JOURNAL OF VIROLOGY, Nov. 1977, p. 436-443
Vol. 24, No. 2

Printed in U.S.A.

Distinctive Protein Requirements of Replication-Dependent and -Uncoupled Bacteriophage T4 Late Gene Expression

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Received for publication 14 April 1977

This paper further explores the relationship of viral DNA replication to bacteriophage T4 late gene expression. It is shown that replication-coupled and -independent late transcription make different qualitative or quantitative demands on phage protein synthesis. In further analysis of these different protein synthesis requirements, experiments were performed with a temperature-sensitive mutant in T4 gene 55 (*ts*553). It is known that the gene 55 product regulates T4 late gene expression and binds to RNA polymerase. In the experiments presented here, it is shown that the temperature sensitivity of the *ts*553 gene 55 protein depends on whether it is involved in replication-coupled or -independent T4 late transcription. This is evidence that the proteins constituting the transcription apparatus interact differently with late transcription units in T4 DNA, depending on whether late transcription is replication coupled or independent.

The expression of the viral late genes during the normal growth cycle of bacteriophage T4 depends on: (i) the action of several virus-coded regulatory gene products (3, 7, 17) that bind to RNA polymerase (19, 22-24), which is ADP ribosylated after phage infection (8, 26), and (ii) the creation of a "competent" DNA template. The chemical nature of DNA competence is not yet worked out but is known to involve interruptions in DNA primary structure (21). Replication normally provides the competent template, but late gene expression can also occur in the absence of replication (14, 20, 21). Our recent work has concentrated on the circumstances for achieving replication-independent late gene expression and on the regulation of late gene expression in the absence of replication. We showed that relative to replication-coupled late protein synthesis, the "uncoupled" protein synthesis is delayed, is temperature sensitive, and depends on the multiplicity of infection. However, replication-uncoupled late gene expression still depends on the function of the three T4 regulatory proteins gp33, 45, and 55 (4, 29, 30).

In the experiments described here, we have probed the relationship of viral proteins to replication-coupled and -independent late gene expression for hitherto unexplored differences. We present evidence that such differences exist. We also present indirect evidence that at least one of the virus-coded RNA polymerase binding proteins undergoes different interactions in replication-dependent and -uncoupled late gene expression.

MATERIALS AND METHODS

Bacterial strains and phage T4 mutants. *Escherichia coli* B^E (*su*$^-$) was used throughout these experiments. CR63 (*su*$_1^+$) served as the permissive host for plating amber phage mutants. Abbreviations used are as follows: *am*, amber; *ts*, temperature sensitive; *pol*, DNA polymerase; *lig*, DNA ligase; *exo*, the DNase function (presumably an exonuclease function) controlled by T4 gp46). The following T4 phage mutants (28) were used: T4D$^+$ (wild type), *ts*P36 (gene 43, *pol*$^-$), *am*E4301 (gene 43, *pol*$^-$), *ts*553 (gene 55), *am*H39X (gene 30, *lig*$^-$), and *am*N130 (gene 46, *exo*$^-$). These mutants were backcrossed at least twice against wild-type T4D. *ts*553 was obtained from J. Pulitzer. It had been checked for its temperature sensitivity, for the absence of second, suppressor mutations in gene 45 (5, 6), and for the temperature insensitivity of DNA replication. A second stock of *ts*553, from our own collection, did not differ in its properties from the above mutant. Multiple mutants were constructed and were checked by liquid complementation according to standard methods.

Media. M9S medium, which is M9 supplemented with 1% Casamino Acids (2), was used for growing phage stocks and for experiments. In certian experiments, protein synthesis was inhibited by adding 200 μg of chloramphenicol (CM) per ml.

Phage infection and manipulation of temperature shift-down experiments. *E. coli* B^E were grown at 37°C to a density of 4×10^8 cells/ml and then transferred to the appropriate temperature 5 to 10 min before phage infection. Cells were infected at a multiplicity of 6 to 10 for those phage mutants that were not defective in DNA synthesis, but a multiplicity greater than 15 was used for infections for replication-defective (DO) mutants (to enhance late gene expression). The protocol for temperature shift-down was

as follows: at various times after infection at 40.5 or 43°C, portions of cultures were either treated with CM 30 s before shift-down or were not so treated. The shift-down was done in this order: 1 volume of ice-cold M9S with or without CM was pipetted into an aerated and prewarmed tube in a 30°C water bath, and then 2 volumes of culture from the higher temperature were immediately poured into the tube. In this manner, the temperature dropped to 29°C within 30 s and returned to nearly 30°C within 1 min.

Other methods. RNA labeling, isolation, and hybridization with separated strands of T4 DNA was done as described or referenced previously (29). Viral DNA synthesis was followed in terms of the incorporation of [^{3}H]thymidine into acid-insoluble material as described previously (32).

Materials. [5-^{3}H]uridine (25 Ci/mol) and [^{3}H]thymidine were obtained from the New England Nuclear Corp. CM was purchased from Calbiochem. Other chemicals were standard reagent grade.

RESULTS

Effect of inhibition of protein synthesis on T4 late transcription. Both phage DNA replication and late gene expression require the synthesis of virus-coded proteins before their initiation (7, 27). In the first experiment, we examined the synthesis of DNA and late RNA in wild-type phage-infected cells when CM was added at various times after infection. In this and the following experiments, the fraction of [^{3}H]uridine-pulse-labeled RNA hybridizing to T4 *r*-strand DNA was used as the measure of the transcription activity of T4 late genes (9, 10).

The kinetics of *r*-transcript synthesis in wild-type phage-infected cells at 30°C are shown in Fig. 1, together with the effect of CM addition at various times after infection. A similar experiment was carried out on the synthesis of viral DNA and is presented in Fig. 2. The results are essentially in agreement with a previous report (17), in which late transcription was analyzed in less detail and by a different method. However, the time scale of late transcription and of replication is advanced here by 1 to 3 min relative to previously published experiments and other experiments in our laboratory. The feature of this experiment that we wish to stress is that the inhibitory effect of CM on *r* transcription is maintained for a much longer time than its inhibitory effect on DNA synthesis. For example, treatment with CM at 8 min after infection still effectively inhibits *r*-strand synthesis but not DNA synthesis. It is not until 15 min after infection that the addition of CM has very little effect on the synthesis of *r*-strand RNA. These results suggest that the first initiation of DNA replication and *r* transcription happen within a relatively short time period but that a longer

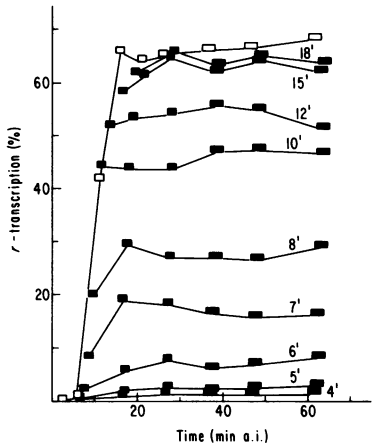

FIG. 1. *Kinetics of r transcription in E. coli B^E infected with T4 wild type: effect of CM. E. coli B^E were infected at 30°C with T4D$^+$ (multiplicity = 8). At various times after infection (a.i.), samples were treated with CM (100 μg/ml), and portions of these samples were pulse-labeled for 2 or 3 min with [^{3}H]-uridine (30 μCi/ml) at various times thereafter. Control samples were not treated with CM. r transcription is expressed as the percentage of total [^{3}H]RNA hybridization specific to the r strand of T4 DNA (i.e., counts per minute hybridizing to r strand DNA divided by the sum of the counts per minute hybridizing to l and r strands). All data in this and subsequent figures have been corrected for the background of radioactivity binding to nitrocellulose filters in the absence of added DNA. The backgrounds ranged from 0.1 to 5% of the radioactive RNA input and were usually within the range of 1 to 2%. Symbols: (□) no CM addition; (■) CM addition at 4, 5, 6, 7, 8, 10, 12, 15, or 18 min.*

duration of protein synthesis is needed for effective and abundant *r* transcription.

In the next experiment, we examined the effects of CM on "processing"-dependent but replication-independent T4 late transcription. Processing is the name that has been given to the as yet incompletely specified structural changes of DNA required for activation of late gene expression (21, 30). The experiment (see Fig. 3) was carried out with *su*$^-$ bacteria infected with *pol*$^-_{am}$-*lig*$^-_{am}$-*exof*$^-_{am}$ T4 phage, which have been the subject of extensive prior analysis (30). It has been previously shown that in such infections, *r* transcription occurs in the absence of DNA synthesis and depends on the multiplicity of infection. In the absence of CM, the onset of *r* transcription (Fig. 3, open symbols) is delayed relative to the wild type (Fig. 1, open symbols), the maximum percent *r* transcription is depressed relative to the wild type, and this maximum relative rate is reached later (cf. 30). No elaborate or extensive differences in the synthesis of

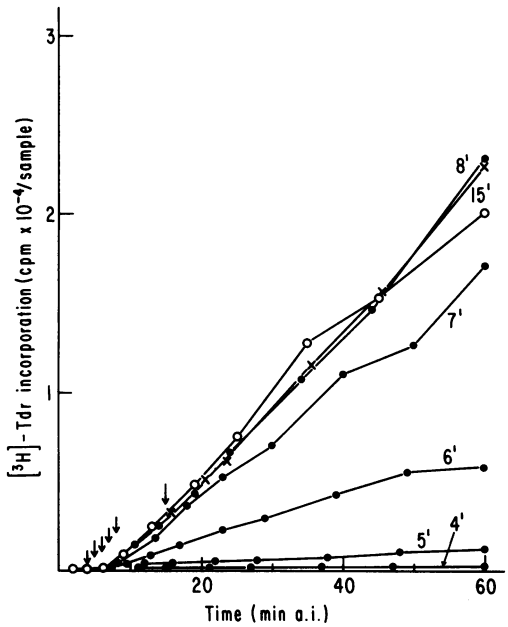

FIG. 2. *Effect of CM on T4 DNA synthesis. E. coli B^E were infected with T4 wild type as described in the legend to Fig. 1, except that [^{3}H]thymidine (10 μCi and 5 μg/ml) and deoxyadenosine (200 μg/ml) were added 1 min after infection (a.i.). Thymidine incorporation into DNA was measured by pipetting 0.07-ml samples of the infected culture onto Whatman 3MM paper disks (2.4-cm diameter). Disks were washed with 5% Cl_3CCOOH and with 95% ethanol as previously described (32). Symbols:* (○) *control, no CM treatment;* (●) *CM-treated cultures except for CM addition at 15 min, which is designated by* (×).

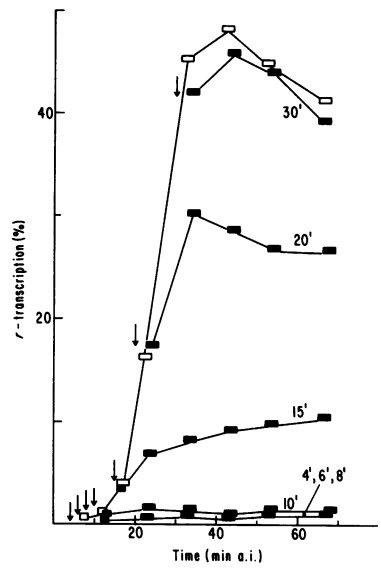

FIG. 3. *Kinetics of r transcription in pol⁻-lig⁻-exof⁻ mutant-infected cells after CM addition. E. coli B^E at 30°C were infected with amE4301-amH39X-amN130 (gene 43⁻-30⁻-46⁻, pol⁻-lig⁻-exof⁻) at a multiplicity of 17. CM treatment and RNA analyses were done as described in Fig. 1. Symbols:* (□) *no CM addition;* (■) *CM added at 4, 6, 8, 10, 15, 20, or 30 min* (↓). *a.i., After infection.*

early proteins during the first 10 min after infection by wild-type phage and by *pol⁻-lig⁻-exof⁻* phage have been noted (cf. 31). If the proteins required for DNA processing were early proteins, they should be made within the first minutes after infection, and DNA processing as well as its concomitant late transcription should thereafter be insensitive to inhibition by CM. We had therefore thought it likely that the delayed onset of *r* transcription might solely reflect the time required for DNA processing. That expectation is not realized: *r* transcription is totally sensitive to CM inhibition for 10 min at 30°C, greatly (greater than 75%) sensitive for 15 min, and substantially sensitive for 20 min. These results are summarized and simplified in Fig. 4, in which the "relative *r* transcription" activity, *R*, has been plotted against the time of CM addition for infections with different T4 mutants. *R* is defined as the ratio of the maximum percent *r* transcription after CM addition (at time *t*) to the maximum *r* transcription in the control experiment to which no CM has been added. The time course, $R(t)$, shows the extent and duration of the inhibitory effect of CM on late transcription. When *R* equals 1, late transcription is insensitive to inhibition by CM.

Data from additional experiments are also summarized in Fig. 4. In these experiments, *E. coli* B^E have been infected at 40.5°C with T4 wild type and with *pol⁻-lig⁻-exof⁻* phage that are either *am* or *ts* in the *pol* gene (gene 43). For one of these infections (pol_{ts}), the primary data are shown in Fig. 5. All of these results show the same features. In the absence of replication, the onset of late transcription is delayed and the sensitivity of *r* transcription to inhibition by CM is prolonged.

The next experiment confirms that different sensitivities to inhibition by CM are, indeed, related to the occurrence of replication. For this experiment, the cells were infected with pol^-_{ts}-lig^-_{am}-$exof^-_{am}$ phage at 40.5°C. Although viral DNA is not replicated at this temperature, the *ts* lesion in P36 is known to be substantially reversible (20). At various times after infection, CM was added, and 0.5 min later each culture was split in two, one part being shifted down to 30°C to permit DNA synthesis while the other remained at 40.5°C. *r* transcription was measured at various times after the shift-down, as described in Fig. 1 and 3. The results are presented

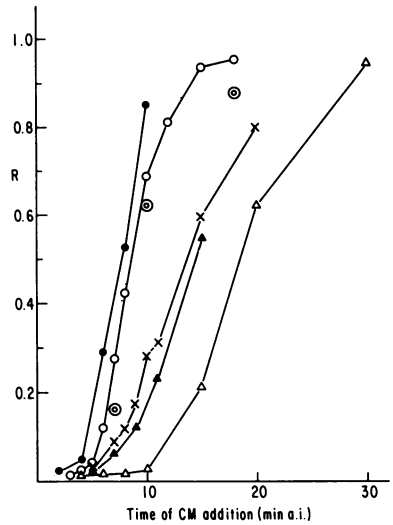

FIG. 4. *DNA replication, CM addition, and the regulation of T4 transcription. Data from Fig. 1, 3, and 5 and from other experiments are summarized in terms of the variation of R, the relative r transcription activity, as a function of time, t, at which CM is added.* $R = [r_{max}(+CM)]/[r_{max}(-CM)]$, *where* $r_{max}(+CM)$ *is the highest level of r transcription attained at any time after CM addition and* $r_{max}(-CM)$ *is the highest level of r transcription attained at any time after infection (a.i.) in the control to which no CM has been added. Symbols: (O and ○)* $T4D^+$ *at 30°C,* $r_{max}(-CM) = 70$ *and 69%, respectively; (●)* $T4D^+$ *at 40.5°C,* $r_{max}(-CM) = 51\%$; (×) *tsP36-amH39X-amN130 at 40.5°C,* $r_{max}(-CM) = 40\%$; (▲) *amE4301-amH39X-amN130 at 40.5°C,* $r_{max}(-CM) = 43\%$; (△) *amE4301-amH39X-amN130 at 30°C,* $r_{max}(-CM) = 48\%$.

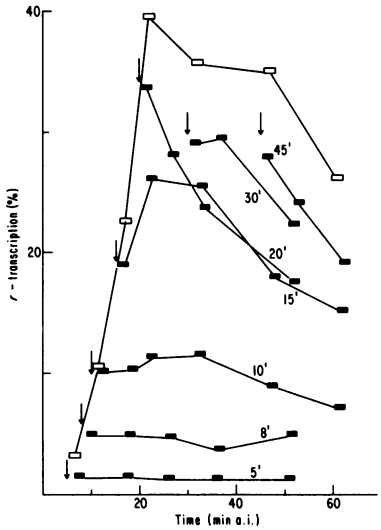

FIG. 5. *Kinetics of r transcription in E. coli* B^E *infected with* pol^-_{ts}-lig^-_{am}-$exof^-_{am}$ *phage. Bacteria were infected at 40.5°C at a multiplicity of 17 and processed as described in the legend to Fig. 1. Symbols as in Fig. 1. CM was added at 5, 8, 10, 15, 20, 30, or 45 min (↓). a.i., After infection.*

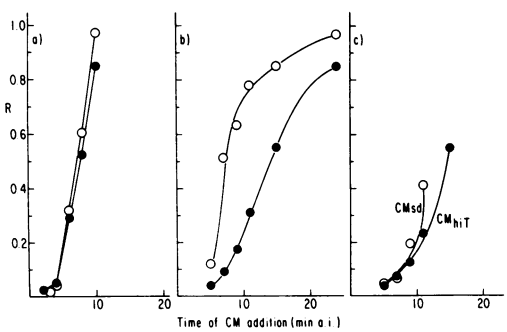

FIG. 6. *Effect of temperature shift-down (40.5°C → 30°C) and of CM on the relative r transcription activity, R, in (a) wild type-, (b) tsP36-amH39X-amN130-, and (c) amE4301-amH39X-amN130-infected E. coli* B^E. *Temperature shift-down was carried out as described in the text. Other procedures are described in the legend to Fig. 1. R is defined in the legend to Fig. 4. Symbols: (○) CM added and shifted down to 30°C* (CM_{sd}); *(●) CM added but held at 40.5°C* (CM_{hiT}). *a.i., After infection.*

in Fig. 6, in terms of the relative *r* transcription activity, *R*, as a function of the time of CM addition. The effect of CM on the quantity, *R*, clearly depends on whether replication is allowed to occur after protein synthesis has been inhibited (Fig. 6b). Controls with wild-type phage in which replication occurs at both the high and low temperature (Fig. 6a) and with pol^-_{am}-lig^-_{am}-$exof^-_{am}$ phage in which replication occurs at neither temperature (Fig. 6c) do not show a comparable effect of temperature shift on *R*. (Figure 6c partly hides some complications which are probably due to temperature sensitivity of replication-uncoupled late transcription [30] and which are further discussed in the next section.)

Gene 55 product. In principle, the effect of CM on late transcription might be due to two causes: (i) induction of antibiotic-induced polarity (1, 12, 16) selectively affecting late transcription (we think that this is unlikely, because antibiotic-induced polarity appears to be eliminated soon after T4 infection as part of what has been called the "early regulatory event" [see 18 for extensive discussion]; (ii) prevention of the synthesis of protein required for *r* transcription. Early and middle viral protein synthesis does not depend absolutely on replication, and replication-coupled late transcription is thought to depend only on early and middle, i.e., prereplicative, proteins. Accordingly, the different CM sensitivities of replication-dependent and -independent transcription might reflect either

a requirement for different proteins or a requirement for different quantities of the same proteins. In the former case, one can conceive of the possibility that replication-independent late transcription is actually strongly dependent on a late protein and is thus involved in a positive feedback regulatory loop. It was this conjecture that prompted the next experiment with a gene 55 mutant. The analysis follows the scheme of the preceding temperature shift-CM experiments but involves the gene 55 mutant *ts*553, which is temperature sensitive for late gene expression (17).

First, we examined the temperature reversibility of *r* transcription in *ts*553-infected bacteria under various conditions. Cells were infected at the nonpermissive temperature of 43°C. Ten or 20 min after infection, the cultures were either treated or not treated with CM and shifted down to 30°C 30 s later. Figure 7 shows the results for *r* transcription of *ts*553 with or without an additional *ts* mutation in the *pol* gene. As already mentioned, *ts*P36 is temperature reversible with respect to viral DNA replication; it is also reversible with respect to *r* transcription (Fig. 7a). At 43°C, *ts*553 is not entirely blocked in *r* transcription but is highly reversible (Fig. 7b). In the double *ts* mutant, *ts*P36-*ts*553, *r* transcription at 43°C is completely blocked (Fig. 7c). Considerable recovery of *r* transcription can be achieved after shift-down into CM (Fig. 7c), although the recovery is incomplete or delayed relative to the recovery of the component single mutants (cf. Fig. 7a and b). Introducing the lig_{am} and $exof_{am}$ mutations does not fundamentally change the picture (Fig. 7d and e). The *r* transcription of the pol^-_{ts}-lig^-_{am}-$exof^-_{am}$ mutant is substantially reversible after 10 or 20 min at 43°C (Fig. 7d). When the *ts*553 mutation is also introduced, recovery of *r* transcription still occurs after shift to the permissive temperature for the *pol* and gp55 function, but recovery (shift-down without CM) is clearly impaired relative to the separate *ts*55 (Fig. 7b) and pol^-_{ts} (Fig. 7a) or pol^-_{ts}-lig^-_{am}-$exof^-_{am}$ (Fig. 7d) cases.

The second part of the experiment shows that in the absence of DNA replication, the reversibility of the *ts*553 mutation is essentially abolished and the capacity for subsequent late transcription is destroyed by prior incubation at 43°C. Holding pol^-_{am}-$gp55^-_{ts}$-infected cells at 43°C for 20 or even 10 min destroys the ability to recover *r* transcription even if protein synthesis is allowed (Fig. 8b). In contrast, 10 or even 20 min of preincubation at 43°C allows some *r* transcription to be retained by pol^-_{am}-infected cells (Fig. 8a). For the pol^-_{am}-lig^-_{am}-$exof^-_{am}$ system of mutants, the situation is identical: preincubation at 43°C for 10 or 20 min does not destroy the capacity for *r* transcription (Fig. 8c). Introduction of the *lig* and *exof* mutations makes the late transcription somewhat more temperature resistant (cf. Fig. 8a and c; see also 30). Nevertheless, the pol^-_{am}-lig^-_{am}-$exof^-_{am}$-$gp55^-_{ts}$ mutant loses its capacity for *r* transcription in the presence or absence of CM within 10 min at 43°C (Fig. 8d). That not only the temperature reversibility, but also the temperature sensitivity, of *r* transcription by the *ts*553 gene 55 product is affected by polymerase mutations is suggested by the simple experiment shown in Table 1. Here, *r* transcription by *E. coli* after infection with pol^-_{am}, pol^-_{ts}, and *ts*553 mutants and double mutants at 37°C has been tested. Under our

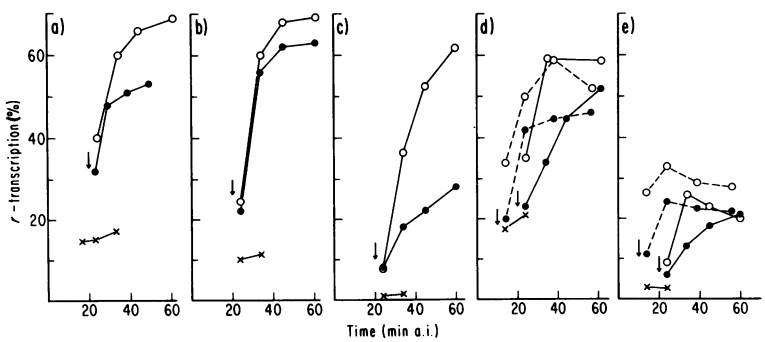

FIG. 7. *Effect of temperature shift-down (43 → 30°C) and CM on r transcription: the ts553 mutation. Cells infected at 43°C (multiplicities of 6 to 10, 21, and 20 for a–c, d, and e, respectively) were either treated or not treated with CM at 10 or 20 min after infection (a.i.) and shifted down to 30°C 0.5 min later (times indicated by arrows). RNA was labeled and analyzed as described in the legend to Fig. 1. Dotted lines refer to cells shifted down at 10 min a.i., solid lines to shift-down at 20 min a.i., and (×) to cells held continuously at 43°C and not treated with CM.* (○) *no CM addition;* (●) *CM addition. The labeling period of RNA was 2 min at 43°C or 3 min at 30°C. Time coordinates are the midpoints of these labeling intervals. (a) tsP36; (b) ts553; (c) tsP36-ts553; (d) tsP36-amH39X-amN130; (e) tsP36-amH39X-amN130-ts553.*

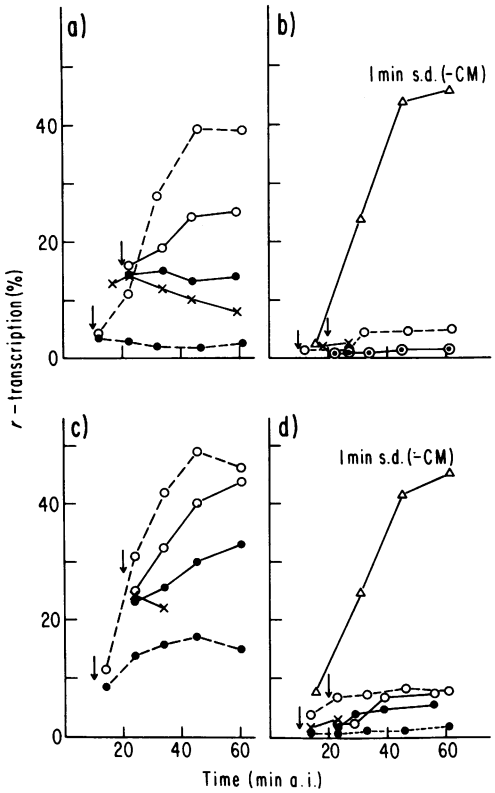

FIG. 8. *Effect of temperature shift-down (s.d.) and of CM on r transcription in the absence of replication: temperature sensitivity of ts553. Experiments were done as described in Fig. 7. Arrows indicate the time of temperature s.d. Symbols as in Fig. 7. In addition, the consequences of s.d. at 1 min after infection (a.i.) without CM (△) are shown in (b) and (d). (a) amE4301; (b) amE4301-ts553; (c) amE4301-amH39X-amN130; (d) amE4301-amH39X-amN130-ts553.*

conditions, DNA synthesis by *ts*P36 is strongly inhibited at 37°C. The percentage of late transcription at these late times after infection and high multiplicities is at a high relative level, as it also is for the pol^-_{am} mutant. *ts*553 is substantially temperature sensitive for late functions only at much higher temperatures (17; that is why the temperature shift experiments of Fig. 7 and 8 have been done at 43°C). For the pol^-_{am}-*ts*553 double mutant, *r* transcription at 37°C is almost abolished, and for the pol^-_{ts}-*ts*553 double mutant *r* transcription is severely depressed relative to the single mutants.

DISCUSSION

In these experiments, we have further examined the requirements for uncoupled, or replication-independent, T4 late transcription. The onset of replication-independent T4 late gene expression had previously been found to be significantly delayed (by about 10 min at 30°C) relative to the wild-type program of gene expression (30), yet it is known that the proteins for replication-coupled late gene expression can be accumulated in the absence of DNA replication (21; Fig. 7b). It is also known that, in the absence of DNA replication, breaks in parental DNA accumulate after infection approximately concurrently with the rise of late transcription. It is therefore conceivable that the delayed onset of uncoupled late gene expression might be determined by the time required for generating the competent DNA template (30). To test that notion, we have looked at the way in which the capacity for late gene expression is acquired (cf. 13, 25). Since that capacity is acquired in part through the synthesis of virus-coded proteins, the sensitivity of replication-uncoupled and -dependent late transcription to inhibition by CM has been compared. The result does not meet the simple expectation that has just been stated. Apparently, the required proteins for late T4 transcription accumulate later in the absence than in the presence of DNA replication (Fig. 1, 3, 4, 5). The temperature shift experiments of Fig. 6 further show that the difference is specific to whether replication does or does not occur.

We conclude that in some unspecified way the protein-nucleic acid interactions of replication-dependent and -independent late gene expression are different. We suspect that these differences arise because the replication-coupled and -independent processing of T4 DNA creates structures of the template that are not identical. Undoubtedly, a wide search among the many cellular and viral proteins that change the struc-

TABLE 1. *Effect of DNA replication on the temperature sensitivity of ts553*[a]

Phage	Time of RNA labeling (min a.i.)	*r* transcription (%)
T4D	30–32	61
	40–42	59
*ts*P36	30–32	45
	40–42	40
*ts*553	30–32	58
	40–42	58
*am*E4301	30–32	36
	40–42	35
*ts*P36-*ts*553	30–32	24
	40–42	14
*am*E4301-*ts*553	30–32	4.0
	40–42	3.6

[a] *E. coli* B^E were grown and infected with phage at 37°C at a multiplicity of 24. RNA was labeled as stated in column 2 and hybridized to *r* strands of T4 DNA. a.i., After infection.

ture of T4 DNA (see references 15 and 28 for recent reviews) would be important. We have barely begun such a search and have not so far found anything striking (data not shown). However, in trying to distinguish possible effects of late proteins on replication-independent late transcription, we have observed one very striking effect involving the functional stability of the gp55 (Fig. 7 and 8, Table 1). gp55 is an RNA polymerase-binding protein (11, 23), and no other interactions for this protein are yet known. The observations on the temperature sensitivity of the *ts*553 gp55 therefore imply that RNA polymerase interacts differently with late transcription units in replicating (I) and nonreplicating (II) competent T4 DNA. The gp55 (*ts*553) is more temperature sensitive in conjunction with late transcription units in DNA II than in DNA I (Table 1). Thermal inactivation of gp55 (*ts*553) with respect to function on DNA II not only is irreversible (Fig. 8b and d), but apparently prevents the interaction of subsequently synthesized, potentially active gp55 with the transcription apparatus on DNA II (Fig. 8b and d, no CM added).

Could the relationship of gp55 to DNA I and II alone account for the different times required to make late transcription of DNA I and II independent of further protein synthesis? That possibility is not excluded by the experiments presented here. However, there is much indirect evidence that many replication proteins interact with the process of transcribing late genes (30). It is not unlikely that some of these might also interact differently with DNA I and DNA II.

ACKNOWLEDGMENTS

This research was supported by a grant from the National Institute of General Medical Sciences (GM 18386). R. Wu gratefully acknowledges a postdoctoral fellowship from the American Cancer Society.

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