The role of bacteriophage T7 gene 2 protein in DNA replication

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ABSTRACT

The in vivo function of the gene 2 protein of bacteriophage T7 has been examined. The gene 2 protein appears to modulate the activity of the gene 3 endonuclease in order to prevent the premature degradation of any newly-formed DNA concatemers. This modulation is not however a direct interaction between the two proteins. In single-burst experiments rifamycin can substitute for the gene 2 protein, allowing formation of fast-sedimenting replicative DNA intermediates and progeny phage production. This suggests that the sole function of the gene 2 protein is the inhibition of the host RNA polymerase and that the latter enzyme directs or promotes the endonucleolytic action of the gene 3 protein.

INTRODUCTION

The replication cycle of bacteriophage T7 has been well documented (1,2,3,4,5). Unit-length, linear DNA is converted to short linear concatemers (5,6,7), which are then condensed to form "flower-structures": compact, fast-sedimenting DNA structures that contain many phage equivalents of DNA (2,4,5). The formation of these replicative intermediates requires the functions of T7 genes 4 (primase) and 5 (DNA polymerase) and has also been reported to require the presence of an active T7 gene 2 protein (8,9). The gene 3 protein, an endonuclease (4,10), has been implicated in the process of maturation of these concatemers (4,11) yielding molecules susceptible to the packaging process.

The gene 2 product (p2), originally referred to as the "I-protein" by Nakada (13), is a protein of 8,500 daltons (12) and has also been shown to inhibit E. coli RNA polymerase in vitro (12,13), thus showing involvement in the "host transcription shutoff" phenomenon exhibited by phage T7 (13). A requirement for an active p2 in the formation of replicative intermediates of T7 DNA has also been described (8). Amber mutants in gene 2, however, grow and produce plaques on a non-suppressing E. coli C strain (14). This result demonstrates that, at least in some bacterial strains,

p2 is dispensible for a productive infection. These studies were initiated in order to elucidate the apparent dual role of p2 (i.e. in the inhibition of host RNA polymerase and in the formation of replicative intermediates) and also to examine the course of productive infection of a T7 gene 2 amber mutant in an *E. coli* C sup^+ strain. The data presented here indicate that the sole function of p2 is to inhibit the host RNA polymerase from binding to DNA. Lack of inhibition of this enzyme results in premature breakdown of replicating DNA catalyzed by the gene 3 endonuclease.

MATERIALS AND METHODS

E. coli B (sup^+), *E. coli* O11' (supE_{44}) and all T7 strains were obtained from F.W. Studier. *E. coli* 7009 (tsnB) was obtained from M. Chamberlin and *E. coli* C (sup^+) from N. Godson. A spontaneous mutant of *E. coli* B was isolated which was resistant to the presence of 100 $\mu\text{g/ml}$ rifamycin. Phage stocks were grown and the double mutant T7 2am_{139} 3am_{29} was constructed by standard techniques (9, 15). In some single burst experiments 100 $\mu\text{g/ml}$ of rifamycin SV (Sigma) was added to the culture 9 minutes after T7 infection.

Replicating DNA was labelled by the incorporation of [^3H] deoxythymidine (^3H -dThd) for 30 seconds at 30°C. For kinetic analysis the culture was treated with trichloroacetic acid, and the acid insoluble radioactive material was quantitated by liquid scintillation spectrometry in a Packard 460C. The size distribution of replicating DNA was determined as previously described (16,17). Analysis of the DNA was by means of a neutral, 5-20% sucrose gradient with a shelf of CsCl/sucrose also as described (16,17). In all analyses the overall recovery of total radioactivity through the extraction and gradient analysis exceeded 80%. Optimal times of sampling for each gradient analysis were predetermined by measuring the kinetics of DNA synthesis. In general, aliquots were taken every two to three minutes throughout the period of thymidine incorporation, and the isolated DNA was subjected to neutral sucrose analysis. Analysis of infections of *E. coli* C strains showed that DNA synthesis persisted for a longer time period than the equivalent infections of *E. coli* B. Thus, the samples taken for gradient analysis extend over a greater range of times. For brevity, only two gradient profiles are shown for each experiment. These represent the size distribution of DNA at the time at which (I) the rate of DNA synthesis is approaching a maximum and (II) just prior to cessation of DNA synthesis. In Figure 1 the kinetics of DNA synthesis are

Figure 1. Sedimentation analysis of pulse-labelled DNA from T7 gene 2am₁₃₉-infected E. coli B (panel A) or E. coli C (panel B). Bacteria were grown at 30°C in M9 salts medium (9) supplemented with 0.2% glucose, 20 µg/ml thymine, and 2 µg/ml thiamine to a density of 2×10^8 cells/ml and were infected with phage at a multiplicity of 10. Inserted figures show the rates of incorporation of [³H] deoxythymidine (³H-dThd) into DNA at various times during an infection. At 12 and 20 minutes post-infection (panels AI and AII, respectively) and at 12 and 34 minutes post-infection (panels BI and BII, respectively). 2.0 ml aliquots were removed from the cultures and incubated for 30 sec with 2.0 µCi of ³H-dThd. DNA was analyzed as described in Materials and Methods. The arrows indicate the sedimentation position of an internal marker of unit-length T7 DNA. The direction of sedimentation is from right to left. Total [³H] cpm recovered from the gradients are as follows: AI. 33,360 cpm, AII. 6,270 cpm, BI. 82,250 cpm, and BII. 13,430 cpm.

shown as inserts to the main figure. The arrows represent the two times from which the gradient profiles are obtained. The other figures are derived from similar studies of the kinetics of ³H-dThd incorporation. In a T7⁺-infection of a permissive host, these times would show in I the maximal presence of fast-sedimenting DNA (though some infections by mutant T7 were delayed by 2 to 3 minutes), and in II its subsequent maturation (16, 17). For a nonproductive infection the analysis of intermediate time points yielded gradient profiles not significantly different to those shown. Similar analyses in productive infections (i.e., T7 2am₁₃₉ grown in E. coli C and the experiments involving rifamycin) yielded a time course for the

formation and maturation of fast-sedimenting DNA equivalent to that of T7⁺ infection (16,17). Times at which aliquots for sucrose gradient analysis were taken are given in the Figure legends.

RESULTS

Studier (14) has reported that T7 amber mutants defective in gene 2 plate on *E. coli* C (sup⁺) with essentially normal efficiency with respect to *E. coli* O11' (supE₄₄). Thus, T7 p2 is nonessential for growth on C strains. To determine whether any differences arise in the production of fast-sedimenting, complex, DNA replication intermediates by a T7 gene 2_{am139} infection of *E. coli* B or *E. coli* C, ³H-labelled intracellular replicating DNA was isolated from these cultures and subjected to neutral sucrose gradient centrifugation. The sedimentation patterns of DNA isolated from 2_{am139}-infected *E. coli* B and *E. coli* C show striking differences. In agreement with the data of Center (8), no fast-sedimenting DNA replication intermediates can be detected in *E. coli* B infected with the 2_{am} (Fig. 1, panel A); however, these intermediates are found following infection of *E. coli* C (Fig. 1, panel B). Such a result could be predicted since T7 2_{am139} productively infects *E. coli* C (sup⁺) strains (14). In addition the rates of ³H-dThd incorporation are also effectively those of T7⁺ infection in *E. coli* C.

In contrast to the results for 2_{am139}-infected *E. coli* B or C strains, infection of either of these strains by T7 3_{am29} leads to the slow formation (relative to T7⁺) of fast-sedimenting replicative intermediates. These intermediates are not, however, subsequently matured to unit-length DNA (Fig. 2), but remain as very high molecular weight species. This is in agreement with the results of Paetkau *et. al.* (16), who showed that the gene 3 endonuclease was required for the processing of replicating DNA. Figure 2 also shows that the *E. coli* C strain used in these experiments is, in fact, non-suppressing because no maturation of fast-sedimenting DNA occurs, thus confirming that p2 is nonessential in infections of *E. coli* C and that the results shown in Figure 1, panel B are not due to suppression of the amber mutation.

It was previously reported that T7 p2 was required for the formation of replicative intermediates (8). In view of the results shown in Figure 1, this cannot in all instances be the case. To further investigate the role of p2 in T7 replication, two formal possibilities were considered: p2 is directly involved in catalyzing the formation of fast-sedimenting

Figure 2. Neutral sucrose gradient sedimentation analysis of pulse-labelled DNA from T7 gene $3am_{29}$ -infected *E. coli* B (panel A) or *E. coli* C (panel B).

The conditions of the experiment are identical to those of Fig. 1. Total [3H] cpm recovered from the gradients are as follows: AI. 40,400 cpm, AII. 17,090 cpm, BI. 25,085 cpm, and BII. 27,540 cpm.

intermediates; in C strains of *E. coli* a host protein directly substitutes for the phage gene product; alternatively, p2 could be involved in maintaining the integrity of replicating DNA structures; in C strains this function is supplied by a host protein. Evidence that indirectly supports this latter possibility was first provided by Center (8), who showed that the intracellular phage DNA resulting from a T7 gene 2^- infection of *E. coli* B was shorter than unit-length. It appeared likely that the breakdown of T7 DNA in a non-permissive T7 gene 2^- infection was due to the presence of an active gene 3 endonuclease. Double amber mutants of T7 were, therefore, constructed which lacked both the gene 2 and gene 3 proteins (T7 $2am_{139} 3am_{29}$, referred to as T7 $_{2-3-}$). Intracellular DNA was thus isolated from T7 $_{2-3-}$ infected *E. coli* B or *E. coli* C and subjected to neutral sucrose gradient analysis. These data are given in Figure 3 and clearly show the stable production of fast-sedimenting replicating DNA. Thus, T7 p2 is not involved in the formation of large replicative DNA; however, at least in *E. coli* B strains, it is required for modulation of

Figure 3. Neutral sucrose gradient sedimentation analysis of pulse-labelled DNA from T7₂₋₃-infected *E. coli* B (panel A) or *E. coli* C (panel B).

The conditions of the experiment are identical to those of Fig. 1. Total [³H] cpm recovered from the gradients are as follows: AI. 18,390 cpm, AII. 12,290 cpm, BI. 16,455 cpm, and BII. 3,235 cpm.

gene 3 endonuclease activity.

T7 p2 is a small polypeptide of 8500 daltons. It has been clearly shown by Nakada and coworkers (13) that this protein inhibits *E. coli* RNA polymerase and is involved in the shut-off of host-mediated transcription. This result has recently been confirmed and extended by DeWynngaert and Hinkle (12), who have also shown that an altered RNA polymerase (*E. coli* 7009) is resistant to p2-mediated inhibition. Furthermore, LeClerc and Richardson (18) showed that the requirement for p2 in an *in vitro* packaging reaction could be obviated by the addition of rifampicin to the reaction mixture. Experiments were, therefore, undertaken to determine whether inhibition of RNA polymerase would render p2 nonessential *in vivo*. Growth of T7 requires the transcription of the leftmost 20% of the T7 genome of the host RNA polymerase; this activity is shut down shortly after infection at approximately the time of onset of DNA synthesis. Therefore, single burst experiments were performed adding rifamycin SV to the culture at about 9 minutes after infection. These results are shown in Table 1. A productive infection by T7 2am₁₃₉ clearly ensues when *E.*

Table 1. Burst sizes of T7 gene 2am₁₃₉ grown in *E. coli* B, B(Rif^R), 7009, C, and O11' with or without rifamycin SV treatment^a.

	B		B(Rif ^R)		7009		C		O11'	
+ Rifamycin	+	-	+	-	+	-	+	-	+	-
	60.0	1.4	1.5	1.6	44.0	2.1	74.4	21.2	37.8	51.8

^aRifamycin SV at 100 µg/ml was added to cultures at 9 minutes after infection with 2am₁₃₉. Phage titers were measured by plating on *E. coli* O11' at 30°C in the absence of rifamycin. Units are progeny phage/cell.

coli B is treated with rifamycin, but not when a spontaneous rifamycin-resistant derivative of *E. coli* B is employed. The small burst of phage seen in untreated *E. coli* B cultures is not sufficient for the generation of visible plaques under normal plating conditions. *E. coli* 7009, which harbors an altered RNA polymerase that is resistant to p2 inhibition (12) and is thus restrictive for T7 growth (19), irrespective of whether p2 is present or not, also becomes susceptible to T7 2am₁₃₉ infection when treated with rifamycin during the eclipse phase of growth.

In the absence of rifamycin, *E. coli* C (sup⁺) supports the growth of T7 2am₁₃₉ but yields somewhat smaller plaques than does a wild type T7 infection (14), an observation which can be partly explained by a reduced burst (Table 1). The growth of T7 2am₁₃₉ on *E. coli* C (sup⁺) can also be enhanced by the addition of rifamycin, implying that the RNA polymerase in this strain also is still somewhat inhibitory for T7 growth. Conversely, in an infection where T7 p2 is active (*E. coli* O11', Table 1 and infection of *E. coli* B or C by T7⁺, data not shown), there is no effect due to the addition of rifamycin.

To confirm that the replicative pathway of T7 occurs by the normal pathway (16) in rifamycin-treated cultures, intracellular DNA was isolated at various times and subjected to sedimentation through neutral sucrose. The results shown for T7 2am₁₃₉ infection of *E. coli* B (Fig. 4) and *E. coli* 7009 (Fig. 5, panel A) indicate that DNA synthesis appears normal if rifamycin is added to the infected cultures 9 minutes after infection. This is in contrast with the data obtained in the absence of rifamycin for *E. coli* B (Fig. 1) or for *E. coli* 7009 (Ref. 20 and Fig. 5, panel B).

DISCUSSION

The gene 2 protein of bacteriophage T7 has been implicated as functioning in phage DNA replication (9,21). This protein has been shown to be

Figure 4. Neutral sucrose gradient sedimentation analysis of pulse-labelled DNA from T7 gene $2am_{139}$ -infected *E. coli* B treated with 100 μ g/ml rifamycin SV at 9 min after infection.

Conditions of the experiment are otherwise as those of Fig. 1 except that samples for analysis were taken at 16 and 30 minutes post-infection. Total [3 H] cpm recovered from the gradients are as follows: I. 49,310 cpm, and II. 26,630 cpm.

Figure 5. Neutral sucrose gradient sedimentation analysis of pulse-labelled DNA from T7 gene $2am_{139}$ -infected *E. coli* 7009 treated with 100 μ g/ml rifamycin SV at 9 min after infection (panel A) or without treatment (panel B).

Conditions of the experiment are otherwise as those of Fig. 1 except that samples for analysis were taken at 18 and 30 minutes post-infection (panel AI, BI and AII, BII, respectively). Total [3 H] cpm recovered from the gradients are as follows: AI. 30,870 cpm, AII. 21,430 cpm, BI. 11,385 cpm, and BII. 22,540 cpm.

identical to the "I-protein", which is involved in the "host shut-off" function of T7 (13). The mechanism of inhibition has recently been shown to be due to the binding of p2 to RNA polymerase (12). This is a negative role for p2 in that it prevents inhibition of phage development by the host RNA polymerase. A positive role for this protein was suggested by Center (8), who implicated p2 as a requirement for formation of replicative DNA intermediates. It was further shown by LeClerc and Richardson (18) that p2 is an essential component for in vitro packaging of T7 DNA, and they purified the protein on that basis. They further showed that the requirement for p2 could be bypassed by adding rifampicin, which inhibits E. coli RNA polymerase, to the in vitro reaction mixture. The function of p2 in the packaging system is also, therefore, one of RNA polymerase inhibition.

In an infection of C strains of E. coli, T7 p2 is nonessential (14). We have shown that, although the burst size of T7 Δ_{139} infection of C strains is somewhat reduced relative to a wild type infection, DNA synthesis proceeds normally, albeit somewhat temporally delayed (Fig. 1, panel B insert), and that replicative intermediates are formed and processed normally. Center (8) has reported that intracellular DNA resulting from a T7 2^- infection of E. coli B is less than unit-length, a result confirmed recently (20). It is unclear why this is not seen in the experiment depicted in Figure 1 or in comparable experiments but may be due to a lack of resolution at the top of the sucrose gradient.

We have also presented evidence that p2 is not actively involved in the formation of replicative DNA in B strains of E. coli, but that its function is rather to prevent premature breakdown of replicating DNA. This nucleolytic breakdown of DNA is catalyzed by the gene 3 endonuclease. In the absence of both p3 and p2, replicative DNA is formed normally but remains stable. Thus, the formation of both linear concatemers and the fast-sedimenting intermediates of Paetkau et. al. (16) appear to require only the functions of genes 4 and 5.

The modulation of endonucleolytic activity of p3 by p2 does not, however, appear to be a direct reaction. This conclusion is based on the fact that addition of rifamycin to an infected culture leads to the normal replication of phage DNA and to progeny phage production (Figs. 4 and 5, Table 1). This result occurs in the presence or absence of active p2 and also with E. coli strains harboring an altered RNA polymerase resistant to p2 inhibition. The effect of rifamycin is, however, confined to inhibition

of RNA polymerase itself because those strains containing a drug resistant enzyme require the presence of active p2 for productive infection (Table 1 and unpublished data using T7⁺). Furthermore, degradation of host DNA (catalyzed by gene 3 endonuclease) occurs normally in the presence of rifamycin (data not shown), indicating that the drug has no effect on the activity of the endonuclease itself.

The following model has been developed to explain the function of p2 in T7 growth. Immediately after infection the left-most 20% of the T7 genome is transcribed by E. coli RNA polymerase. One of the products of this transcription is a T7-specific RNA polymerase that transcribes the remainder of the genome (21,22,23). Inhibition of host-catalyzed transcription is normally achieved largely by means of p2, which prevents further binding of the host RNA polymerase to promoter sites on the DNA (12). In the absence of p2, binding of RNA polymerase persists, which could provide a recognition site for the gene 3 endonuclease, which then cleaves the DNA. This would explain the predominant cutting of the DNA at the left-most end of the genome (20), where the strong promoters for E. coli RNA polymerase occur. The requirement for active p2 for productive growth can be obviated by the addition of rifamycin to cells. Rifamycin binds to the β subunit of RNA polymerase but does not prevent the enzyme from binding to DNA (24). It appears reasonable, therefore, that the gene 3 endonuclease can also recognize the β subunit, and, if the RNA polymerase is bound to T7 DNA, uncontrolled cleavage of that DNA occurs. In a wild type host, rifamycin can effectively compete with the gene 3 endonuclease for a common binding site on RNA polymerase. This competition is abolished if rifamycin cannot bind to the enzyme, as in a rpoB mutant that is rifamycin resistant (Table 1). Other rpoB mutants exist, e.g. E. coli 7009 (tsnB) and BR3 (12,20) which are rifamycin sensitive but which are resistant to the inhibitory action of p2 and are restrictive for T7 growth. A T7 infection of at least one of them (7009) can be made productive by the addition of the drug during the infection.

This model predicts that E. coli C strains harbor an altered β subunit of RNA polymerase such that the p3 endonuclease of T7 recognizes it at only a much lower efficiency than a B or K12 enzyme. Infection by T7 gene 2am mutants in C strains is productive, as premature cleavage of the T7 DNA does not occur. The long-standing problem of how the gene 3 endonuclease activity of T7 is moderated so that it specifically degrades only host and not T7 DNA, a property which it shows in vitro (25), may be at least partly

resolved by an analysis of its interaction with RNA polymerase and of the latter's control by the gene 2 protein.

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Abbreviations: p2, gene 2 protein; p3, gene 3 protein; dThd, deoxythymidine

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