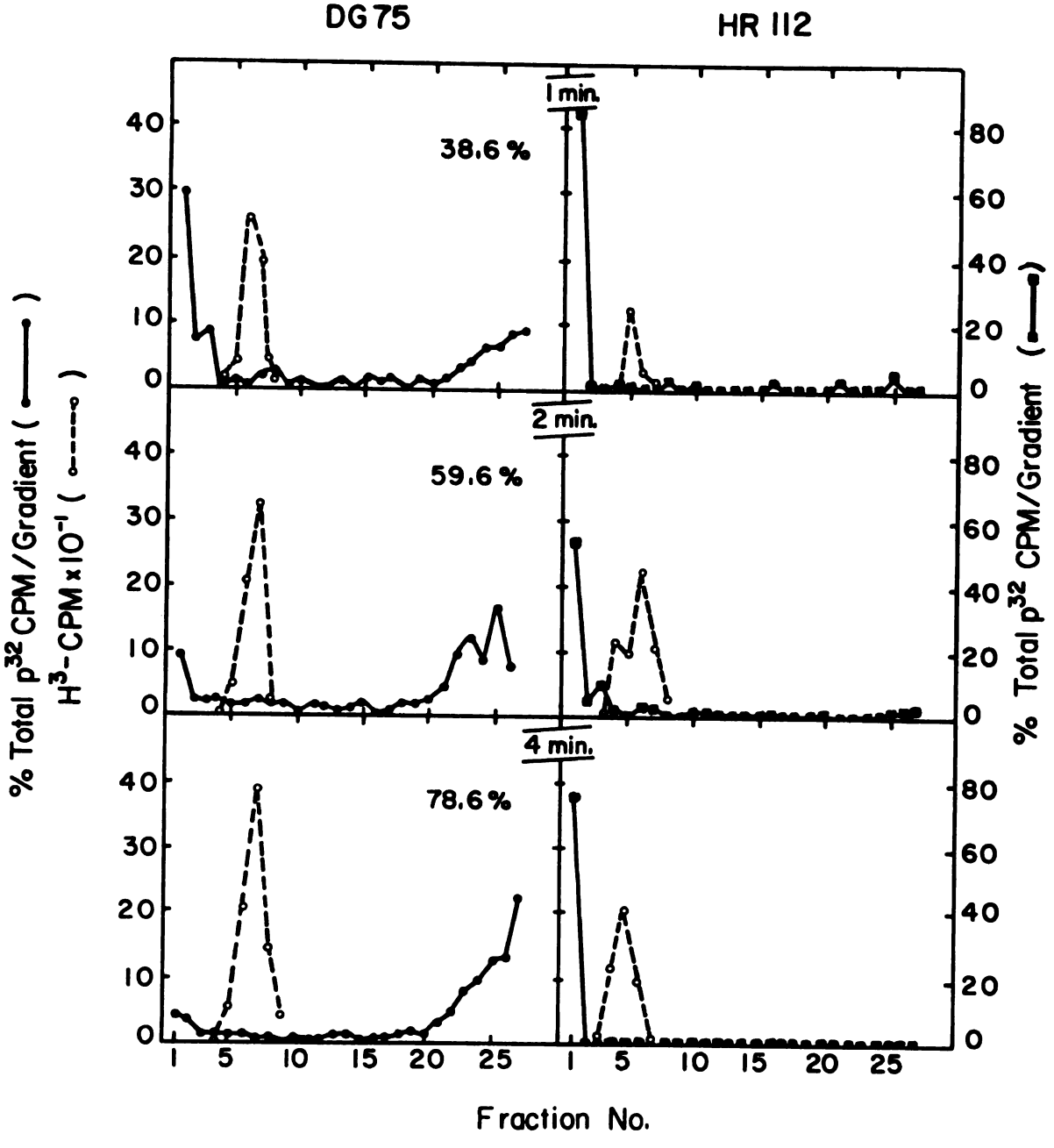
DG 75
HR 112
1 min.
38.6 %
2 min.
59.6 %
4 min.
78.6 %
% Total p32 CPM/Gradient ( )
H3-CPM x 10-1 ( )
% Total p32 CPM/Gradient ( )
Fraction No.

Figure 2

Figure 3. T4 early gene expression on degraded DNA *in vivo*. *Escherichia coli* HR112 and DG75 were grown in M9AA at 30 C. The bacteria (4 x 10^8/ml) were infected with T4 am E10, HA57 at an MOI of 10. T4 proteins were labeled as described in Materials and Methods at the times indicated. HR112 (a,c,e); DG75 (b,d,f).

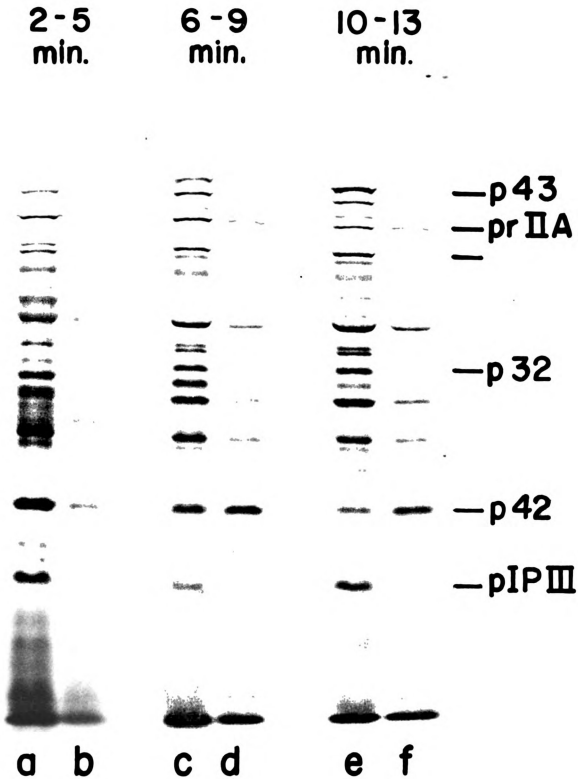

Figure 3

Figure 4. Anti-late RNA made on degraded T4 DNA *in vivo*. *Escherichia coli* HR112 and DG75 were grown in 100 ml of M9S at 30 C to 4 x 10^8/ml. Each culture was infected with T4 am E10, HA57 at an MOI of 5. At 5 minutes after infection, they were poured over an equal volume of ice and the RNA was extracted. The assay was done with 0.5 μg of ^3H (17 to 20 minute) RNA per tube. Total CPM added = 4039; background (without unlabeled RNA) was 137 CPM. (O) HR112 (am su^-, rgl^-); (●) DG75 (am su^-, rgl^+).

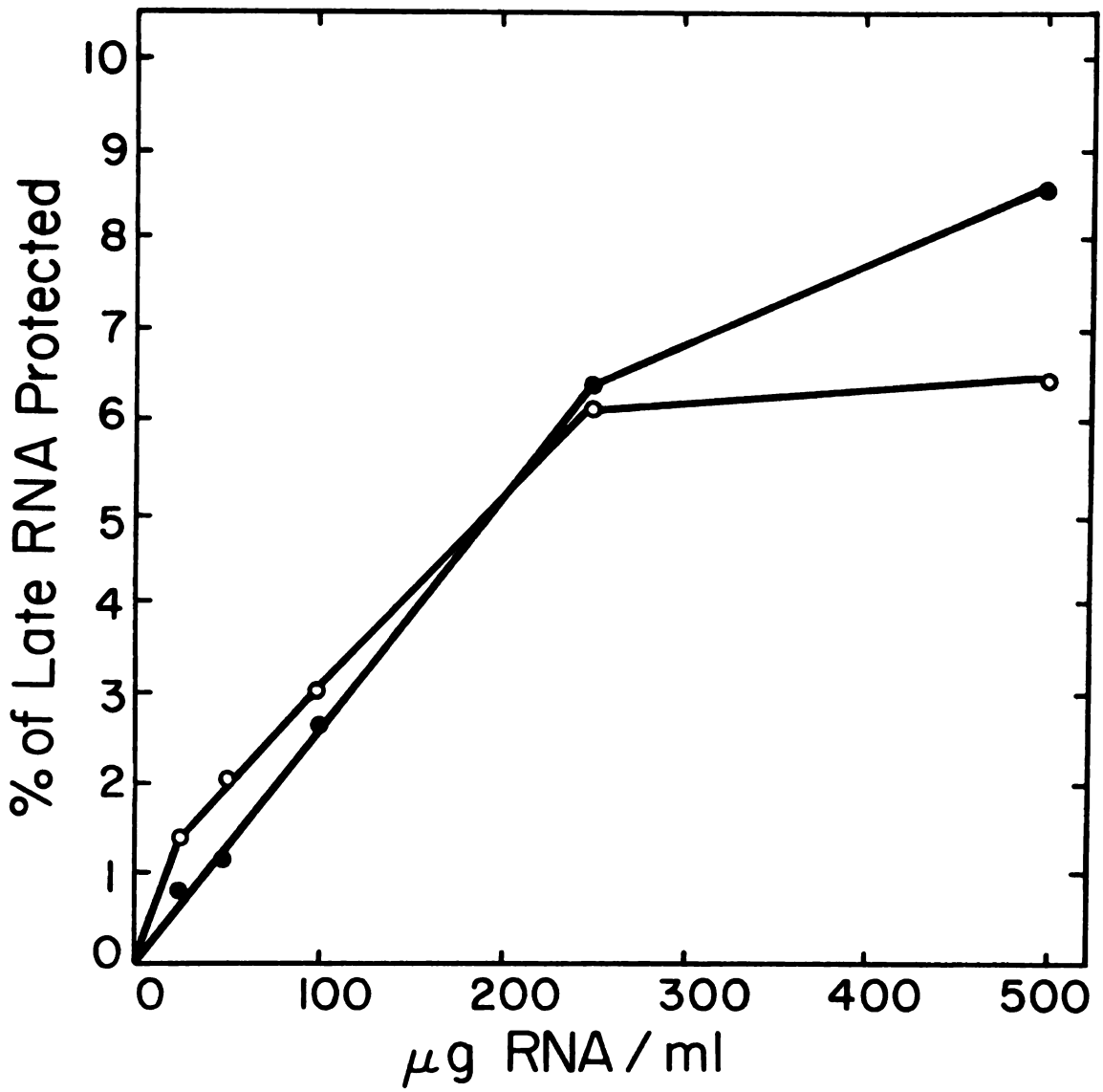
% of Late RNA Protected
0
1
2
3
4
5
6
7
8
9
10
0
100
200
300
400
500
μg RNA / ml

Figure 4

included). Since unglucosylated DNA is degraded to small fragments less than 2.5% of the size of the intact genome by the time anti-late synthesis begins (2 minutes at 30 C), it seems unlikely that it is made by a "run through" mechanism from early promoters.

We also attempted to synthesize anti-late RNA on sheared DNA templates *in vitro*. The results from an experiment using intact and sheared DNA for the *in vitro* synthesis of anti-late RNA are shown in Table 2. *Escherichia coli* B RNA polymerase holoenzyme was used at DNA excess to decrease the number of nonspecific initiations

Table 2. *In vitro* synthesis of anti-late on intact and sheared T4 DNA

	^{3}H-Complementary RNA made *in vitro*[a]					
	In vivo RNA (250 µg/ml)	RNase	Intact DNA CPM	%	Sheared DNA CPM	%
1	---	---	3018	---	983	---
2	---	+	178	---	140	---
3	5 min	+	273	3.3[b]	197	6.8
4	20 min	+	364	6.5	191	6.0

[a] *In vitro* RNA was synthesized in the presence of ^{3}H-UTP as described by Milanesi et al. (1970) and extracted as described by Brody et al. (1970), including the DNase step. The assay procedure was that of Geiduschek and Grau (1970).

[b] Percentage of added label (1) after background (2) subtraction.

(Milanesi et al., 1970). As previosuly demonstrated (Geiduschek and Grau, 1970), anti-late RNA is made *in vitro* on the intact DNA template as noted by the greater protection with 20 minute *in vivo* RNA. Differences between the published amounts synthesized and those reported here are probably due in part to the fact that rifampicin was not used here and therefore reinitiations could occur. Since this should result in more copies of early mRNA, the concentration of anti-late RNA may be diluted, especially if the majority of the anti-late transcripts *in vitro* are made by a "read through" as suggested by the data of Geiduschek and Grau (1970). If this is true, then we would not expect as much anti-late RNA synthesis on the sheared DNA.

Annealing with 5 minute RNA is a measure of the symmetry of transcription (Brody et al., 1970). As expected, the symmetry increases on the sheared template (3.3 vs. 6.8%). Since, however, there is no significant difference in the amount of labeled RNA protected by the 5 or 20 minute *in vivo* RNA, we conclude that anti-late RNA either is not made on the sheared template or is made in such low amounts that it is not detectable in our assay. Although the background levels are high, this experiment was repeated with different polymerase and DNA preparations with similar results.

Despite reports demonstrating the fidelity of *in vitro* T4 transcription (Brody et al., 1970; Brody and Geiduschek, 1970; Milanesi et al., 1969, 1970; O'Farrell and Gold, 1973b), it may be very different than *in vivo* transcription. For example, Cohen et al. (1974) reported substantial differences in the time of synthesis of deoxynucleotide kinase. Synthesized immediately *in vitro*, the

message is not synthesized until 2 minutes after infection *in vivo*. If our hypothesis is correct, anti-late RNA synthesis may also reflect differences in *in vivo* and *in vitro* transcription reactions, since *in vitro* it is probably made to some extent by a "runthrough" mechanism.

(c) Anti-Late RNA is Made in the Presence of Rifampicin. When rifampicin is added to the infected bacteria 1 minute after infection, the synthesis of delayed early gene products occurs but the gene products are made in lower amounts and some, those made exclusively from P_Q promoters, are not made at all (O'Farrell and Gold, 1973a). Anti-late RNA is made under these conditions but in reduced amounts (Figure 5). We interpret the lower slope of the treated sample to mean that less of particular areas (analogous to promoter proximal regions) are being made per unit time in the treated culture. This is a reasonable assumption knowing that rifampicin blocks the initiation but not the elongation of transcription (Wehrli and Staehelin, 1971). It also implies that initiations can occur in the late region on the l-strand. The fact that a saturation concentration of RNA has not been reached at 500 μg/ml indicates that there is a considerable portion of the late region being transcribed (Notani, 1973).

(d) Effect of a Host RNA Polymerase Mutant on Anti-Late RNA Synthesis. Rif^R-2 is a mutation to rifampicin resistance which also inhibits the growth of T4 bacteriophage (Snyder, 1973). Although the major block to phage synthesis is on late transcription, many other defects occur during infection. These include delays in

Figure 5. The effect of rifampicin on anti-late RNA synthesis. *Escherichia coli* B_E was grown in M9S at 30 C and infected with $T4^+$ at an MOI of 5. At 1 minute after infection, one half of the culture (125 ml) was transferred to a flask containing sufficient rifampicin to give a final concentration of 100 µg/ml. At 5 minutes the cultures were poured over ice and the RNA extracted. The assay tubes contained 0.2 µg ^3H (17 to 20 minute) RNA. Total CPM added = 2963; background (without added unlabeled RNA) was 46 CPM. (O) RNA from untreated culture; (●) RNA from rifampicin treated culture.

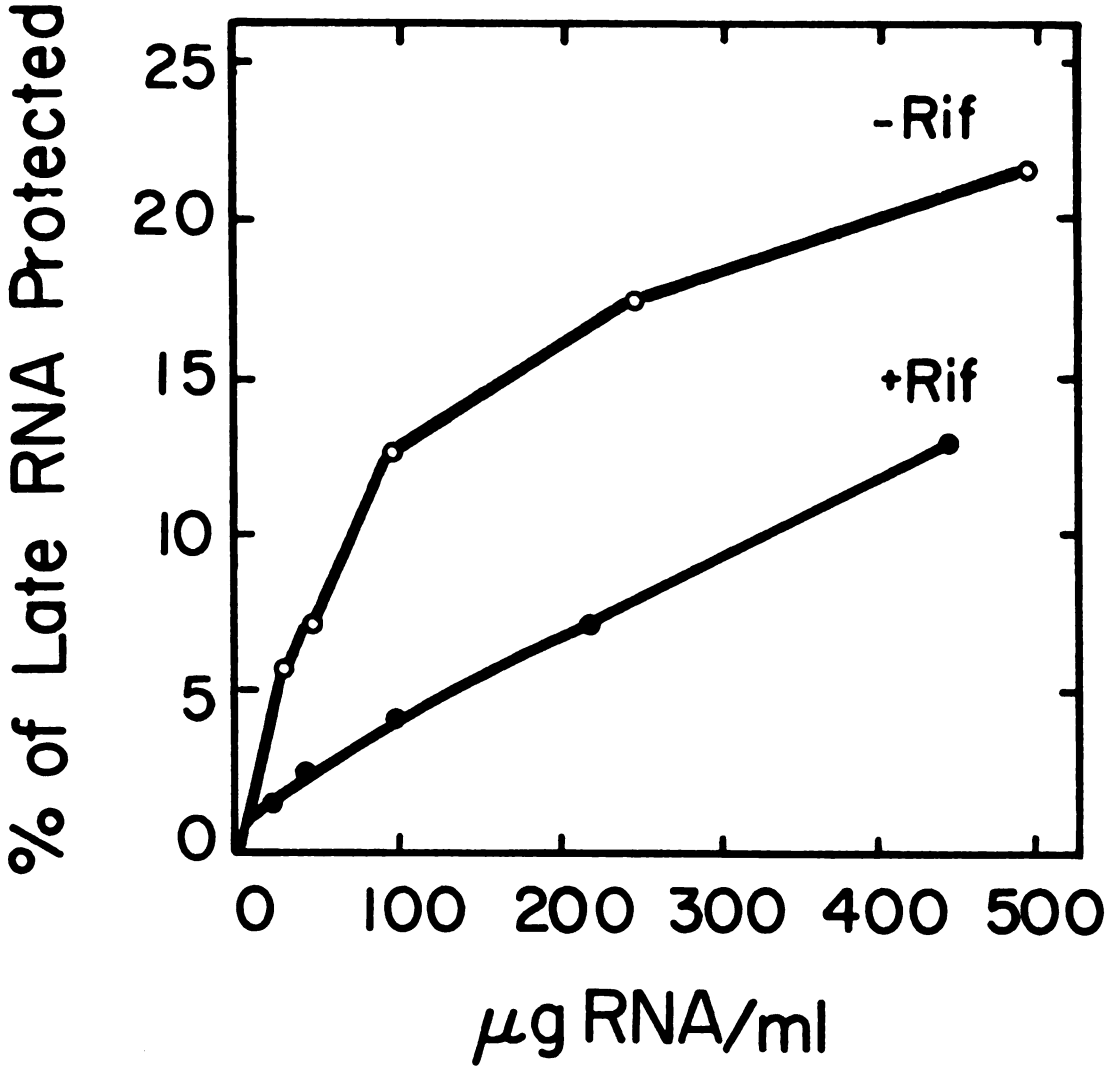
% of Late RNA Protected
25
20
15
10
5
0
-Rif
+Rif
0
100
200
300
400
500
μg RNA/ml

Figure 5

a) replication, b) shutoff of host macromolecular synthesis and c) early gene expression (Snyder, 1972; Snyder and Montgomery, 1974; Figure 6). The autoradiograms in Figure 6 are of labeled proteins from T4 am N122 (gene 42) infected DG75 and DG75 Rif^{R}-2. DNA negative (DO) phage were used because of the defect in replication on Rif^{R}-2 which may itself affect early gene expression.

All phage specific proteins appear with a lag ranging from 1 to 2 minutes for some to 4 minutes for others. The gene 43 product may be a special case since there is a 7 to 8 minute delay before it is synthesized in Rif^{R}-2. We have no explanation for this at this time; however, gene 43 has been shown to have autoregulatory control (Russel, 1973). The overall lag in early gene expression is probably not simply due to the lower rate of amino acid incorporation since the delays are too long and different gene products appear to be affected to varying extents independent of their molecular weight. In any event, if the delay in appearance of the respective polypeptides is due to an altered early transcription pattern, this may also be reflected in the synthesis of anti-late RNA.

Complementary RNA is made in Rif^{R}-2 2 to 3 minutes after it is made in the parental bacteria (Figure 7). The percentages reported here are low since anti-late RNA makes up only 1 to 2% of the phage RNA (see also Notani, 1973). However, repeated assays (3) from separate experiments gave the same results. Additional controls were done with 500 µg/ml of 5 minute RNA. These were all equivalent to background controls without added unlabeled RNA. In the earliest

Figure 6. SDS polyacrylamide gel electrophoresis analysis of early gene products made on T4 am N122 infected *E. coli* DG75 and DG75 Rif^R-2. The procedure is described in Materials and Methods. The proteins were labeled for 1 minute intervals with ^{14}C-leucine. The rate of incorporation of label was the same throughout the interval examined for the respective cultures, although the rate in DG75 Rif^R-2 was approximately 76% of that in DG75 (data not shown).

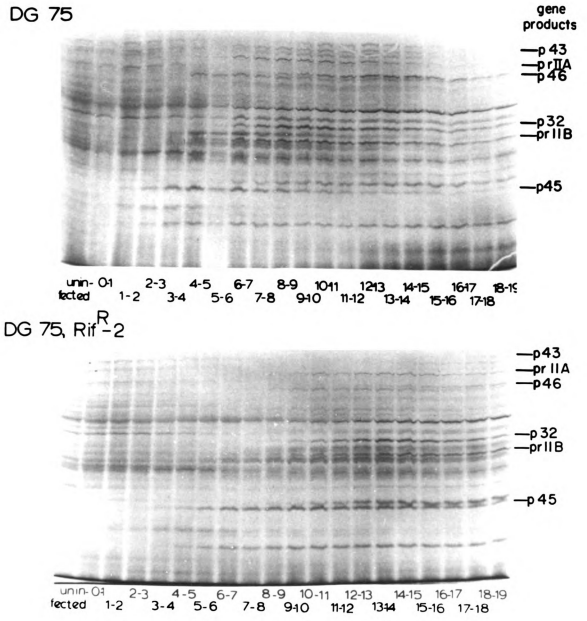
DG 75
gene products
p43
prIIA
p46
p32
prIIB
p45
unin-fected
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
DG 75, Rif R-2
p43
prIIA
p46
p32
prIIB
p45
unin-fected
0-1
1-2
2-3
3-4
4-5
5-6
6-7
7-8
8-9
9-10
10-11
11-12
12-13
13-14
14-15
15-16
16-17
17-18
18-19
Time After Infection (min)

Figure 6

Figure 7. The time of anti-late RNA synthesis in T4 infected *E. coli* K803 and K803 Rif^R-2. The bacteria were grown to 4 x 10^8/ml at 27 C and infected at an MOI of 5. At the times indicated, 10 ml samples were labeled with 10 µCi and 1 µg/ml of ^{3}H-uridine and stopped by pouring over ice. The RNA was extracted as described in Materials and Methods. Annealing reactions were run with 20 minute RNA as described by Geiduschek and Grau (1970). Each ^{3}H labeled RNA was assayed at a concentration of 5 µg/ml. Total CPM added and background (without unlabeled RNA) were: for K803 (O), 2 to 4 min, 8641 and 31 CPM; 5 to 7 min, 39,121 and 74 CPM; 8 to 10 min, 39,296 and 216 CPM; 11 to 13 min, 40,304 and 140 CPM; for K803 Rif^R-2 (●), 2 to 4 min, 21,945 and 107 CPM; 5 to 7 min, 20,146 and 67 CPM; 8 to 10 min, 17,561 and 95 CPM; 11 to 13 min, 18,019 and 113 CPM.

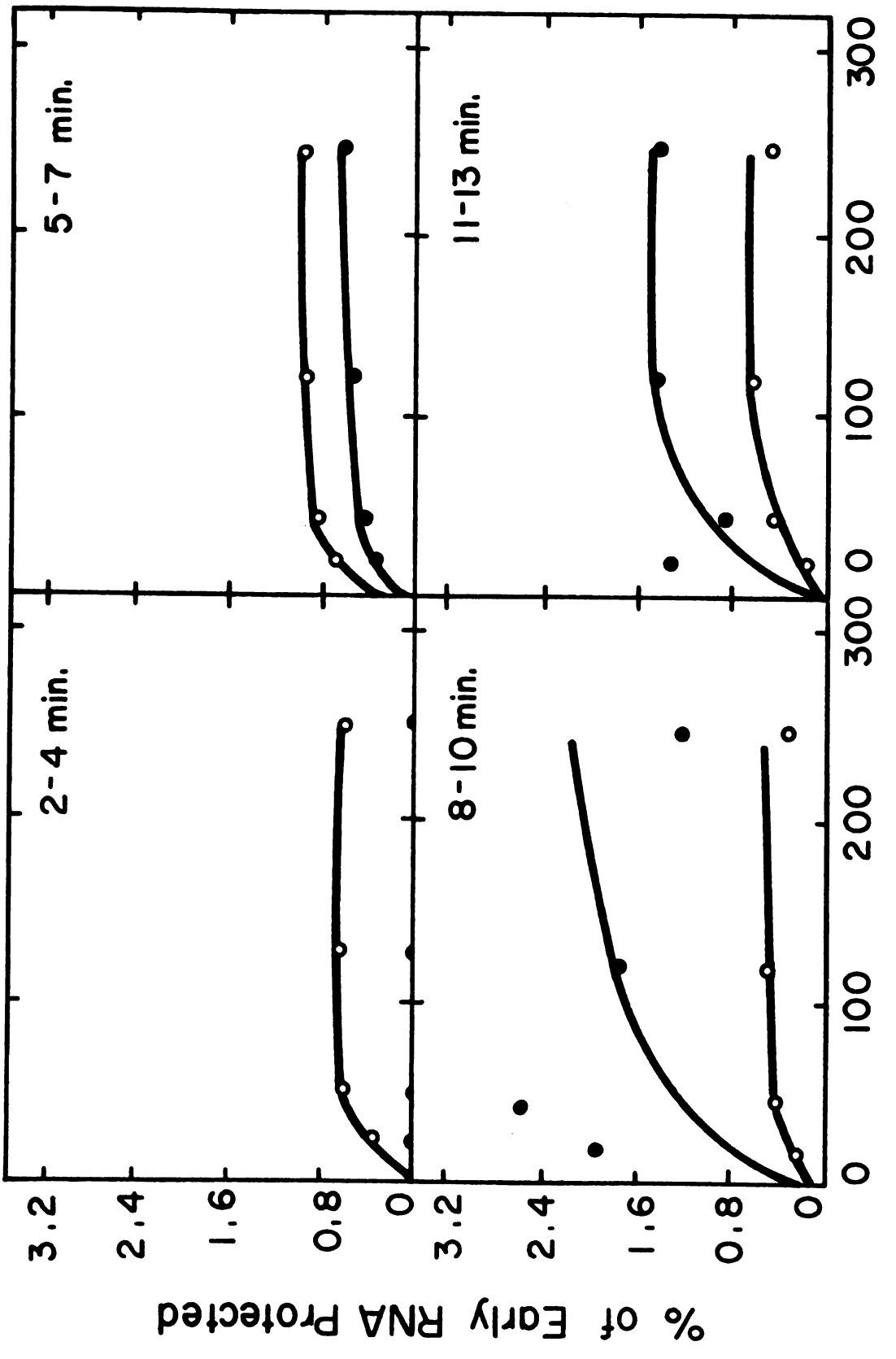
% of Early RNA Protected
3.2
2.4
1.6
0.8
0
2-4 min.
5-7 min.
8-10 min.
11-13 min.
0
100
200
300
0
100
200
300
μg 20 min. RNA / ml

Figure 7

pulse, 2 to 4 minutes, no anti-late RNA is detected in Rif^R-2 (Figure 7). At 8 to 10 minutes and 11 to 13 minutes after infection a greater percentage of the RNA being made in Rif^R-2 is anti-late. This might be expected if there is normally a shift from one transcriptional mode to another that is delayed or occurs inefficiently on Rif^R-2. This is also reflected in the SDS gels (Figure 6) where some proteins continue to be made in the mutant after they have stopped in the parent.

Since Rif^R-2 is defective in host shutoff, it is possible that anti-late RNA may be obscured in the assay because both host and phage RNA were being transcribed. We repeated the experiment, therefore, using unlabeled RNA from the infected mutant and assaying for the ability to protect a 17 to 20 minute labeled RNA (Figure 8). There is essentially no late complementary RNA at 4 minutes after infection. By 12 minutes, anti-late RNA made in Rif^R-2 and its parent protect equivalent amounts of the late RNA. The greater sensitivity of this procedure is also more satisfying since we do not have to contend with background levels approaching 50% of the assay values. The apparent rapid decrease in the amount of anti-late RNA in K803 at 12 minutes is due to the synthesis of late mRNAs (Notani, 1973). Since late messenger is not made in Rif^R-2, there is no comparable interference with the assay at this time.

(e) Effect of Phage Mutants on Anti-Late RNA Synthesis. Several T4 mutants have recently been isolated which have altered prereplicative gene expression. One of these, T4 tsG1, was originally isolated and characterized by Mattson et al. (1974). This mutant

Figure 8. Anti-late RNA synthesis on T4 infected *E. coli* K803 and K803 Rif^R-2. The bacteria were grown at 27 C and infected at an MOI of 5. At the times indicated, the cultures were poured over ice. The RNAs were extracted and assayed as described in the legend of Figure 5. Total CPM added = 3634; background (without unlabeled RNA) was 59 CPM. (○,●) and (□,■) are parallel experiments; (○,□) K803, (●,■) K803 Rif^R-2.

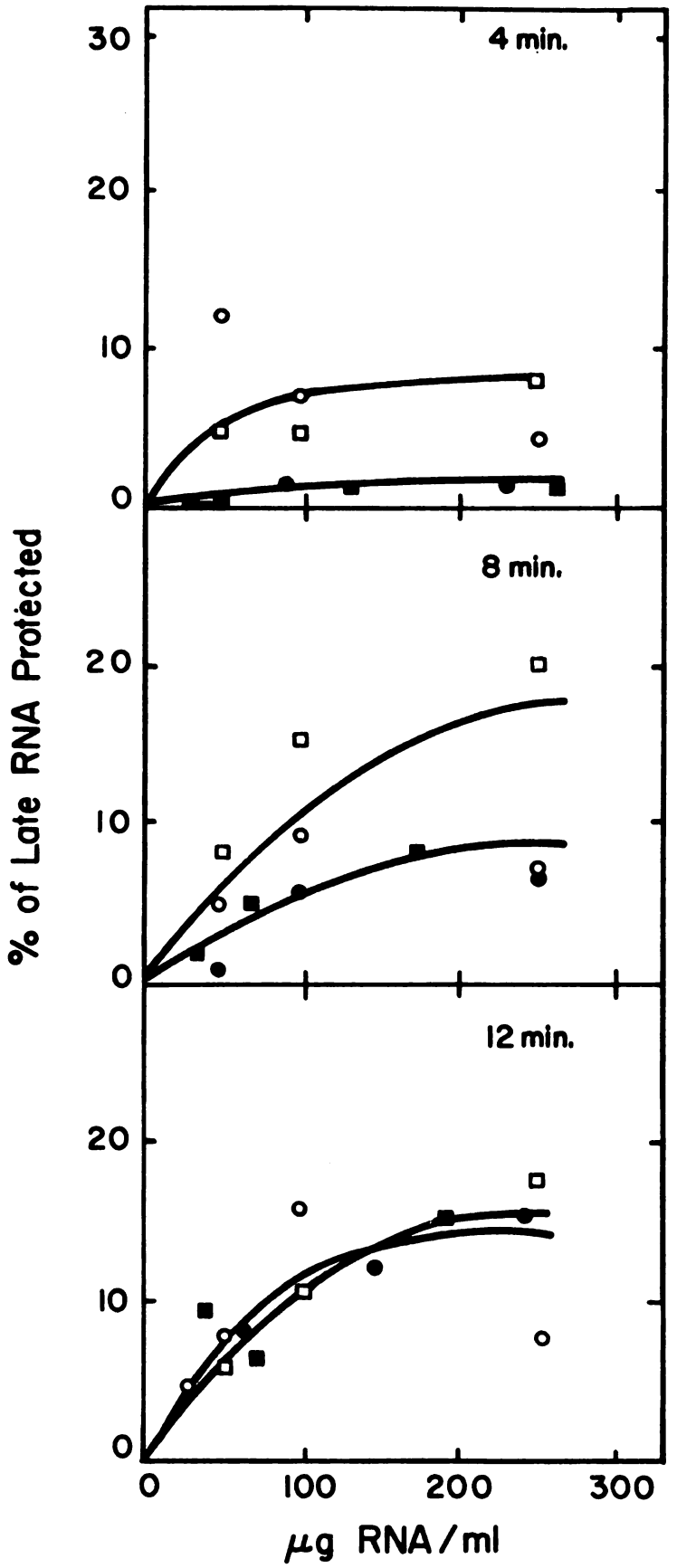
4 min.
8 min.
12 min.
% of Late RNA Protected
μg RNA/ml
0
10
20
30
100
200
300

Figure 8

begins making early gene products at the normal times at 41 C, but many are made in greatly reduced amounts. The gene 32 product is made later than normal while the rIIA protein is synthesized for a protracted interval. Hercules and Sauerbier (1975) showed that genes 43 and 45 each have two promoters, one proximal and one distal. In tsG1 only the distal promoter is recognized early in infection. Anti-late RNA synthesis in tsG1 at 41 C is shown in Figure 9. It is made within 2 minutes after infection but in reduced amounts. In addition, there is never sufficient anti-late RNA to protect the equivalent maximum of that protected by wild type. The apparent rapid decline in amount of anti-late RNA in the wild type infection after 6 minutes is probably due to the synthesis of late mRNA as mentioned earlier. Since events are happening so quickly at this temperature, it is desirable to in some way obviate this complexity and thereby facilitate our interpretation of the data. We repeated the above experiment, therefore, using the double mutant tsG1, am E10 (mot$^-$, gene 45). T4 am E10 does not make DNA or late gene products in a su$^-$ host. At 43 C, anti-late RNA is made for the same time interval in the am E10 and am E10, tsG1 infected bacteria (Figure 10). There is, however, much less made in the double mutant. This seems to be due to the exclusion of certain anti-late regions from being transcribed, although the result could be affected by a decrease in the efficiency of anti-late transcription or perhaps the rate of transcription in general.

The second T4 mutant studied and reported here is Acl9rev2. This was isolated in L. Gold's laboratory. It is a revertant of an rIIB frame shift mutation which does not grow on *E. coli* BE at

Figure 9. Anti-late RNA synthesis in T4 tsG1 infected *E. coli* B_E at 41 C. The procedure is the same as described in the legend of Figure 8. The sample times are indicated. Total CPM added = 2272, background (without unlabeled RNA) was 45 CPM. (O) $T4^+$; (●) T4 tsG1.

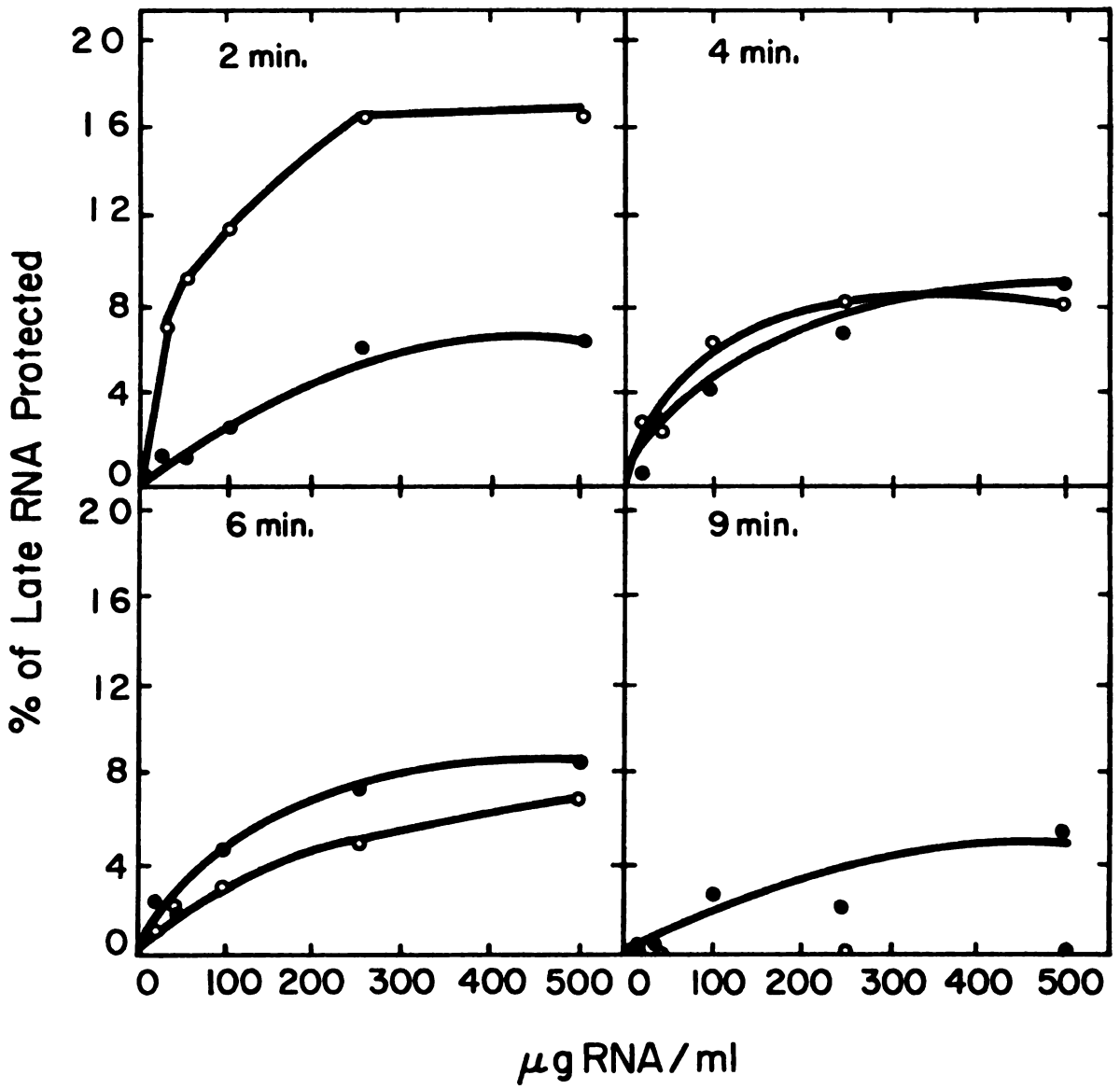
2 min.
4 min.
6 min.
9 min.
% of Late RNA Protected
0
4
8
12
16
20
0
100
200
300
400
500
μg RNA / ml

Figure 9

Figure 10. Anti-late RNA synthesis in T4 tsG1, am E10 infected *E. coli* B_E at 43 C. The procedure was the same as described in the legend of Figure 8, except the MOI was 10. The sample times are indicated. Total CPM added = 4039, background (without unlabeled RNA) was 137 CPM. (O) T4 am E10; (●) T4 tsG1, am E10.

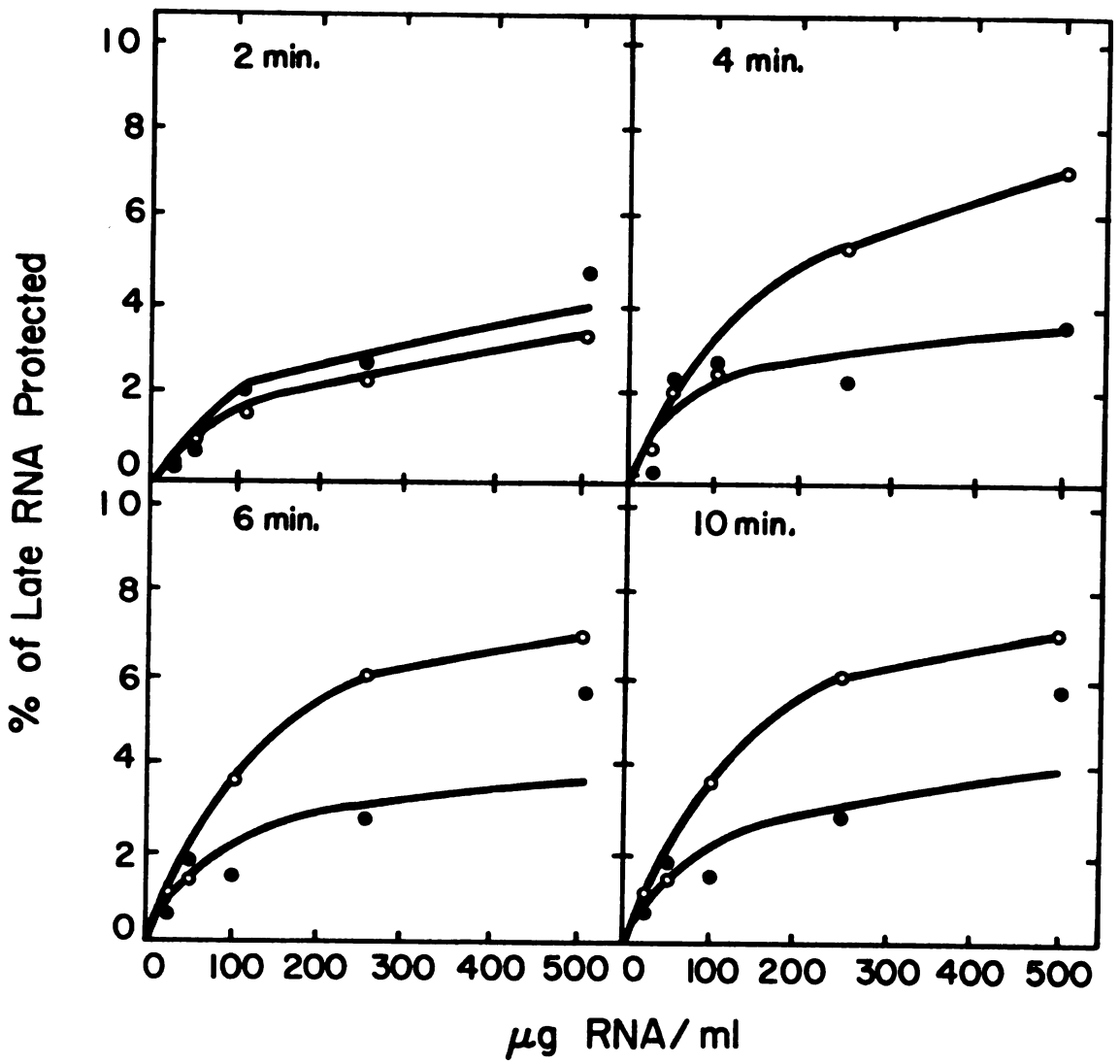
2 min.
4 min.
6 min.
10 min.
% of Late RNA Protected
μg RNA/ml
0
2
4
6
8
10
0 100 200 300 400 500

Figure 10

23 C (Mary Anne Nelson and L. Gold, personal communication). At this temperature, Acl9rev2 makes slightly less delayed early proteins (such as P43 and P45) and does not make quasi-late proteins (such as P32) (M. Nelson and our unpublished observations). Anti-late RNA synthesis is also affected in Acl9rev2 (Figure 11). At early times there is less of the complementary RNA, although it continues to be made at least 20 minutes after infection. This may be a reflection of the defect in promoter recognition in Acl9rev2. We shall reserve the discussion of this point for later.

A third phage mutant, T4 SP62, was also examined. This phage has a mutation in the reg A gene which, when accompanied by a mutation inhibiting replication, results in the overproduction of early gene products. Many early enzymes, for example, are synthesized for up to an hour at 37 C (Wiberg et al., 1973; Karam and Bowles, 1974). We constructed an am N122, reg A double mutant and compared its anti-late RNA synthesis with am N122 and reg A strains (Figure 12). Anti-late is made at the same time in all these infections and, more importantly, there is no increase in the amount made after 6 minutes. This was repeated with a second double mutant, SP62, am N55 (reg A, gene 42) with the same results. The apparent rapid decrease in the amount of complementary RNA in the single mutant SP62 after 6 minutes is due to the synthesis of late mRNA.

All previous work (Wiberg et al., 1973; Karam and Bowles, 1974; Trimble and Maley, 1976) indicates reg A affects the translational control system of T4. This is also indicated by the absence of any effect on anti-late RNA synthesis. If this RNA species is not translated (see discussion), its regulation would logically seem to

Figure 11. Anti-late RNA synthesis in T4 Acl9rev2 infected *E. coli* B_E. The bacteria were grown and infected at 23 C at an MOI of 5 using the procedure outlined in the legend of Figure 8. Total CPM added = 2277; background (without unlabeled RNA) was 45 CPM. (○) $T4^+$; (●) T4 Acl9rev2.

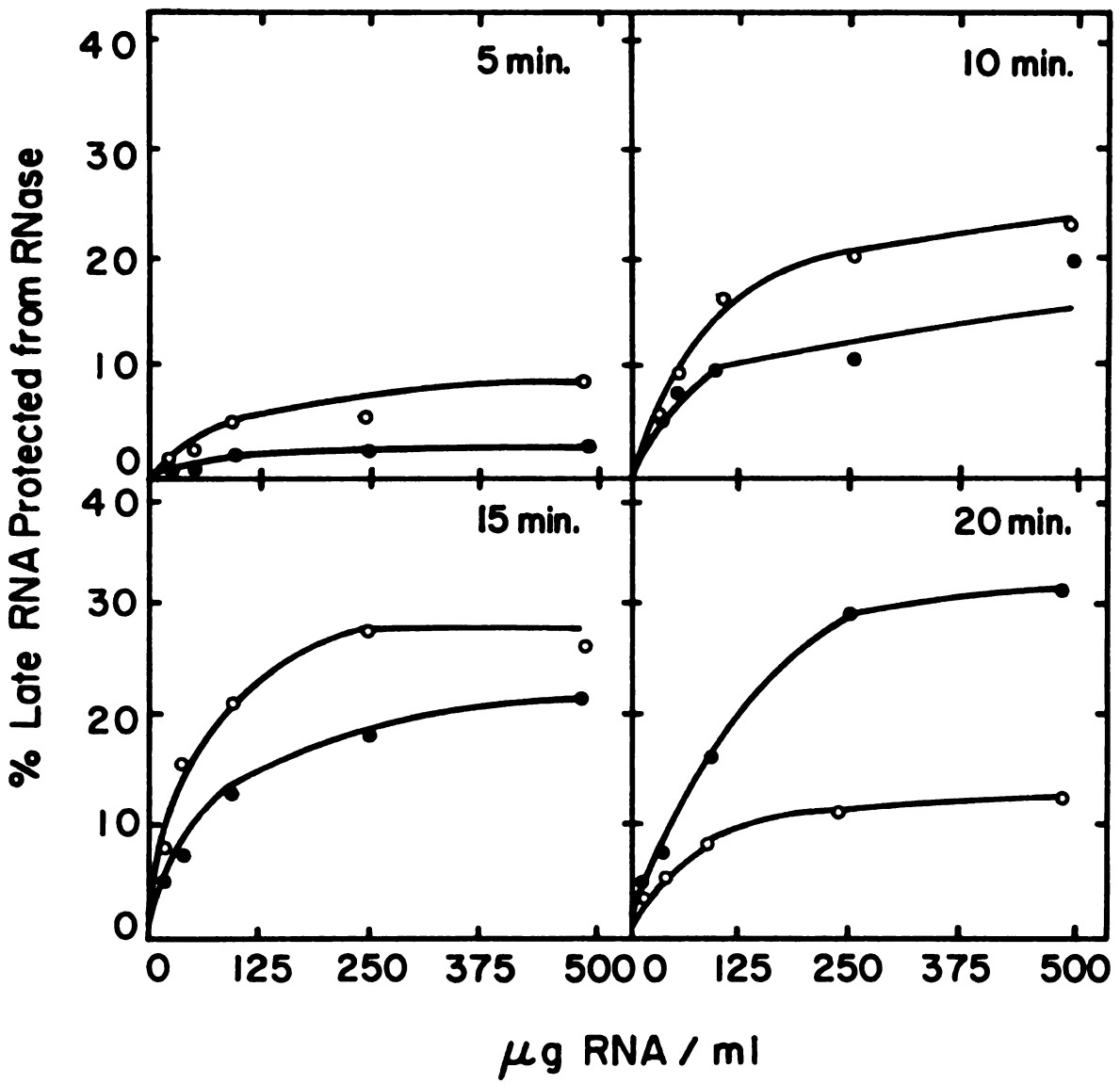
5 min.
10 min.
15 min.
20 min.
% Late RNA Protected from RNase
0
10
20
30
40
0
125
250
375
500
μg RNA / ml

Figure 11

Figure 12. Anti-late RNA synthesis in T4 SP62, am N122 infected *E. coli* B_E. The bacteria were grown and infected at 37 C. The procedure was the same as described in the legend of Figure 8. The sample times are indicated. Total CPM added = 4406; background (without unlabeled RNA) was 82 CPM. (○) T4 SP62 (reg A); (●) T4 am N122 (gene 42); (■) T4 SP62, am N122.

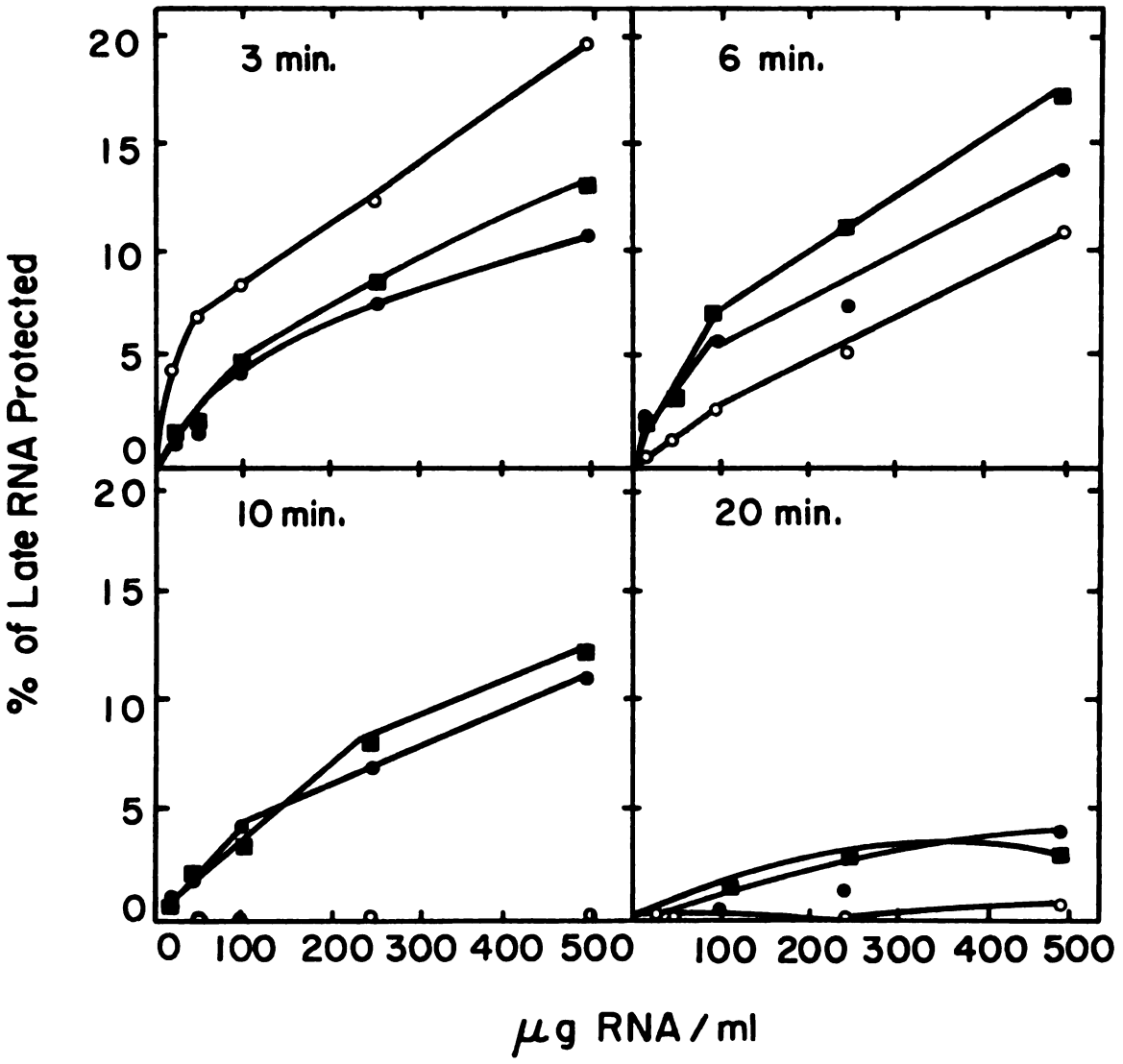
3 min.
6 min.
10 min.
20 min.
% of Late RNA Protected
0
5
10
15
20
0 100 200 300 400 500
100 200 300 400 500
μg RNA / ml

Figure 12

be at the level of transcription. The examination of anti-late RNA therefore could offer another criterion for distinguishing between translational and transcriptional regulation.

DISCUSSION

Anti-late RNAs are unique T4 transcription products. Since they are made on such an extensive portion of the late region (Notani, 1973), they must be essentially anti-sense polynucleotides. We presume that they are not translated into large polypeptides, despite the fact that they are as large as late mRNA (compare Figure 1 and Ricard and Salser, 1975). This presumption is based on the probability of the chance occurrence of ribosome initiation sites and termination codons for a randomly ordered (anti-sense) RNA sequence. It might be mentioned in this regard that the minus strand of the RNA phage, R17, could not bind ribosomes and consequently could not be translated *in vitro* (Swartz et al., 1969). In this report, we have examined anti-late RNA as a unique species in the early temporal scheme of T4 transcription.

We conclude from this work that anti-late RNA is regulated by or at least is responsive to the same control mechanism(s) as delayed early mRNAs. The evidence for this is the following: 1) The synthesis of anti-late RNA is sensitive to chloramphenicol (Notani, 1973). 2) It is made in the presence of rifampicin added at 1 minute after infection. 3) It is made in the same temporal order as the DE class. At 30 C, its synthesis is initiated at 2 minutes and stops at approximately 6 minutes (Notani, 1973). 4) In 3 different situations where T4 early gene expression is altered or

defective, anti-late synthesis is also altered or defective. These will be discussed further below.

Anti-late RNA was made on degraded DNA *in vivo*, but not to any measurable extent on sheared DNA *in vitro*. This could be explained if there are inherent differences in the *in vivo* and *in vitro* anti-late transcription processes. In at least one other instance (Cohen et al., 1974), a difference has been reported. This may in turn explain the different conclusions drawn by Geiduschek and Grau (1970) and Notani (1973) regarding the synthesis of anti-late RNA. It should be noted that the former authors did point out that *in vitro* there was a very rapid synthesis of complementary RNA up to 3 minutes with continued synthesis at a slower rate for an hour. Initiations were restricted to the first minute of synthesis. This is consistent with Notani's and our *in vivo* data if one assumes the initial burst of anti-late RNA synthesis was due to initiations in the late region on the l-strand and that the continued slow synthesis was due to "read through" from the early regions.

Anti-late RNA synthesis is at least partially refractory to rifampicin at 1 minute after infection, analogous to delayed early mRNA. O'Farrell and Gold (1973a) interpreted the insensitivity of delayed early genes to rifampicin added at 1 minute after infection as indicative of their transcription as promoter distal cistrons from pre-early promoters. There is now considerable evidence that many, if not most, prereplicative genes are transcribed as polycistronic messages (Brody et al., 1970; Milanesi et al., 1969, 1970; Black and Gold, 1971; Witmer, 1971; O'Farrell and Gold, 1973a; Hercules and Sauerbier, 1974). This suggested a passive control

of delayed early transcription dependent upon the topography of the particular cistrons (O'Farrell and Gold, 1973a,b). It does not seem likely, however, that anti-late RNAs are made by a passive mechanism because this would necessarily require an "immediate early" anti-late RNA, and none has been detected (Notani, 1973, and our data). The antibiotic refractory state of the polymerase in the late region may indeed be due to previously initiated transcription complexes, but if these were actively synthesizing before 2 minutes, it should be possible to detect the complementary RNA.

In the same context, anti-late RNA synthesis is sensitive to chloramphenicol at the time of infection (Notani, 1973). This also argues against fairly large "immediate early" anti-late RNA subspecies which should by analogy be insensitive to the antibiotic and consequently be synthesized in sufficient amounts to be detected in the assays. Taken together, these data suggest a strong polarity effect on anti-late RNA transcription. This will be discussed in more detail after considering the genetic evidence for a delayed early type of transcription control.

Four different T4 infection systems which result in altered early gene expression were examined for changes in the expression of anti-late RNA. In the bacterial mutant, $\underline{\text{Rif}^{R}\text{-2}}$, the synthesis of prereplicative gene products is delayed. Immediate early species were seen on SDS gels 1 minute after they were made in the parental strain. The difference for many of the delayed early class was 3 to 4 minutes. In addition, the proteins were synthesized for longer intervals (Figure 6; Snyder and Montgomery, 1973; Montgomery,

unpublished observations). In the same fashion, anti-late RNA is made after a delay. Yet by 12 minutes at 28 C, there is as much of the complementary RNA in the mutant as in the parental strain. Although it may be possible to explain these results by a general reduction in the rate of transcription, they are more likely due to a defective regulatory capability by the Rif^R-2 polymerase. The transcriptional capacity of the polymerase in the bacteria is not appreciably altered by the mutation (Snyder, unpublished observations). Also, it was shown in pulse label experiments that after 8 minutes the anti-late RNA in Rif^R-2 makes up a greater percentage of the total RNA being synthesized than in the parental strain (Figure 7).

In addition to the bacterial mutant, we also examined anti-late RNA synthesis in 3 phage mutants with defects in the regulation of early gene expression. T4 AC19rev2 phage do not make quasi-late gene products on *E. coli* B_E at 23 C (M. Nelson and L. Gold, personal communication). An analysis of early proteins by SDS polyacrylamide electrophoresis showed a reduction in the amounts of many early proteins. Some, such as P32, were not made even as late as 30 minutes after infection. At the restrictive temperature, anti-late RNA was made at a lower rate initially but by 20 minutes there was sufficient complementary RNA to protect as much of the late ^{3}H-RNA as was protected by the wild type control. This is in contrast to what was seen in tsG1 infections. In tsG1, early gene expression at 41 C was marked by a reduction in the amounts of many of the early gene products (e.g., P43, P45, and rIIB), but they were made at the same time as they are made by normal phage (Mattson

et al., 1974). T4 tsG1 differs from Ac19rev2 in that P32 is made (at later times) and rIIA is overproduced. That is, the P_E to P_Q shift is made by tsG1 after a delay, but is not made by Ac19rev2. As for anti-late production, it appears at the same time in tsG1 as in the control cultures but in reduced amounts throughout infection. This would be consistent with a shift in promoter recognition, for example, from P_E to P_Q, if anti-late RNA initiation sites have P_E like promoters.

Finally, we looked at anti-late RNA synthesis in a reg A mutant. There was no difference between SP62, am N122 and am N122. All published evidence indicates a post-transcriptional regulation of early gene products (Wiberg et al., 1973; Karam and Bolle, 1974; Trimble and Maley, 1976). Our data not only support a translational control model for reg A but in addition may be another means for distinguishing between translational and transcriptional control mechanisms.

All of the data presented here support the theory of an anti-late RNA control mechanism similar to and possibly the same as that responsible for delayed early gene expression. A controlled anti-late RNA synthesis also suggests the possibility of their being functional molecules. This possibility has already been mentioned by others (Wu et al., 1973). The hypothetical functions include a role in translation, transcription or replication. Notani (1973) calculated that 81% of the l-strand late region is transcribed by 5 minutes after infection. Young (1975) confirmed extensive (up to 90%) transcription of the l-strand early. If this were due to initiation errors as suggested by Notani (1973), it would seem

reasonable that they are not made stoichiometrically. This in turn might *a priori* allow one to exclude the possibility that anti-late transcripts are functional molecules.

We can calculate the number of copies of anti-late RNA using previous published (Notani, 1973) data and following the procedure of Kasai and Bautz (1969). In the first 5 minutes after infection, T4 mRNA amounts to 3.3 to 5% of the total cellular RNA (Landy and Spiegleman, 1968; Kasai and Bautz, 1969; Brody and Geiduschek, 1970). Since anti-late RNA constitutes 2% of the T4 mRNA (Notani, 1973), it would be 0.066 to 0.1% of the total RNA. We find that there is 3 mg of RNA per 4×10^{10} bacteria. If the total T4 genome is 1.66×10^{5} base pairs (Kim and Davidson, 1973) and the late region is 40% of the T4 genome, the late region would be 66.4 kilobases. From this, we calculate that 1.2 to 2 copies of the l-strand late region is present per cell during the first 5 minutes of infection. Even assuming that at an MOI of 5 (as we used in most of the experiments reported here), there are 5 genomes per cell, this would be 0.24 to 0.4 copies per T4 genome. Since anti-late RNA is made for approximately 4 minutes and has a half-life comparable to mRNA (Notani, 1973), it would seem reasonable that essentially the entire l-strand late region is transcribed for each genome. This does not, of course, in any way prove that these molecules are functional, but the possibility cannot be excluded.

In another sense, the regulation of anti-late RNA synthesis may be fortuitous. That is, they may be the result of transcriptional errors or the by-products of some unknown phage process. From either point of view, the regulated synthesis of complementary RNA

provides a different insight into the understanding of T4 transcriptional regulation. We will discuss this further with respect to the present understanding of T4 early gene expression.

The original suggestions of a defect in termination (Geiduschek and Grau, 1970) or overlapping transcription segments (Jurale et al., 1970) do not fit more recent data (see above). The possibility of initiation errors (Notani, 1973) has not been excluded, but this in itself does not explain why the complementary RNA is made only on the DNA l-strand and only early during the infection. It has already been shown that RNA polymerase binds very quickly to early promoters (Guha et al., 1971; Salser et al., 1970; Oleson et al., 1969). We suggest that this is also the case in the late region since anti-late RNA synthesis is refractory to rifampicin at 1 minute after infection. This implies that once the polymerase binds it also initiates or in some way becomes resistant to the antibiotic. If initiation does occur, transcription in all probability is severely limited; that is, there is a strongly polar effect on RNA synthesis in the late regions. This is indicated by the fact that complementary RNA is not detected before 2 minutes. There is no analogous immediate early type of anti-late RNA made. The polarity effect is also suggested by the sensitivity of anti-late transcription to chloramphenicol.

If we pursue the analogy to delayed early transcription control and the extreme polarity on anti-late RNA, it would also seem necessary to hypothesize some sort of termination factor or intrinsic termination site where the transcription complexes would be poised until released by an antitermination. This is of course the same

model proposed for the synthesis of delayed early mRNA (Schmidt et al., 1970; Brody and Geiduschek, 1970; Milanesi et al., 1970; Brody et al., 1970; Schachner et al., 1971). It is also consistent with the rapid (2 to 4 minutes) synthesis of such an extensive region of the T4 genome (Notani, 1973).

One candidate for the termination factor is rho (Roberts, 1969). *In vitro,* rho has been shown to limit the size of mRNA (Travers, 1970; Richardson, 1970) and prevent the synthesis of specific mRNAs (Jayaraman, 1970). One gene not transcribed in the presence of rho was that for lysozyme. This may be of particular interest here since lysozyme is a late gene product that is transiently synthesized early in infection and is coded for on the DNA l-strand (Bautz et al., 1966). Schmidt et al. (1970) attributed this transient early synthesis to read through (antitermination?) of transcription from early genes. Recently, Richardson et al. (1975) and Ratner (1976) have shown that rho is coded for by the suA gene. The suA mutation suppressed the polarity effect of chloramphenicol on the transcription of the trp operon (Morse, 1970). It does not, however, suppress the polarity effect of chloramphenicol on early gene expression in T4 (Baros and Witmer, 1975; Witmer et al., 1975; Young, 1975). This argues against the involvement of rho in the termination process.

Another possible termination factor, kappa, has been isolated and studied (Schafer and Zillig, 1973). This has been shown to have a different reactive site than rho, while both form quaternary termination complexes of DNA, RNA, termination factor and RNA polymerase (Schafer and Zillig, 1973).

If anti-late RNA is made from "terminated" complexes, an anti-terminator would be required for their transcription. This may be analogous to the N gene product of lambda (Roberts, 1970), although this seems to act only from specific promoters (the juggernaut model, Adhya et al., 1974; Freidman et al., 1976). A more general type of anti-terminator may be needed for anti-late RNA synthesis. One possibility is some host factor already present in the bacteria at the time of infection but initially unavailable for anti-termination activity. If, for example, a ribosome associated factor also acted to relieve termination but was only available for the latter when the ribosomes were dissociated, this would coordinate the control of transcription with translation. Such a mechanism has been suggested as being responsible for the polarity effect of chloramphenicol on delayed early genes (Morse and Cohen, 1975). This antibiotic stops elongation, but not initiation or polysome formation (Cremer, 1974; Gurgo et al., 1969). Amino acid analogs, amino acid starvation or potassium depletion, however, do not induce polarity, although protein synthesis is stopped (Lembach and Buchanan, 1969; Morse and Cohen, 1975; Baros and Witmer, 1975). In these cases, the ribosomes are not stable but dissociate in the cell. If one assumes that anti-late RNA synthesis is under the same polarity constraints (they are chloramphenicol sensitive) and they are not translated, then it cannot be ribosome movement per se that relieves the polarity (Baros and Witmer, 1975; Witmer et al., 1975). It might also be mentioned that ribosome associated proteins have been shown to have a stimulatory effect on transcription *in vitro* (Leavitt et al., 1972, 1974).

In summary, we have provided evidence for the regulation of anti-late RNA synthesis by or at least responsive to the same mechanism as delayed early mRNA. We suggest that the study of anti-late RNA offers a different approach to the examination of early transcriptional control in T4. It also may be used as another criterion for distinguishing between transcriptional and translational control early in infection. This, together with the increasing number of reports of complementary RNA, makes the characterization of anti-late RNA a timely and hopefully fruitful effort.

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APPENDIX

APPENDIX

Some of the experiments described in Article 2 of this dissertation are predicated on the fact that different mutations and treatments result in an altered T4 early gene expression. Since we have used different bacteria and/or different conditions than have been reported in the literature, the following SDS polyacrylamide gels were run. In all cases, the results confirm published or unpublished results obtained in other laboratories. The procedure, essentially the same for all the gels, was outlined in Article 2.

Figure 1 is an SDS-polyacrylamide electropherogram of the T4 proteins made on *E. coli* B_E at 30 C with and without rifampicin (100 µg/ml) added at 1 minute after infection. The pulse time was 2 to 12 minutes. As reported by O'Farrell and Gold (1973), many early bands (e.g., p43, p46) appear in reduced amounts. Others such as the rIIA protein continue to be made. The explanation for this is that, since the synthesis of later appearing mRNA transcripts is prevented, there is less competition for the translational machinery and the earlier transcripts continue to be translated. Note that p32, a "true" quasi-late, is not made in the presence of rifampicin added at 1 minute after infection.

T4 tsG1 has a mutation in the mot gene (modifier of transcription) (Mattson et al., 1974). Figure 2 is the SDS-polyacrylamide electropherograms of T4 progeins made by the tsG1 phage. In Figure 2A,

Figure 1. SDS polyacrylamide gel electropherogram of T4 proteins made in the presence of rifampicin added at 1 minute after infection. *Escherichia coli* B_E was grown to 4 x 10^8/ml in M9AA at 30 C. Tryptophan (10 µg/ml) was added before infecting the bacteria at an MOI of 10. The 10 ml culture was divided at 1 minute after infection and one half treated with 100 µg/ml of rifampicin. ^{14}C-Leucine (312 mCi/mMole) was added in 3 equal volumes (0.05 ml) containing 5 µCi at 2, 5, and 8 minutes. The incorporation was stopped at 12 minutes by pouring the cultures over ice. The samples were prepared and 12.5% SDS slab gel run as described by Studier (1973).

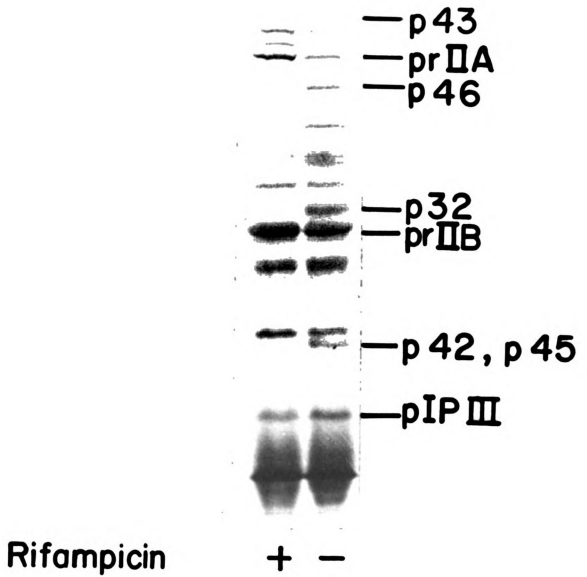
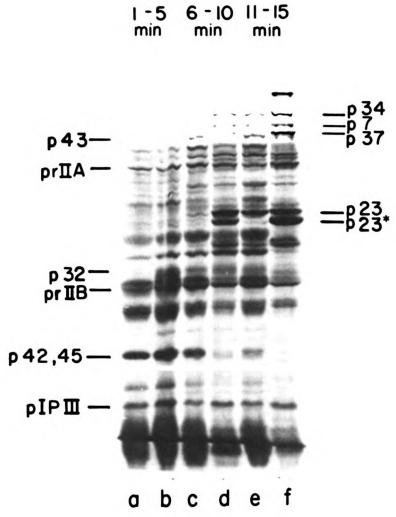


Figure 1

Figure 2. SDS polyacrylamide gel electropherogram of T4 tsG1 gene products made at 43 C. *Escherichia coli* B_E was grown in M9AA at 43 C and infected at an MOI of 10. At the times indicated, 1 ml of the culture was labeled with ^{14}C-leucine (2 µCi/ml; 312 mCi/mMole). The rest of the procedure was the same as described in the legend of Figure 1. A. T4 tsG1 (a,c,e) and T4$^+$ (b,d,f); early gene products are indicated on the left, late gene products on the right. B. T4 am E10 (a,c,e) and T4 am E10, tsG1 (d,e,f).


1 - 5 min
6 - 10 min
11 - 15 min
p43
prIIA
p32
prIIB
p42,45
pIPIII
p34
p7
p37
p23
p23*
a b c d e f

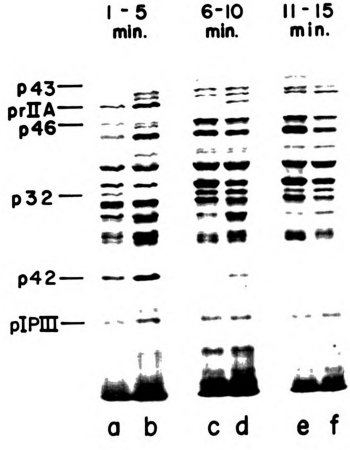

1 - 5 min.
6-10 min.
11 - 15 min.
p43
prIIA
p46
p32
p42
pIPIII
a b c d e f

Figure 2

tsG1 is compared with $T4^+$ and in 2B, the double mutant tsG1, am E10 is compared with am E10. In both cases, the bacteria used were *E. coli* B_E (am su^-) and the labeling was done at 43 C.

Most of the early gene products appear at the normal time but are made in reduced amounts. Others, such as p32, are delayed. In addition, some T4 proteins are overproduced. See, for example, the p rIIA and p42 bands.

Finally, we include the SDS-polyacrylamide gel analysis of T4 Acl9rev2 (Figure 3). This strain was isolated by M. Nelson and L. Gold (personal communication) as a revertant of an rIIB frame shift mutant. It is both cold sensitive and heat sensitive on lambda lysogenic bacteria. In addition, it is cold sensitive on *E. coli* B_E. The block in Acl9rev2 early gene expression is complete for the true quasi-late genes. The gene 32 product, for example, is not made even in the 30-39 minute interval. There is also a demonstrable difference in the production of p46 and p43.

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Studier, W. F. 1973. Analysis of bacteriophage T_7 early RNAs and proteins on slab gels. J. Mol. Biol. 79: 237-248.

Figure 3. SDS polyacrylamide gel electropherogram of T4 Acl9rev2 gene products made at 23 C. The procedure was the same as described in the legend of Figure 2. T4 Acl9rev2 (a,c,e); $T4^+$ (b,d,f).

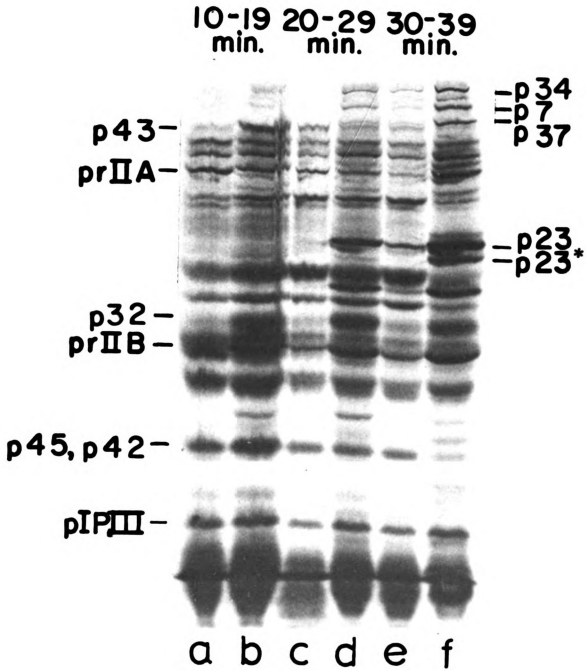
10-19 min.
20-29 min.
30-39 min.
p43
prIIA
p32
prIIB
p45, p42
pIPIII
p34
p7
p37
p23
p23*
a b c d e f

Figure 3