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Regulation of Expression of Late Genes of Bacteriophage T5

G. CHINNADURAI AND D. J. McCORQUODALE
Institute for Molecular Biology, University of Texas at Dallas, Dallas, Texas 75230

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Amber mutants of bacteriophage T5 defective in gene C2 have been characterized. The product of this gene is required for the normal turn-on of synthesis of late RNA's and proteins, and, apparently, for the normal continued synthesis of early RNA's and proteins during late stages of infection. The inability of nonpermissive cells to synthesize any proteins, either late or early, during the late period after infection with a C2-mutant is not due to premature lysis of the infected cells, to a depletion of the cellular energy supply, or to degradation of phage DNA at late times. A possible role for the product of gene C2 in early and late transcription of the viral genome is suggested.

After infection of permissive cells of *Escherichia coli* by bacteriophage T5, the expression of the phage genome is controlled in a well-defined temporal sequence. Three classes of phage-specific proteins are synthesized in three distinct time periods after infection (18). The "pre-early" proteins, which appear to be involved mainly with the establishment of the phage infection, are synthesized from 1 to 8 min after infection and have been studied in some detail (1, 16-20). They are coded by the 8% section of T5 DNA that is initially transferred to host cells, and they are responsible for the degradation of host DNA, the shut-off of synthesis of host RNA and proteins, the shut-off of their own synthesis, and the completion of transfer of phage DNA to the host cell. After about 5 min of infection a second class of proteins, the "early" proteins, begins to be synthesized (18). It appears that the delay in the turn-on of synthesis of early proteins is due to the unique mode of transfer of T5 DNA. Since pre-early genes, which are in the 8% segment of DNA that is initially transferred to host cells, are physically in the cell first and must be expressed before the remaining phage DNA containing early and late genes can be transferred to the host cell, a delay between the synthesis of pre-early and early proteins must occur. The rate of synthesis of early proteins decreases considerably about 25 min after infection, and we will describe a negative control mechanism for this shut-off in a subsequent publication (G. Chinnadurai and D. J. McCorquodale, manuscript in preparation). Synthesis of late proteins begins at about 13 min after infection and continues until lysis (18). The delay in expression of late genes is due to a requirement for certain early proteins (22). In this investigation we describe an amber mutant defective in an early gene whose product is required for the normal turn-on of synthesis of late proteins. This product is also required for maintenance of expression of early genes for the normal period of time.

MATERIALS AND METHODS

Bacteria and phage. *E. coli* F, a nonpermissive (su^-) host for amber mutants of T5 (11) was used in this study. The amber mutants in gene C2 were isolated in this laboratory (11, Mullaley and McCorquodale, unpublished data).

Growth and infection of bacteria. Bacteria were grown in maleate-glucose-salts medium (14) to 2×10^8 cells/ml and were harvested by centrifugation. The cells were washed with maleate-salts buffer containing 10^{-3} M $CaCl_2$, resuspended at 5×10^9 cells per ml in the same buffer, and incubated at 37 C for 30 min with slight aeration to exhaust residual nutrients. At the end of this incubation the cells were infected with an average of 5 to 6 phages per bacterium. After 15 min at 37 C for adsorption of the phage, the suspension was diluted 10-fold into maleate-glucose-salts medium containing 10^{-3} M $CaCl_2$ (MGM-Ca) and aerated vigorously. Phage growth is initiated at the time of this dilution, which is designated zero time for each infection.

Pulse labeling of proteins for electrophoresis. Pulse labeling of proteins was carried out essentially as described by McCorquodale and Buchanan (18) except that in the present study the proteins were labeled with ^{14}C-reconstituted protein hydrolysate (final concentration = 0.2 μCi/ml). Pulse-labeled samples were suspended in 0.05 M Tris-chloride at pH 6.8, containing 1% SDS, 1% mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue, and the samples were prepared for electrophoresis as described by Studier (32). Whenever problems of solubilization occurred, the samples were sonically treated for 15 s.

Electrophoresis and autoradiography. Electrophoresis of radioactive proteins was performed in polyacrylamide gel slabs containing sodium dodecyl sulfate (SDS) (25, 31, 32), and using a discontinuous buffer system that also contained SDS (13). Electrophoresis of proteins in 10% gels was carried out for 8 to 10 h at 10 mA. The slabs were fixed with 12.5% trichloroacetic acid for 30 min and stained with RDS-L Coomassie blue protein stain concentrate. The slabs were destained by diffusion into 7.5% acetic acid in 5% methanol. Drying of gels and autoradiography, using Kodak DF-85 X-ray film, were performed according to Fairbanks, Levinthal, and Reeder (9).

Enzyme assays. Samples (10 ml) of infected bacteria were taken at various times after infection and poured over 5 g of cracked ice. The samples were centrifuged for 10 min at 5,000 × *g*, the pellet was resuspended in 2 ml of 0.05 M Tris-chloride buffer at pH 7.4 containing 0.01 M 2-mercaptoethanol, and the cells were disrupted at 0 C with a Branson sonifier (6 periods of 15-s sonic treatment with intervals of 1 min between periods). These preparations of disrupted cells were used for the determination of deoxyribonucleoside monophosphokinase and lysozyme activities.

Measurements of dTMP kinase activity were performed according to the method of Fessler et al. (10) as modified by McCorquodale and Buchanan (18). The reaction mixture contained 0.04 ml of 1.0 M Tris-chloride buffer at pH 7.4, 0.02 ml of 0.2 M $MgCl_2$, 0.02 ml of a solution containing 100 μmol of ATP per ml adjusted to pH 7.4, 0.06 ml of a solution containing 5.0 μmol of ^{14}C-dTMP (specific activity = 4×10^6 counts per min per μmol), and 0.05 ml of a preparation of disrupted cells, in a total volume of 0.5 ml. Activity is expressed as units of dTMP kinase, where one unit is defined as the amount of enzyme catalyzing the conversion of 1 μmol of deoxyribonucleoside-5'-monophosphate to its di- and triphosphates per hour under the conditions of the assay. Lysozyme activity was determined as described by Sekiguchi and Cohen (26). One unit of lysozyme activity is defined as the amount of enzyme which gives ΔOD_{450}/min = 0.01.

Serum blocking power. Ten milliliters of the infected cultures were taken at various times after infection, and the cells were sedimented, resuspended in 2 ml of maleate-salts buffer containing 10^{-3} M $CaCl_2$, and disrupted by sonic treatment as described above for enzyme assays. Serum-blocking power (SBP) was assayed according to DeMars (7). A disrupted cell preparation (0.1 ml) was mixed with a dilution (10^{-4}) of T5 antiserum, the volume was made up to 0.8 ml with maleate-salts buffer containing 10^{-3} M $CaCl_2$, and the mixture was incubated at 46 C for 12 h. T5 (0.2 ml at 5×10^7 PFU/ml) was added to the mixture as the tester phage to measure the residual antiserum and the mixture was incubated at 46 C for another 2 h. Suitable dilutions were made and the viable phages were measured. The results were converted to phage equivalents per milliliter in the undiluted lysate by comparing with a standard graph prepared by using T5 A1·*am*H27, which does not yield plaques on *E. coli* F (su^-).

Incorporation of ^{3}H-leucine into acid-insoluble proteins. Samples (1 ml) of phage-bacterium complexes in MGM-Ca were removed at various intervals after phage growth began and rapidly mixed with 0.1 ml of MGM-Ca containing 1.0 μCi of ^{3}H-leucine (5 μg). After 2 min of incubation at 37 C, 1.0 ml of chilled 10% trichloroacetic acid was added. The acid-insoluble proteins were collected on nitrocellulose membrane filters (0.45 μm) and the radioactivity was counted in a liquid scintillation counter.

Isolation of RNA and DNA for hybridization. Pulse-labeled RNA used for hybridization with T5 DNA was prepared as described by Sirbasku and Buchanan (27). Portions (20 ml) of phage-infected cells were taken at various times after infection, mixed with ^{3}H-uridine (28 Ci/mmol) to give a final concentration of 12.5 μCi per ml, and incubated for 2 min at 37 C. The labeled cells were sedimented, resuspended in 2 ml of 0.01 M Tris buffer at pH 7.5 containing 0.01 M NaCl, 0.005 M $MgCl_2$, and 200 μg of lysozyme per ml, and were incubated for 10 min at 0 C. SDS was added to yield a final concentration of 2%, and the mixture was warmed at 37 C until it became clear. The lysed mixture was brought to pH 5.2 with sodium acetate buffer (final concentration = 0.02 M), and the RNA in it was extracted by shaking with an equal volume of distilled phenol for 3 min at 60 C. The mixture was chilled and centrifuged for 15 min at 4,000 × *g* and 4 C. The aqueous phase was extracted twice more with hot phenol, and the RNA was precipitated by adding 0.5 volume of saturated NaCl and 3 volumes of ethanol and letting the mixture stand overnight at −18 C.

T5 DNA was isolated by phenol extraction of phage preparations that had been purified by isopycnic banding in CsCl.

Incorporation of ^{3}H-thymidine into DNA. For the determination of cumulative incorporation of ^{3}H-thymidine into DNA, 3.5 μCi of ^{3}H-thymidine per ml (2 μg/ml), 250 μg of deoxyadenosine per ml, and 5 μg of uracil per ml were added to the MGM-Ca in which the phage-bacterium complexes were diluted to initiate phage growth. Samples (1 ml) were withdrawn at various times starting at 5 min after infection, and brought to 5% trichloroacetic acid with 1 ml of cold 10% trichloroacetic acid. Acid-insoluble materials were collected on nitro-cellulose membrane filters, and were washed, dried, and counted in a liquid scintillation counter.

To determine the *rate* of DNA synthesis, 1-ml samples of phage-bacterium complexes were pulse labeled by mixing with 0.1 ml of MGM-Ca medium containing 0.25 μCi of ^{3}H-thymidine (0.5 μg), 200 μg of deoxyadenosine, and 4 μg of uracil, and incubating for 2 min at 37 C. Acid-insoluble materials were collected and radioactivity was counted as in the case of cumulative labeling of DNA.

Determination of ATP levels. Acid-soluble ATP pools in infected cells were extracted by the method of Ramirez and Smith (24) and measured by the luciferin-luciferase assay (21).

Sources of reagents. All radioactive chemicals were purchased from Schwarz Bio Research, Inc., Orangeburg, N. Y. Nitrocellulose membrane filters (0.45 μm pore size, 24 mm diameter) were purchased from Schleicher & Schuell, Inc. Glass fiber filters, grade 984H, were purchased from Reeve Angel, Clif-

ton, N. J. RDS-L Coomassie blue protein stain concentrate was purchased from Canalco, Inc., Rockville, Md. Firefly extract was a gift from John Jagger.

RESULTS

When su⁻ cells are infected with T5⁺, a sequential synthesis of pre-early, early, and late proteins is induced. When su⁻ cells are infected with an amber mutant of T5 defective in gene C2 (*am* M142a), the synthesis of pre-early proteins is normal, the synthesis of early proteins is induced but prematurely shut off, and the synthesis of late proteins is barely detectable (Fig. 1A, B, 2, Table 1).

The effect of a mutation in gene C2 on the overall pattern of protein synthesis is shown in Fig. 1B. Band L9 is not a structural protein of the phage, but since it is synthesized from about 15 min after infection until lysis, it is classified as a late protein (see ref. 33). A brief synthesis, but at a reduced rate, of bands L9 and L8 is seen. Band L12 is very faint. In cells infected with wild-type phage (Fig. 1A), band L13 is composed of an early polypeptide (E7) and a major head (late) polypeptide; these two can be resolved in 15% gels (results not shown). In cells infected with the C2-mutant, the amount of synthesis of L13 is so small, that E7 is easily distinguishable from it (Fig. 1B). The virtual absence of synthesis of late proteins is also observed with two other mutants defective in gene C2, namely, C2·*am*H41a (Fig. 1C) and C2·*am*H55 (results not shown).

When gene C2 is defective, the synthesis of lysozyme and SBP is very low, whereas the synthesis of the early enzyme, dTMP kinase, begins at the same time and rate as in a wild-type infection, but the rate decreases markedly after about 15 min (Fig. 2A, B, C). This in accordance with the autoradiographic patterns of Fig. 1B, and with the overall rate of protein synthesis indicated in Fig. 3.

The autoradiogram of Fig. 1B also indicates that the synthesis of early proteins is apparently normal up to about 15 min after infection with a mutant defective in gene C2, but is then shut off. The pulse labeling data (Fig. 3) confirms this, in that the rate of incorporation of ^{3}H-leucine into total protein is similar to that in a wild-type infection up until 12 to 13 min after infection, but then decreases drastically.

In Fig. 1B, a band is labeled AF (for amber fragment) since we believe that it is an N-terminal fragment of the intact polypeptide chain coded by gene C2 and therefore truncated at the site corresponding to the amber codon in the C2 gene of mutant *am*M142a. Extracts from cells infected with T5⁺, with C2·*am*H41a, or with

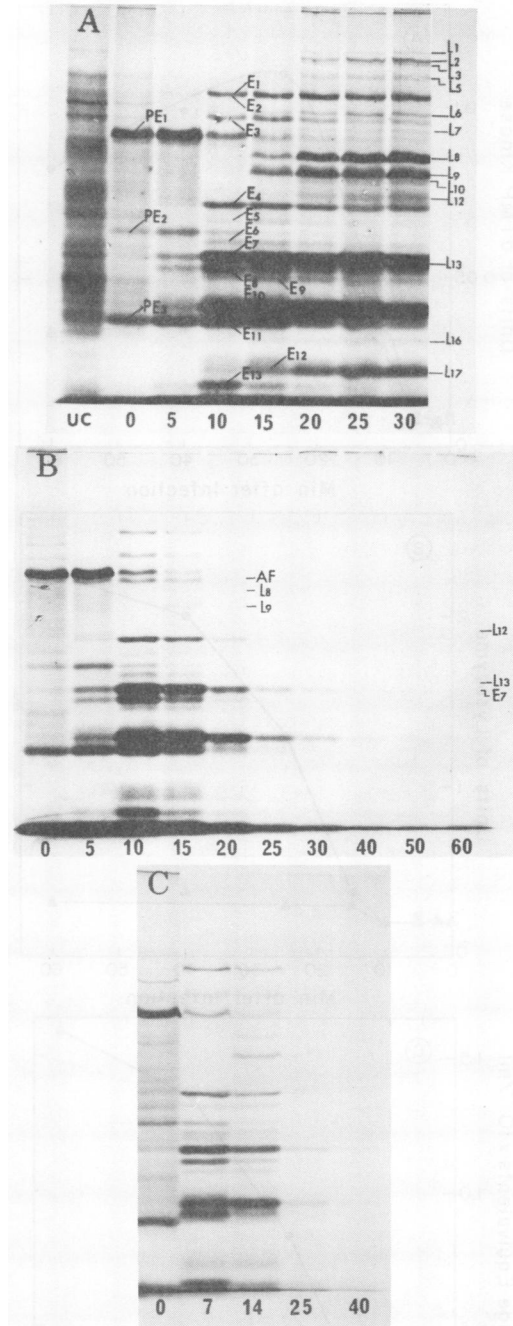

FIG. 1. *Autoradiograms of pulse-labeled polypeptides extracted from su⁻ cells infected with (A) T5⁺, (B) C2·amM142a, and (C) C2·amH41a, after separation in 10% SDS-polyacrylamide gels. The number below each autoradiogram indicates the time after infection at which a 3-min period of labeling was begun. The designations UC and AF refer to uninfected cells and amber fragment, respectively.*

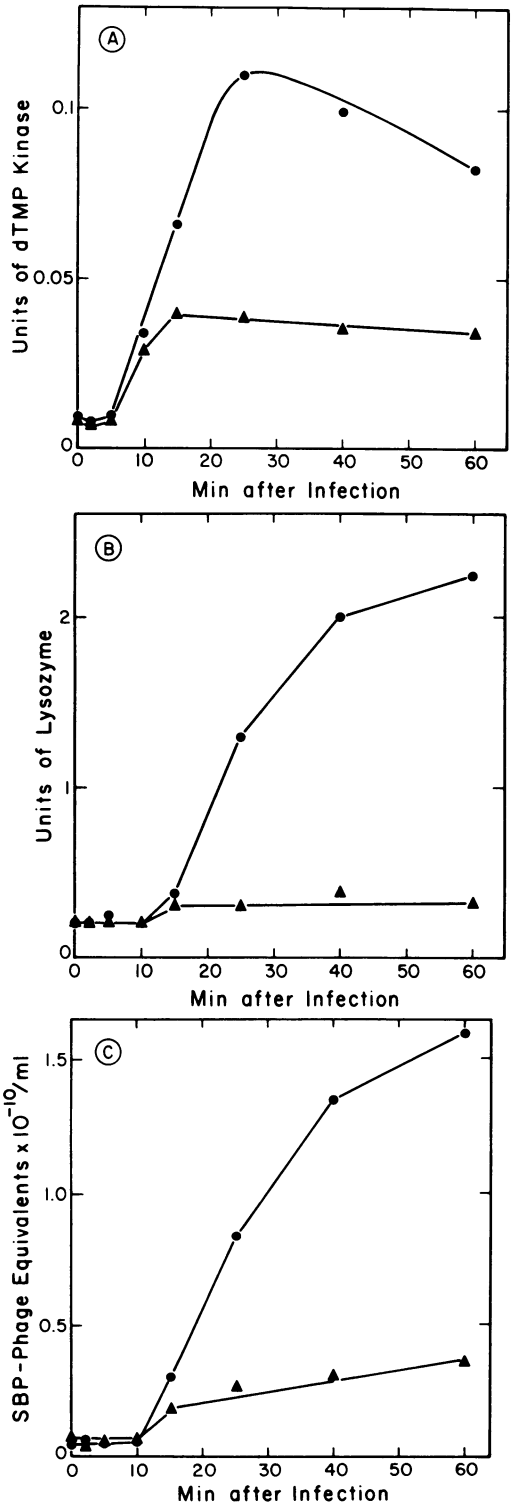

FIG. 2. *Early and late protein synthesis after infection of E. coli F (su^-) with T5+ (●) and C2·amM142a (▲). A, Deoxynucleoside monophosphokinase activity; B, lysozyme activity; C, serum blocking power.*

TABLE 1. *Presence of various phage-induced late-proteins in su^- cells infected with amber mutants in gene C2*[a]

Band no.	Component	Present or absent
L1	Not identified	Absent
L2	Tail	Absent
L3	Not identified	Absent
L5	Tail	Absent
L6	Tail	Absent
L7	Tail	Absent
L8	Major tail	Brief synthesis at reduced rate
L9	Polypeptide required for head morphogenesis	Brief synthesis at reduced rate
L10	Not identified	Absent
L12	Head	Faint band seen
L13[b]	Major head	Faint band seen
L16	Tail	Absent
L17	Head	Absent

[a] Identification of late T5 proteins has also been reported by Zweig and Cummings (33). The bands that are not listed here and that have intermediate numbers can be seen when ^{14}C-leucine is used for labeling.

[b] See text.

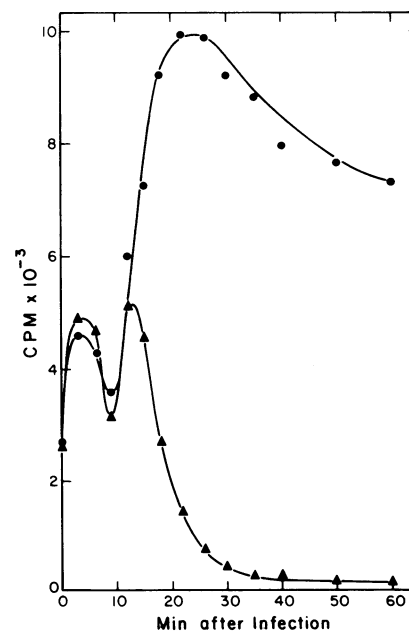

FIG. 3. *Incorporation of ^{3}H-leucine into acid-insoluble proteins in nonpermissive cells infected by T5+ (●) and C2·amM142a (▲).*

C2·*am*H55 do not contain the polypeptide corresponding to this band. If band AF is indeed an amber fragment of the product of gene C2, the intact product of this gene must be higher in

molecular weight than the amber fragment, which corresponds to a molecular weight of about 50,000. This must mean that the intact product of gene C2 is either polypeptide E1 or E2, since E3 is present in cells infected either with $T5^+$ or with amber mutants in gene C2. Preliminary results indicate that E2 is the product of gene D9, which codes for a T5-specific DNA polymerase with a molecular weight of 96,000 (29). Polypeptide E1, with a molecular weight similar to polypeptide E2, is therefore most likely the product of gene C2.

As measured by the rate of incorporation of ^{3}H-uridine into RNA that can hybridize with T5 DNA, the rate of RNA synthesis in cells infected with a mutant defective in gene C2 is similar to that in cells infected with $T5^+$ up to about 15 min after infection. After 15 min the rate decreases slightly in cells infected with a C2 mutant, whereas it increases considerably in cells infected with wild-type phage (Fig. 4). In the mutant-infected cells, the significant rate of RNA synthesis after 15 min is in contrast to the substantial decrease in the rate of protein synthesis after 15 min (Fig. 3), but we do not know if this RNA synthesized after 15 min is functional (see Discussion).

Phage DNA synthesis is initiated in cells infected with C2·*am*M142a at the same time and rate as in cells infected with $T5^+$, but after about 15 min of infection the rate of DNA synthesis is reduced (Fig. 5). This is in agreement with the cumulative incorporation of ^{3}H-thymidine into phage DNA where the incorporation increases more slowly after 15 min (Fig. 6). When the ^{3}H-thymidine that is incorporated into DNA up to 21 min after infection is chased with a large excess of unlabeled thymidine, the ^{3}H-labeled DNA is not degraded (Fig. 6). This demonstrates that degradation of phage DNA is not the cause of the premature shut-off of synthesis of early proteins, nor of the failure to turn on the synthesis of late proteins.

In order to eliminate other possible causes for the defects in the synthesis of proteins displayed by C2-mutants, we have measured ATP levels, and tested for possible premature lysis of cells infected with a C2-mutant. The determinations of ATP levels indicate that energy metabolism is not impaired in cells infected with mutants defective in gene C2. Indeed, there is an increase at late times in the level of ATP in such cells when compared to cells infected with $T5^+$ (Fig. 7). This is not unexpected since there is a reduced drain on the ATP pool because the synthesis of late proteins is virtually absent and the synthesis of phage RNA and DNA is much reduced in cells infected with a C2-mutant. Optical density measurements (Fig. 8) indicate that su^- cells infected with *am*M142a do not lyse, whereas such cells infected with $T5^+$ lyse at the usual time of about 45 min. Hence, we conclude that premature lysis is not the cause of the defects in synthesis of early and late proteins.

DISCUSSION

We have identified an early gene (C2) of T5 that is required for the normal turn-on of synthesis of late RNAs and proteins. Amber mutants in this gene render the phage incapable of inducing the synthesis of late RNAs and proteins in su^- cells. This incapacity is due to a lack of the product of gene C2, and not to premature lysis of the infected cells, or to a depletion of energy in the host cells, or to

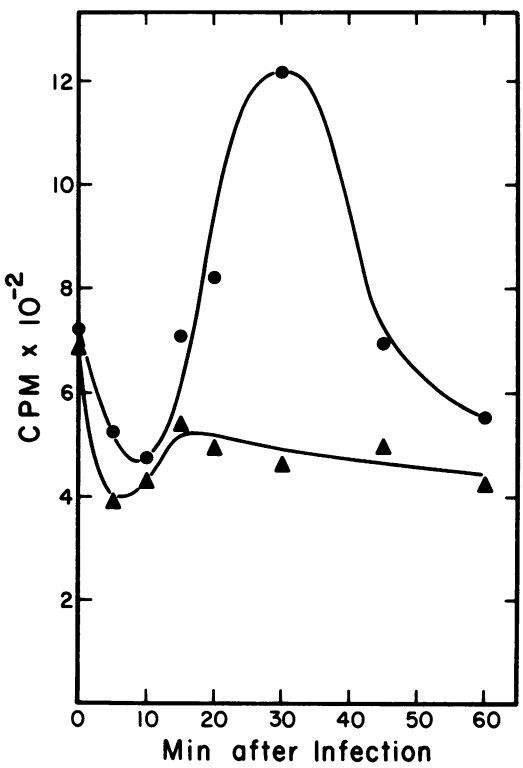

FIG. 4. *Rate of incorporation of ^{3}H-uridine into T5-specific RNA in nonpermissive cells infected with $T5^+$ (●) and amM142a (▲). For determinations of the rate of incorporation of ^{3}H-uridine into T5-specific RNA, samples of RNA pulse labeled for 2 min at different times of infection were extracted as described under Materials and Methods and subjected to exhaustive hybridization analysis as described by Landy and Spiegelman (15). Results are reported as CPM per μg of total RNA (along the ordinate).*

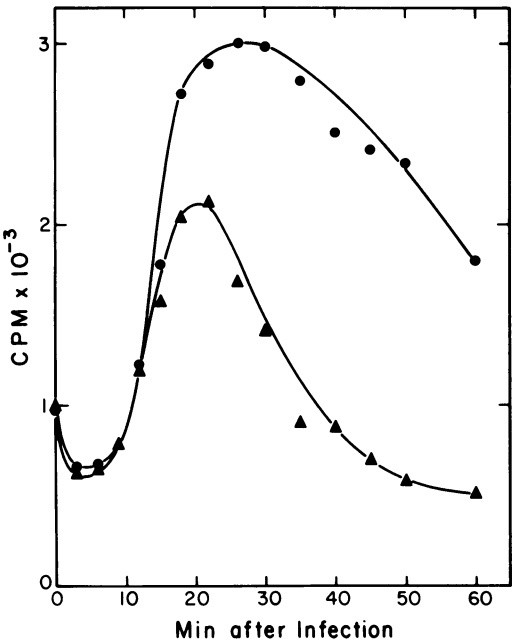

FIG. 5. *Rate of incorporation of ^{3}H-thymidine into DNA in nonpermissive cells infected with T5$^+$ (●) and C2·amM142a (▲).*

degradation of phage DNA at late times. In cells infected with a C2-mutant we do see a few late proteins but in much reduced amounts (Table 1, Fig. 1, 2).

The duration of synthesis of early RNAs and proteins is also aberrant in cells infected with amber mutants defective in gene C2. Normally, early proteins are synthesized throughout the late period but at rates that start decreasing by 20 to 25 min after infection. By contrast, the synthesis of early proteins is shut off virtually completely by about 15 min after infection of su$^-$ cells with C2 amber mutants (Fig. 1B, C). It may therefore be that the product of gene C2 is required not only for turning on the synthesis of late RNAs and proteins, but also for the continued synthesis of early RNAs and proteins during the late period of infection.

The decrease in the rate of DNA synthesis after 15 min of infection with a C2-mutant (Fig. 5, 6) is probably due to the premature arrest of synthesis of early proteins. Crawford (6) has shown that the synthesis of phage DNA in T5-infected cells is arrested if chloramphenicol is added at any time during DNA synthesis.

In T5-infected cells the periods of synthesis of three classes of phage-specific messenger RNAs correspond to the periods of synthesis of three classes of proteins (18, 22, 28). This indicates that the synthesis of T5-specific proteins is regulated mainly at the transcriptional level. The results reported in this paper show that the virtual absence of expression of late genes and the shortened period of expression of early genes in cells infected with a C2-mutant are also explained by transcriptional events.

Moyer and Buchanan (22) have previously shown that transcription of late T5 genes requires certain early proteins. We have recently shown that one of these early proteins is a nuclease coded by gene D15 that presumably modifies intracellular T5 DNA so that transcription of late genes can occur (Chinnadurai and McCorquodale, Proc. Nat. Acad. Sci. in press). In this investigation, we show that another of these early proteins is the product of

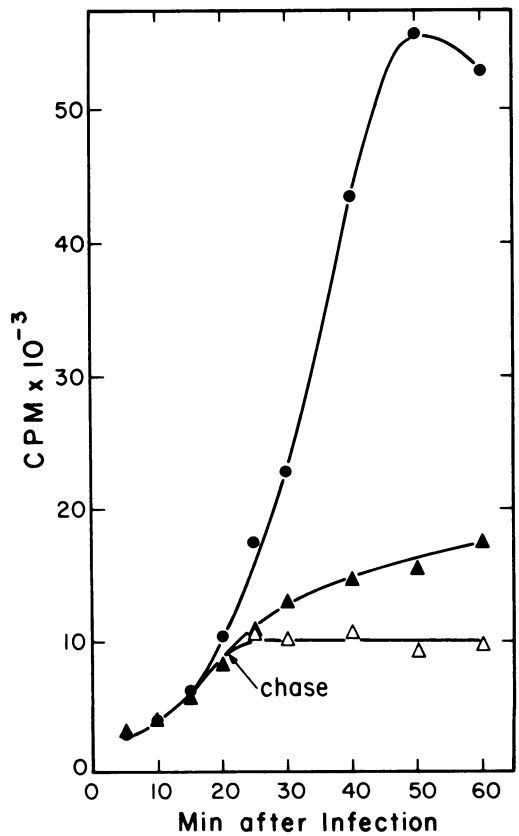

FIG. 6. *Cumulative incorporation of ^{3}H-thymidine into DNA in nonpermissive cells infected with T5$^+$ (●) and C2·amM142a (▲). The symbol Δ indicates phage DNA that was labeled from 5 to 21 min after infection with C2·amM142a, and then chased by addition of a 1,000-fold excess of unlabeled thymidine.*

gene C2, and this product functions in the process of transcription. Since our earlier studies demonstrated that at least that portion of the host RNA polymerase which is sensitive to rifampin is required for all essential transcription of the T5 genome (2), since we know that the RNA polymerase from uninfected cells cannot efficiently transcribe late genes in vitro (23; Bremer, personal communication), and since the polypeptide of gene C2 has a molecular weight (96,000) similar to that of the sigma factor of the host, *E. coli* (5) (see Results), we believe it is reasonable to propose that the product of gene C2 is a T5-specific sigma factor that replaces the host-specific sigma factor in the RNA polymerase in infected cells so that it can transcribe late genes from modified T5 DNA. This proposal now has experimental support in that H. Bujard (personal communication) has shown that RNA polymerase isolated at late times from cells infected with $T5^+$ and labeled with ^3H-leucine from 6 to 10 min after infection contains ^3H-labeled sigma factor that comigrates with ^{14}C-labeled sigma factor from uninfected cells. This finding does not yet prove that gene C2 is the structural gene for a T5-specific sigma factor, but it strongly supports this proposition.

R. W. Moyer (personal communication) has informed us that the RNA polymerase isolated at late times from cells infected with $T5^+$ has the same specificity for transcription of pre-early and early genes as does the RNA polymerase from uninfected cells. This finding presents us with an explanation for the premature shut off of synthesis of early RNAs and proteins in cells infected with a C2-mutant, as follows. During the period of expression of early genes, the core is modified in order to dissociate the host sigma factor and to increase the affinity of the core for the T5-specific sigma factor. Transcription of early genes is therefore initially carried out by the unmodified host RNA polymerase, but later by the T5-modified RNA polymerase, which contains the T5-specific sigma factor. If, however, no T5-specific sigma factor is available (as proposed in the case of infection with a C2-mutant), the modified core enzyme, having ejected the host sigma factor, would have no sigma factor with which to function properly. The net result would be that the transcription of early genes, which begins at early promoter sites, would cease prematurely, and the proper transcription of late genes would never begin. Whatever RNA is synthesized would be transcribed by a modified core enzyme that would be expected to have lost its ability to recognize promoter sites, and might therefore synthesize biologically incompetent RNA. This situation would result in a pattern of protein synthesis just as we report for cells infected with a C2-mutant.

Our current view of the transcription of late T5 genes is therefore that the products of at least two early genes are required. One of these genes (C2) probably codes for a T5-specific sigma factor that replaces the host sigma factor

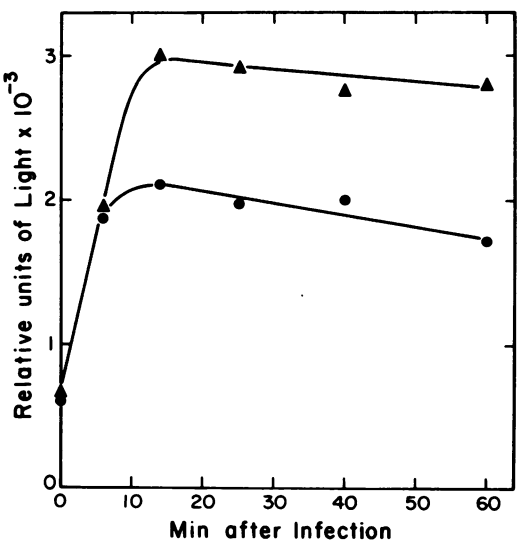

FIG. 7. *Levels of adenosine triphosphate in non-permissive cells infected with $T5^+$ (●); and with C2-amM142a (▲). The levels are expressed as relative units of light produced in the luciferin-luciferase assay.*

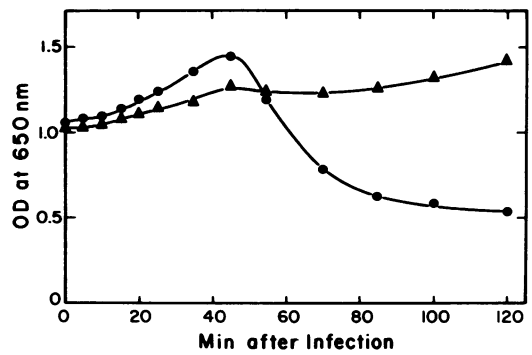

FIG. 8. *Lysis of nonpermissive cells infected by T5. Samples were taken at various times after dilution into MGM-Ca (Materials and Methods) and the optical density was determined at 650 nm. Symbols: ●, cells infected with $T5^+$; ▲, cells infected with C2-amM142a.*

of the RNA polymerase, whereas the other gene (D15) codes for a nuclease that modifies the T5 DNA so that late transcription can occur. It may be that the product of a third early gene modifies the core enzyme in such a way that the host sigma factor dissociates from it and the T5-specific sigma factor binds to it. Alternatively, the product of this third gene may modify the host sigma factor itself in order to dissociate it from its core, which would then remain unmodified.

These requirements for late T5 transcription are similar to those for late T4 transcription. The latter requires a state of the T4 DNA different from the state of parental DNA (4), as well as the products of at least two early genes (33 and 55), (3, 8) that modify the existing RNA polymerase (30). Certain differences between the two systems exist, however. For example, continual T4 DNA replication is required in order to maintain the proper state of the DNA for late T4 transcription, whereas T5 DNA replication is not required for late T5 transcription (12).

Currently, we are isolating the product of gene C2 in order to study its effect on in vitro transcription systems.

ACKNOWLEDGMENTS

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