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Inadequate inhibition of host RNA polymerase restricts T7 bacteriophage growth on hosts overexpressing *udk*

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Summary

Overexpression of *udk*, an *Escherichia coli* gene encoding a uridine/cytidine kinase, interferes with T7 bacteriophage growth. We show here that inhibition of T7 phage growth by *udk* overexpression can be overcome by inhibition of host RNA polymerase. Overexpression of gene *2*, whose product inhibits host RNA polymerase, restores T7 phage growth on hosts overexpressing *udk*. In addition, rifampicin, an inhibitor of host RNA polymerase, restores the burst size of T7 phage on *udk*-overexpressing hosts to normal. In agreement with these findings, suppressor mutants that overcome the inhibition arising from *udk* overexpression gain the ability to grow on hosts that are resistant to inhibition of RNA polymerase by gene 2 protein, and suppressor mutants that overcome a lack of gene 2 protein gain the ability to grow on hosts that overexpress *udk*. Mutations that eliminate or weaken strong promoters for host RNA polymerase in T7 DNA, and mutations in T7 gene *3.5* that affect its interaction with T7 RNA polymerase, also reduce the interference with T7 growth by host RNA polymerase. We propose a general model for the requirement of host RNA polymerase inhibition.

Introduction

Phage biology has been a focus of numerous studies for the last century. The relative simplicity of phage, their fast growth rate and the ease of their genetic manipulations as compared with eukaryotic viruses, make them an attractive target for research. Yet, not a single phage has been fully characterized. For example, approximately half of the 56 genes of the very well-studied bacteriophage T7 have no known function. In addition, the function of many genetic elements, such as the repetitive direct repeats in both ends of the T7 genome, remains unknown (Dunn and Studier, 1983). Significant issues such as what molecular mechanisms control the production of phage components during the phage life cycle, and what molecular mechanisms dictate the timing of each step of the phage infection remain unanswered. In this report, we aim to elucidate the mechanism by which a recently identified host gene, *udk*, interferes with T7 phage growth, and to build a model concerning the requirement for host RNA polymerase inhibition at late stages of T7 phage growth.

We recently used a system-wide approach to determine the genetic relations between T7 phage and its host, *Escherichia coli* (Qimron *et al.*, 2006). We identified four host genes that inhibit T7 growth when overexpressed. One of the identified genes, *rcsA*, activates capsule synthesis genes and induces capsule formation (Gottesman and Stout, 1991). The capsule formation, in turn, prevents T7 growth, presumably by masking the lipopolysaccharide that serves as a ligand for the receptor of T7 phage. The mechanism by which two of the other genes, *hsdR* and *dgt*, restrict T7 growth were previously characterized (Studier, 1975; Mark and Studier, 1981; Beauchamp and Richardson, 1988). *hsdR* encodes the restriction subunit of EcoK1 that is inhibited upon T7 phage infection by the binding of gene product (gp) 0.3. *dgt* encodes a nucleotide triphosphatase that hydrolyses dGTP and when overexpressed dramatically reduces the dGTP pool. T7 gp1.2 can overcome the overexpression of *dgt* by binding to and inhibiting its product. The fourth identified gene, *udk*, encodes uridine/cytidine kinase, an enzyme that converts uridine and cytidine into their respective ribonucleoside monophosphates, UMP and CMP. The most efficient phosphate donors for this reaction are GTP and dGTP. The *udk* gene is nonessential to *E. coli*, and is part of the salvage pathway for pyrimidine ribonucleotides (Keseler *et al.*, 2005). The mechanism by which overexpression of this gene restricts growth of the T7 phage is the subject of this study.

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We previously showed that *udk* overexpression only inhibits T7 phage growth when the T7 gp0.7 is inactive (Qimron *et al*., 2006). Gp0.7 is a 43 kDa protein kinase that phosphorylates various host proteins including ribosome components, ribonuclease III and E, and the β and β′ subunits of *E. coli* RNA polymerase to create cellular conditions favourable for T7 phage growth; i.e. stable transcripts, preference towards phage protein translation, and reduced host RNA polymerase activity (Molineux, 2005). Gene *0.7* is not essential for T7 phage growth on fast-growing hosts, but becomes essential under suboptimal conditions such as elevated temperature, limited nutrients or the presence of phage-inhibitory plasmids (Hirsch-Kauffmann *et al*., 1975; Gomez and Nualart, 1977). Gp0.7 phosphorylates a specific threonine (T1068) of the host RNA polymerase β′ subunit leading to an increase in transcription termination (Severinova and Severinov, 2006). This role of gp0.7 in host RNA polymerase inhibition is shared with the T7 phage-encoded gp2 that inhibits host RNA polymerase by binding to the β′ subunit and thus preventing recognition of host promoters by the sigma70 subunit (DeWyngaert and Hinkle, 1979; Nechaev and Severinov, 1999).

Beside *udk*-overexpressing hosts, two other *E. coli* strains, BR3 and Y49, render gene *0.7* essential for T7 phage growth (Studier, 1973; Shanblatt and Nakada, 1982). These hosts encode a mutant host RNA polymerase (rpoC-E1158K substitution) that is resistant to gp2 inhibition and therefore require the inhibitory activity of gp0.7 on the host RNA polymerase for phage T7 growth (Nechaev and Severinov, 1999). We show here that overexpression of *udk* leads to an inability of T7 gp2 to adequately inhibit the host RNA polymerase, thus necessitating the presence of gp0.7. Thus, *E. coli* strains overexpressing *udk* have the same phenotype with regard to gene *0.7* as do strains BR3 and Y49. Mutations in gene *2* that increase the inhibition of host RNA polymerase by gp2 overcome the inhibition of T7 growth in cells overexpressing *udk*. Interestingly, mutations in the very strong promoters for *E. coli* RNA polymerase as well as mutation in gene *3.5*, which encodes T7 lysozyme, also can suppress the inhibition of phage growth by overexpressed *udk*. Gp3.5 is known to interact with T7 RNA polymerase to affect its activity and modulate its pauses at certain sites (Zhang and Studier, 2004). These results, taken together, allow us to propose a model to explain the necessity for inhibition of the host RNA polymerase during T7 infection.

Results

Rationale

Escherichia coli K-12, B or C strains overexpressing *udk* restrict growth of T7 phage lacking gene *0.7* (T7Δ*0.7*) from 30- to 100-fold (Qimron *et al*., 2006, Table 1); the growth of wild-type T7 phage on these strains is only slightly affected (Table 1). A similar phenotype in which wt-T7 phage grow albeit poorly on *E. coli* strains while T7Δ*0.7* phage are severely impaired has been observed with strains BR3 (Studier, 1973) and Y49 (Shanblatt and Nakada, 1982). These two strains contain a mutation in the host RNA polymerase that renders it resistant to inhibition by gp2. Gp2 binds to the β′ subunit of RNA polymerase and renders it inactive (Nechaev and Severinov, 1999). Gp0.7 is a protein kinase that inhibits the host RNA polymerase and is essential for T7 phage to grow on *E. coli* BR3 and Y49 whose RNA polymerase is not sufficiently inactivated by gp2 of the phage. It is thus appealing to speculate that the inability of T7Δ*0.7* to grow on *E. coli* strains overexpressing *udk* is likewise a consequence of the inability of gp2 to adequately inhibit the host RNA polymerase.

Table 1. Plating efficiencies of T7 phage on different *E. coli* strains.

E. coli strain[a]	wt-T7[b]	T7Δ*0.7*[b]
K12; induced	0.38 ± 0.02[c]	< 0.01
K12; uninduced	1	1
B; induced	0.51 ± 0.20[c]	< 0.01
B; uninduced	1	1
C; induced	0.13 ± 0.06	0.03 ± 0.01
C; uninduced	1	1

a. All indicated strains are transformed with a plasmid encoding *udk* under the control of the lac promoter. Induced cells were grown for 45 min in LB medium containing 1 mM IPTG, prior to plating with the indicated phage, whereas uninduced cells were grown in LB medium in the absence of IPTG.
b. Numbers represent the EOP of the indicated phage on the indicated cells relative to the EOP on the uninduced cells of the same strain. Average and SD of at least three experiments are shown.
c. Small plaques.

T7 *phage suppressors that permit growth on cells overexpressing* udk

To test the hypothesis above, we carried out a suppressor screen to identify mutations in T7 phage that can overcome the inhibition of T7Δ*0.7* growth arising from *udk* overexpression. We anticipated that suppressor mutants would arise with mutations enhancing gp2 activity. For example, *E. coli tsnB* having a RNA polymerase that is resistant to inhibition by gp2 cannot support the growth of phage T7, but mutations in gene *2* can allow for T7 infection and growth (suppressors termed T7β) presumably by restoring the ability of the gp2 to inhibit RNA polymerase (Schmitt *et al*., 1987). To select for suppressors, we isolated plaques of T7Δ*0.7* that were able to grow on hosts overexpressing *udk*. Phages obtained from the infection were used to infect fresh cultures of *udk*-overexpressing hosts for three cycles. In order to test whether the isolated suppressors can compensate for the lack of inhibition of host RNA polymerase by gp2, we examined their growth

Table 2. Mutations in T7Δ*0.7* suppressors that overcome *udk* overexpression.

Suppressors	Gene affected	Mutation(s)	Residue substitution(s)
udk-overexpressing and BR3 hosts[a]	Gene *2*	8972 T → A/8978 A → G	H25R/F27Y
		8986 C → T	P30S
		8998 G → A	E34K
		9041 T → C	V48A
		9044 C → G	R49P
	Gene *3.5*	10797 C → T	R30C
		10807 G → T	R33L
		10809 C → A	Q34K
		10887 G → A	R60Q
udk-overexpressing host only	Gene *2*	9031 T → C	W45R
		9031 T → G	W45G
		9031 T → G	W45G
		9050 G → A	G51D
		9054 T → C	F52S

a. Suppressors to BR3 are plated with approximately 1000-fold better efficiency than T7Δ*0.7* on *E. coli* BR3, but plate with approximately 1000-fold lower efficiency as compared with their EOP measured on *E. coli* B.

on *E. coli* BR3. Approximately 20% of these suppressors produced plaques on *E. coli* BR3. The efficiency of plating (EOP) of these suppressors on *E. coli* BR3 was still much less (approximately 1000-fold) than on B strain; nevertheless, it was 1000-fold greater than was the parental T7Δ*0.7* on BR3. We selected nine of these suppressor phages and another five suppressor phages that were unable to produce plaques on *E. coli* BR3 for further study (Table 2). Sequence analysis of the 14 selected suppressor phage reveals that 13 have a single amino acid residue change in either gp2 or gp3.5, while the other has two amino acid residue changes. The mutations in gene *3.5* are all found in the group of suppressors that both allow T7 to grow on cells overexpressing *udk* and on *E. coli* BR3. The mutations in gene *3.5* are not surprising in that the gp3.5 is known to bind to T7 RNA polymerase and affect its promoter selectivity. Thus, all of the suppressor mutations appear to affect transcription either by the host or by phage RNA polymerase.

Suppressor mutations are dispersed throughout gene *2* (Table 2). We assume that the gp2 encoded by these suppressors are more potent than the wt-gp2. This assumption is supported by the fact that 20% of the gene *2* mutants also suppress for a phenotype related to the lack of host RNA polymerase inhibition (*E. coli* BR3). In fact, two of the suppressor mutations we identified (gp2-F27Y, gp2-W45R/G) are identical to those described previously for T7β phage that were isolated based on their ability to suppress for the decreased ability of wt-gp2 to inhibit the host RNA polymerase (Schmitt *et al.*, 1987). Furthermore, as will be shown in the next sections, overexpression of wt-gp2 can overcome the inhibition of T7 phage growth imposed by overexpression of *udk*, suggesting that the identified suppressors share a similar mechanism, decreasing the amount of host RNA polymerase that does not have gp2 bound to it.

Because the T7 suppressors identified above were derived from a T7 phage lacking gene *0.7*, and therefore have reduced ability to inhibit host RNA polymerase, it is possible that the suppressor phenotype compensates for reduction in host RNA polymerase inhibition that is derived from the lack of gene *0.7*, and not to overcoming the *udk* overexpression inhibition of phage growth. To rule out this possibility, we isolated a suppressor mutant for the phenotype arising from the *udk* overexpression by starting with a wt-T7 strain, which encodes gene *0.7*, and thus is not deficient in host RNA polymerase inhibition. Although wt-T7 will grow on the host overexpressing *udk*, the slight growth inhibition gives selective advantage to suppressors overcoming the phenotype arising from *udk* overexpression. If indeed the *udk* inhibition of phage growth and not the lack of gene *0.7* is responsible for suppressors acquiring mutations that increase the host RNA polymerase inhibition, then we expected to also identify mutations in either gene *2* or gene *3.5* on a suppressor T7 that does encode gene *0.7*. As expected, the mutant isolated had a mutation in gene *2* (gp2-F52S), indicating that the other suppressors identified likely evolved to compensate for the phenotype arising from overexpression of *udk* and not because of the lack of gene *0.7*.

The more intriguing suppressor mutants are those with mutations in gene *3.5* (Table 2). Most of the substitutions, or variants thereof, that we have identified in gp3.5 had been previously identified as substitutions that render the T7 RNA polymerase less sensitive to inhibition by T7 gp3.5 (Cheng *et al.*, 1994). Residues R30, R33 and Q34 of gp3.5 were all identified in that screen as well as G59 that is adjacent to residue R60 mutated in our screen. As all of the gene *3.5* suppressors also grow on *E. coli* BR3, it seems likely that they suppress for global defects in the ability of T7 to inhibit the

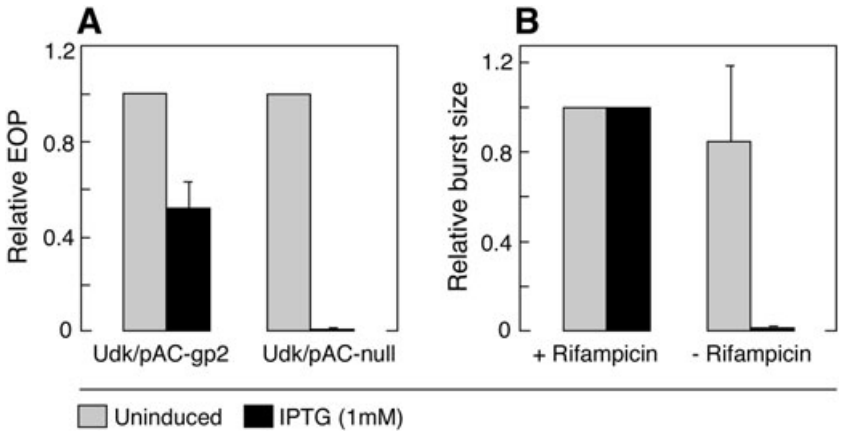

Fig. 1. Effect of exogenous RNA polymerase inhibition on T7 phage growth on cells overexpressing *udk*.
A. *E. coli* K-12 harbouring the *udk* plasmid were aerated for 45 min either with 1 mM IPTG or in its absence (uninduced). Cells carried a plasmid encoding gene *2* (pAC-gp2) or a control plasmid (pAC-null). Approximately 100 PFU of T7Δ*0.7* were mixed with the cells in top agar, and overlaid on LB-agar plates. Plaques were counted after 5 h. Relative EOP indicates the number of plaques of T7Δ*0.7* on the induced cultures compared with the number of plaques on the uninduced cultures.
B. *E. coli* K-12 harbouring the *udk* plasmid were aerated for 60 min either with 1 mM IPTG or in its absence (uninduced). Cells were infected at an OD_{600} of 0.1 (5×10^7 cfu ml^{-1}) with T7Δ*0.7* at a Moi of 5. After 2 min 0.5 ml of the cells was centrifuged for 1 min at 13 000 r.p.m., the supernatant was removed, and the pellets were resuspended in 0.5 ml of fresh medium. After 9 min, rifampicin in DMSO at a final concentration of 200 μg ml^{-1} (+ rifampicin) or DMSO alone (– rifampicin) were added to the cells. Cells were further incubated at 37°C for 1 h and the PFU was measured by plating dilutions of the lysates on a bacterial lawn. Relative burst size corresponds to the burst size on rifampicin-treated cultures relative to the burst size on untreated cultures. All experiments were carried out at least twice; the average and SD are shown.

host RNA polymerase. In the next sections we show that gene *3.5* suppressors can overcome the lack of gene *2* as well as the combined lack of genes *0.7* and *2*.

Exogenous inhibition of host RNA polymerase restores T7 phage growth on udk*-overexpressing cells*

To further test whether overexpression of *udk* results in inadequate inhibition of host RNA polymerase, we examined the growth of T7Δ*0.7* when gene 2 is overexpressed. Hosts overexpressing *udk* were transformed with either gene *2* expressing plasmid under a T7 promoter (pAC-gp2) or with a control plasmid (pAC-null). Both plasmids have an origin of replication that is compatible with the *udk* encoding plasmid. The indicated cells were infected with T7Δ*0.7* and the EOP was measured. As can be seen in Fig. 1A, cells harbouring the control pAC-null plasmid, restrict T7Δ*0.7* growth 100-fold upon *udk* overexpression (1 mM IPTG), as compared with cells harbouring the control plasmid that does not overexpress *udk* (uninduced). In contrast, cells harbouring the pAC-gp2 plasmid restrict T7Δ*0.7* phage growth only about twofold upon *udk* overexpression (1 mM IPTG) as compared with cells that do not overexpress *udk* (uninduced). Thus, enhancing the inhibition of the host RNA polymerase in *udk*-overexpressing host by overexpression of gene *2* from a plasmid restores T7Δ*0.7* phage growth, and suggests that T7Δ*0.7* is restricted from growing on hosts overexpressing *udk* because of inefficient inhibition of the host RNA polymerase.

One concern is that the multicopy presence of gene *2* prior to infection may reduce the *udk* accumulation in the host. For example, host RNA polymerase might be inhibited because of leakiness of gene *2* expression, and therefore the results could represent only the reduction of *udk* expression and not the inhibition of the host RNA polymerase by gene *2*. However, gene *2* is under control of the T7 promoter, and is only overexpressed upon T7 phage infection and synthesis of T7 RNA polymerase. Nevertheless, we set up a more conclusive experiment in which the host RNA polymerase is unquestionably inhibited only after infection. In this experiment, cells are infected at a high multiplicity of infection (Moi), the unadsorbed phages are removed, and rifampicin or dimethylsulphoxide (DMSO) (control) is added (Fig. 1B). The addition of rifampicin after infection selectively inhibits the host RNA polymerase and can thus substitute for gp2 (LeClerc and Richardson, 1979). The burst size is calculated by counting the progeny phage produced and dividing by the number of infected bacteria. When no rifampicin is added, the burst size of T7Δ*0.7* is approximately 100-fold lower on cells overexpressing *udk* (1 mM IPTG) as compared with cells not expressing *udk* (uninduced). The addition of rifampicin restores burst size on *udk*-overexpressing-hosts to nearly the burst size of normal hosts (uninduced). These results, taken together, demonstrate that the sole reason T7Δ*0.7*

cannot grow on *udk*-overexpressing hosts is insufficient inhibition of the host RNA polymerase. The results suggest that the phage gp2 alone is not sufficient for host RNA polymerase inhibition on hosts overexpressing *udk*. Under these conditions the phage gp0.7 becomes essential, presumably because of its phosphorylation of the β′ subunit of the host RNA polymerase that renders it less active (Severinova and Severinov, 2006).

Suppressors overcoming the lack of host RNA polymerase inhibition also overcome inhibition by overexpression of udk

We have shown that *udk* overexpression prevents T7Δ*0.7* phage growth because of insufficient inhibition of host RNA polymerase. Mutations in the phage genome that enhance host RNA polymerase inhibition, as well as the addition of exogenous host RNA polymerase inhibitors, can overcome this effect. Thus, it is likely that suppressors that compensate for the lack of gene *2* and hence overcome the requirement for host RNA polymerase inhibition would be able to suppress for *udk* overexpression as well. To test this hypothesis, it is necessary to construct T7 phage lacking both gene *0.7* and gene *2*. Gene *2* must be deleted in order to isolate suppressor mutants that grow without gene *2*, and gene *0.7* must be deleted if these mutants are to be tested on *udk*-overexpressing-hosts. If gene *0.7* is not deleted, then its expression would mask the inhibition arising from *udk* overexpression. Consequently, gene *2* suppressor mutants expressing gene *0.7* would be able to grow on hosts overexpressing *udk* regardless of the suppression mutation. In order to construct the double deletion mutant, T7Δ*0.7*Δ*2*, we made use of two selection markers. The design of such a phage was enabled by the recent finding that the *E. coli* gene *cmk* is essential for T7 phage growth (Qimron *et al*., 2006). T7Δ*0.7*Δ*2* construction is described in *Experimental procedures*. In order to select for suppressors that can overcome the lack of gene *2*, T7Δ*0.7*Δ*2* phage was plated on *E. coli* B cells and 11 suppressor mutants were isolated and tested for growth on cells overexpressing *udk*. We arbitrarily chose to test seven of them and all seven of the T7Δ*0.7*Δ*2* suppressor mutants could also suppress the phenotype arising from the overexpression of *udk* (Fig. 2). The EOP of these suppressors on K-12 AG1 cells overexpressing *udk* is significantly higher than that determined with the parental T7Δ*0.7*Δ*2* and of that of T7Δ*0.7* alone. In fact their EOPs are comparable with that of wt-T7 on hosts overexpressing *udk*. These results unequivocally show that suppressor mutations overcoming the lack of host RNA polymerase inhibition can also overcome inhibition observed by overexpression of *udk*.

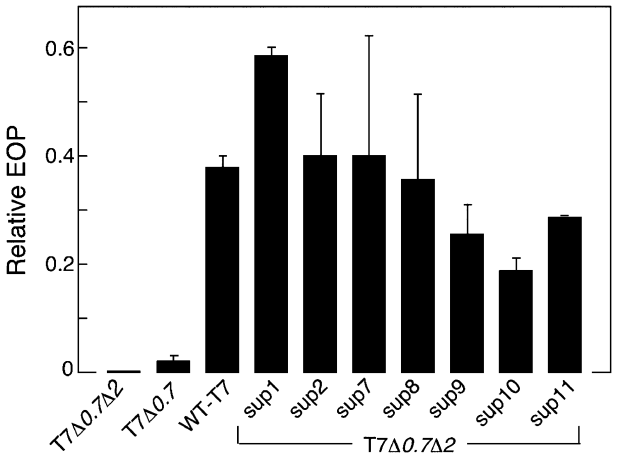

Fig. 2. Growth of suppressors that are deleted for T7 genes *0.7* and *2* on *udk*-overexpressing cells. T7 phage suppressors that overcome the lack of T7 genes *0.7* and *2* were isolated as described in *Experimental procedures*. *E. coli* K-12 AG1 cells harbouring the *udk* plasmid were aerated for 45 min with IPTG induction. Approximately 100 PFU of each suppressor were mixed with the cells in top agar, and overlaid on LB-agar plates. Plaques were counted after 5 h. The EOP on the induced cultures was calculated relative to its EOP on the uninduced cultures. Suppressor phage indicated under each bar are listed in Table 3. Experiments were carried out twice; the average and SD are shown.

Suppressors overcoming lack of host RNA polymerase inhibition map to E. coli *promoters and to gene* 3.5

Interestingly, as Table 3 shows, all suppressors of T7Δ*0.7*Δ*2* lack all the major early *E. coli* promoters: A0, A1, A2 and A3. Some of the deletions extend to other nonessential genes as well, but it is clear that when both gene *0.7* and gene *2* are missing, mutations arise that eliminate the major *E. coli* RNA polymerase promoters and thus reduce considerably the loading of the host RNA polymerase on T7 DNA. Interestingly, one of the suppressor mutants (T7Δ*0.7*Δ*2*-sup11) also acquired a mutation in gene *3.5*. The amino acid substitution in gp3.5 has been shown to result in weaker T7 RNA polymerase/gp3.5 interactions (Cheng *et al*., 1994). As mentioned above, approximately 30% of suppressors to *udk* overexpression map to mutations in gene *3.5* (Table 2). The mutations result in gp3.5 that have weaker interaction with T7 RNA polymerase.

We next isolated suppressor mutants that overcome a less severe phenotype than T7Δ*0.7*Δ*2*, namely the lack of gene *2* alone (T7Δ*2*). Similar procedure as described above was used to isolate suppressor phage that encode gene *0.7* but lack gene *2*. The suppressors that overcome the lack of gene *2* are summarized in Table 4. Similar mutants in gene *3.5*, having weaker interaction with T7 RNA polymerase, arose to overcome the lack of gene *2* alone. This result is not surprising as the effect of deletion

Table 3. Mutations in suppressors that lack T7 genes *0.7* and *2*.

Suppressor	Mutation(s)[a]	Genes/elements affected
T7Δ*0.7*Δ*2*-sup1	Δ235–1942	A0, ΦOL, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup2	235 T → A; Δ496–3117	A0, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup3	235 T → A; Δ495–949	A0, A1, A2, A3 promoters, and gene *0.3*.
T7Δ*0.7*Δ*2*-sup4	224 C → A; Δ469–470; Δ577–1958	A0, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup5	Δ235 T → A; 464 G → A; Δ577–1958	A0, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup6	256 T → C; Δ367–1435	A0, ΦOL, A1, A2, A3 promoters, and genes *0.3–0.4*.
T7Δ*0.7*Δ*2*-sup7	Δ233–2020	A0, ΦOL, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup8	Δ233–245; Δ467–1113	A0, A1, A2, A3 promoters, and gene *0.3*.
T7Δ*0.7*Δ*2*-sup9	235 T → A; Δ288–2014	A0, ΦOL, A1, A2, A3, B promoters, and genes *0.3–0.6B*.
T7Δ*0.7*Δ*2*-sup10	235 T → A; Δ337–809	A0, A1, A2, A3 promoters.
T7Δ*0.7*Δ*2*-sup11	Δ226–1980; 10807 G → A	A0, ΦOL, A1, A2, A3, B promoters, and genes *0.3–0.6B*; Gp3.5-R33H

a. Most of the indicated deletions occurred through a cross-over in short direct repeats. The extent of the deletion is reported with the repeat included in the left side of the deletion.

of gene *2* on the growth of T7 phage is similar to that observed when T7 infects strains overexpressing *udk*. In both cases the inhibition of *E. coli* RNA polymerase is inadequate. In addition to the gene *3.5* suppressors, the screen revealed mutations in the *E. coli* promoters A0, A1, A2 or A3. These mutations are similar to those we have obtained when isolating suppressors for T7Δ*0.7*Δ*2*. The last group of mutations is dispersed in two major clusters in the left region of T7 DNA. We do not know how these mutations are beneficial for overcoming the lack of gene *2*.

Discussion

During productive T7 phage infection, the host RNA polymerase must be inhibited. The reason this inhibition is required is unknown. One strategy to understand this phenomenon is to identify hosts in which the RNA polymerase cannot be inhibited, and T7 suppressors that grow on these hosts.

We show here that hosts overexpressing *udk* do not support T7 phage growth because the phage cannot adequately inhibit the host RNA polymerase. Conditions that exogenously inhibit the host RNA polymerase, such as overexpression of gene *2,* or the addition of rifampicin at the time of phage infection, can restore T7 phage growth on hosts overexpressing *udk*. This phenotype is similar to that of a large group of T7 resistant mutants, *tsnB* (Chamberlin, 1974). *E. coli tsnB* mutants encode RNA polymerase that is completely resistant to inhibition by T7 gene *2*, and therefore these cells do not support T7 phage growth. Indeed, several T7Δ*0.7* suppressor mutants isolated on cells overexpressing *udk* could also suppress T7Δ*0.7* on the host strain BR3, a *tsnB*-like

Table 4. Mutations in suppressors that lack T7 gene *2*.

Suppressor	Suppressor class	Mutation(s)[a]	Genes/elements affected
T7Δ*2*-sup1	Host promoters	235 T → A; 489 C → T; Δ606–730	A0, A1, A2, A3 promoters
T7Δ*2*-sup2	Host promoters	716 G → A; 1183 T → C	A3 promoter; Gp0.3-Y78H
T7Δ*2*-sup3	Host promoters	480 T → C; 737 T → C	A1, A3 promoters
T7Δ*2*-sup4	Host promoters	235 T → A; 489 C → T; Δ616–730	A0, A1, A2, A3 promoters
T7Δ*2*-sup5	Host promoters	714 T → C	A3 promoter
T7Δ*2*-sup6	Host promoters	714 T → C	A3 promoter
T7Δ*2*-sup7	Host promoters	Δ606–730; 1921 C → T; 10797 G → A	A2, A3 promoters; Gp0.6B-E96E; Gp3.5-R30H
T7Δ*2*-sup7	Gene *3.5*	Δ606–730; 1921 C → T; 10797 G → A	A2, A3 promoters; Gp0.6B-E96E; Gp3.5-R30H
T7Δ*2*-sup8	Gene *3.5*	10888 G → A	Gp3.5-R60Q
T7Δ*2*-sup9	Gene *3.5*	1096 G → A; 10822 A → G	Gp0.3-A58T; Gp3.5-E38G
T7Δ*2*-sup2	Others	716 G → A; 1183 T → C	A3 promoter; Gp0.3-Y78H
T7Δ*2*-sup7	Others	Δ606-730; 1921 C → T; 10797 G → A	A2, A3 promoters; Gp0.6B-E96E; Gp3.5-R30H
T7Δ*2*-sup9	Others	1096 G → A; 10822 A → G	Gp0.3-A58T; Gp3.5-E38G
T7Δ*2*-sup10	Others	1867 G → A	Gp0.6B-W77-stop codon
T7Δ*2*-sup11	Others	1807 insert A	Gp0.6B-G60-frameshift
T7Δ*2*-sup12	Others	1866 G → A	Gp0.6B-W77-stop codon
T7Δ*2*-sup13	Others	1921 C → T	Gp0.6B-E96E
T7Δ*2*-sup14	Others	1867 G → A	Gp0.6B-W77-stop codon

a. Most of the indicated deletions occurred through a cross-over in short direct repeats. The extent of the deletion is reported with the repeat included in the left side of the deletion.

mutant on which gene *0.7* is essential for T7 phage growth. Nevertheless, a major difference between the *udk*-overexpressing-hosts and the members of the *tsnB* or the *tsnB*-like group is that whereas all of the known *tsnB* group members encode a RNA polymerase resistant to gene *2* inhibition, the *udk*-overexpressing-host encodes a wt-RNA polymerase. A phenotype in which the host RNA polymerase is not inhibited sufficiently is also manifested in phage T7Δ*2* growth on normal hosts. Not surprisingly, suppressor screens that we carried out for mutants that can grow on *udk*-overexpressing-hosts yielded comparable mutations as those we identified for suppressors for the lack of T7 gene *2*. Furthermore, suppressors that could suppress for a major deficiency in host RNA polymerase inhibition, the lack of both gene *0.7* and gene *2*, could also suppress for the phenotype arising from overexpression of *udk*.

The nature of the suppression mutations that we have identified on hosts overexpressing *udk*, as well as the suppressor mutations we have identified for T7Δ*2* and T7Δ*0.7*Δ*2*, are particularly interesting. Three major suppressor mutations were identified: (i) mutations in the *E. coli* early promoters on the T7 genome, (ii) mutations in the gene encoding T7 lysozyme (gp3.5) that reduce the interactions of the lysozyme with T7 RNA polymerase and (iii) mutations in gene *2* that presumably enhance the activity of gene *2*.

Why is the inhibition of host RNA polymerase required for T7 phage growth? This question is still unanswered. However, it is known that the major defect when the host RNA polymerase is not inhibited is in DNA concatemer formation and processing. For example, during T7Δ*2* infection, no high-molecular-weight T7 DNA is found in the cells, corresponding to a defect either in concatemer formation or in premature breakdown (Center, 1975; Mooney *et al*., 1980).

Models have been proposed to account for how a deficiency in ability to inhibit host RNA polymerase interferes with T7 phage growth, and we propose another possibility. Our model is based, in part, on the suppressor mutants described in this paper that overcome the requirement for inhibition of the host RNA polymerase. The fact that suppressor mutations are found in all of the *E. coli* early promoters in T7Δ*0.7*Δ*2* unequivocally demonstrates that host transcription on T7 DNA at late stages of T7 infection is detrimental for T7 phage growth. Earlier findings showed that T7Δ*2* or T7Δ*0.7*Δ*2* infections produced genomes whose left ends were degraded (Center, 1975; DeWyngaert and Hinkle, 1980). These results taken together suggest that this selective degradation of T7 DNA at the left end is caused by host RNA polymerase-transcription.

According to the current model, suggested by Zhang and Studier (2004), the formation of the right end of T7 DNA is initiated by the pausing of T7 RNA polymerase. The site of this pausing was shown experimentally to occur at a unique 7 bp sequence immediately downstream of the concatemer junction (Lyakhov *et al*., 1998). The pausing of T7 RNA polymerase then recruits the terminase–prohead complex, including the products of genes *10*, *18* and *19*. This recruitment, in turn, induces a DNA break by the terminase (gp18 and gp19) adjacent to the pause site. As outlined in Fig. 3, we broaden this model to include the requirement for host RNA polymerase inhibition in this process. We propose that in the absence of proper inhibition of host RNA polymerase, the host RNA polymerase transcribes the early region late in the infection after the terminase and prohead are synthesized. This transcription constitutes a 'road-block' to transcription of T7 RNA polymerase, which proceeds fivefold faster than *E. coli* RNA polymerase (Molineux, 2005). These 'road blocks' cause pausing or slowing down of the T7 RNA polymerase near the host RNA polymerase, and consequently recruit the prohead-packaging machinery that cleaves the left end of T7 DNA aberrantly. The model explains why mutations in gene *3.5* that reduce the interaction of gp3.5 with the T7 RNA polymerase are beneficial when host RNA polymerase is inadequately inhibited. The gene *3.5* mutations reduce the pausing of the T7 RNA polymerase behind the host RNA polymerase thus reducing the catastrophic recruit of the packaging machinery. According to this model, degradation of the left end of the T7 genome can only take place in the presence of genes *10*, *18* and *19*. Indeed, DeWyngaert and Hinkle (1980) showed that on *tsnB* hosts, selective degradation of the left end requires the presence of genes *10* and *19* (requirement for gene *18* was not tested in their study). In our model, overexpression of *udk* results in insufficient host RNA polymerase inhibition, and consequently in more collisions between the two RNA polymerases that lead to selective degradation of the left end of T7 DNA. One possible mechanism for this activity is that *udk* overexpression leads to a surplus in RNA precursors in the cell. Consequently, the host RNA polymerase initiates more chains and thus becomes less susceptible to inhibition by gp2. It is still not clear why the *udk* enzyme overexpression inhibits T7 phage growth as opposed to other ribonucleoside kinases, whose overexpression does not inhibit T7 phage growth.

T7 is a very potent phage. Only a few defence mechanisms of the host are not overcome by the phage: (i) eliminating the adsorption of the phage to the host by means of capsule formation or phage-ligand modifications, (ii) eliminating the phage ability to inhibit the host RNA polymerase, (iii) eliminating host *trxA* production and (iv) eliminating host *cmk* production (Qimron *et al*., 2006). By elucidating the mechanism of inhibition of T7 phage growth by hosts overexpressing *udk*, we can classify this phenotype in the second group of defence mechanisms of the host. In order to produce a more potent phage to a

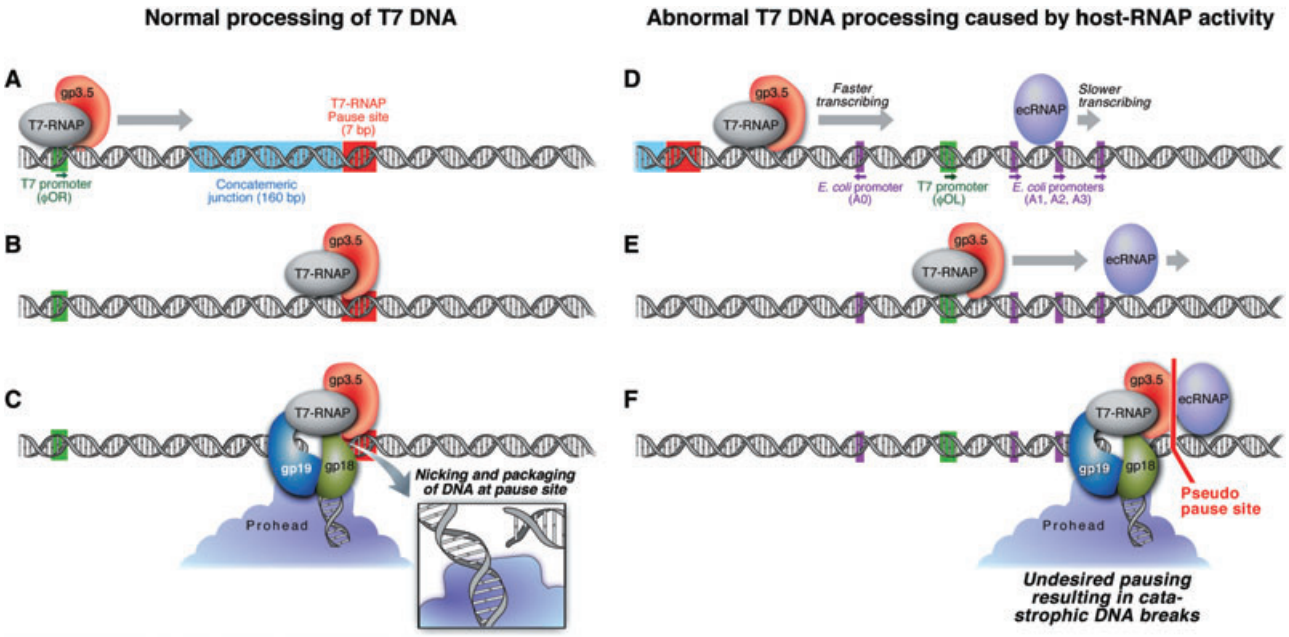

Fig. 3. Model for the requirement of the inhibition of host RNA polymerase during T7 phage growth. The left panels depict the processes occurring during normal processing of T7 DNA. T7 RNA polymerase transcribes from the ΦOR promoter (A), and pauses at a unique site immediately downstream to the concatemer junction (B). This pause, enhanced by gp3.5, recruits the prohead and the terminase complex to nick the DNA and thus produce a T7 end for packaging (C). The right panels depict the processes in the absence of sufficient inhibition of host RNA polymerase. Transcription by T7 RNA initiates either from the ΦOR or from the ΦOL promoters (D and E). Transcription by the slower host RNA polymerase from the strong *E. coli* promoters on the left end of the T7 DNA forms a 'roadblock' to T7 RNA polymerase. This blockage causes the T7 RNA polymerase to pause at a pseudo-pause site, and thus recruits the terminase and prohead complex, resulting in truncated left ends (F).

wide range of *E. coli* strains, a gene, in addition to gene *0.7* and gene *2*, that inhibits the host RNA polymerase could be inserted into the T7 genome. A modified T7 phage encoding such a gene along with the *trxA* and *cmk* genes should theoretically overcome all the known defence mechanisms of an infected host. These points should be considered in designing a bacteriophage for antibacterial therapy.

Experimental procedures

Materials

Luria–Bertani (LB) broth, LB agar, and granulated agar were purchased from EMD (San Diego, CA). Chloramphenicol was purchased from American Bioanalytical (Natick, MA). Rifampicin and Jumpstart polymerase chain reaction (PCR) reaction mix were purchased from Sigma-Aldrich (St Louis, MO). DNA purification kits were purchased from Qiagen (Valencia, CA). Elongase PCR kit was purchased from Invitrogen (Carlsbad, CA).

Bacterial strains and phages

The *udk*-overexpressing *E. coli* K-12 AG1 and the *E. coli* K-12 BW25113 *cmk* strains were provided by Hirotada Mori from the Nara Institute of Science and Technology, Nara, Japan. *E. coli* K-12 AG1 is a derivative of DH1: *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* ($r^{K-}m^{K+}$), *supE44*, *relA1* (Kitagawa *et al.*, 2005). *E. coli* K-12 BW25113 is: *lacI*^{q}, *rrnBT14*, Δ*lacZWJ16*, *hsdR514*, Δ*araBADAH33*, Δ*rhaBADLD78* (Baba *et al.*, 2006). *E. coli* lacking *cmk* and *trxA* is a derivative of *E. coli* K-12 BW25113 *cmk* with its *trxA* gene replaced by a tetracycline-resistance gene. The *trxA* gene replacement was carried out in this study as described (Datsenko and Wanner, 2000). *E. coli* B and *E. coli* C strains are from our lab collection. *E. coli* BR3 is a derivative of *E. coli* B strain and was provided by William F. Studier (Brookhaven National Laboratory, Upton, NY). The T7 phages used are from our lab collection or constructed in this study. Bacteria and phage were grown at 37°C, unless indicated otherwise.

Plasmids construction

pAC-null and pAC-gp2 were constructed as follows. A plasmid backbone containing the p15A origin of replication along with the tetracycline-resistance gene were prepared from pACYC184 using the restriction enzyme BsaA1 (producing blunt ends). This vector replaced the ampicillin-resistance gene and the origin of replication of pET19b that were cleaved out using the restriction enzyme EcoRV (producing blunt-ends) to yield pAC-null. Gene *2* was amplified from the wt-T7 and cloned into pET19b at the NdeI and XhoI

restriction sites to yield pET19b-gp2. The fragment containing gene *2* was then cleaved from pET19b-gp2 using EcoRV and cloned into pACYC184 vector digested by BsaA1 to yield pAC-gp2. pAC-null and pAC-gp2 encode a null gene or gene *2* under the T7/lac promoter, and carry the p15A origin of replication along with the tetracycline-resistance gene. The p15A origin of replication is compatible with the colE1 origin of replication of the *udk*-encoding plasmid. The *udk* plasmid was purified from clone 19-B9 of the ASKA collection (Kitagawa *et al*., 2005). It encodes the *udk* gene under the T5/lac promoter and carries a chloramphenicol-resistance gene.

Efficiency of plating assays

Bacteria overexpressing *udk* were grown overnight in LB medium containing 35 μg ml^{-1} chloramphenicol, diluted 1:1 in LB medium containing 35 μg ml^{-1} chloramphenicol, with or without the indicated concentration of IPTG, and aerated for 45 min. One millilitre of the culture was then mixed with approximately 100 PFU of the indicated T7 phage in top agar (0.7% agar in LB). The mixture was overlaid on LB plates, and after 5–15 h the plaques were counted. The EOP was determined relative to the uninduced culture by dividing the number of PFU obtained on the induced culture by the number obtained in the uninduced culture.

Restoration of burst size by the addition of rifampicin

Bacteria overexpressing *udk* were grown overnight in LB medium containing 35 μg ml^{-1} chloramphenicol, diluted 1:10 in LB medium containing 35 μg ml^{-1} chloramphenicol with or without 1 mM IPTG, and aerated for 1 h at 37°C. Cells were infected at an OD_{600} of 0.1 (5×10^7 cfu ml^{-1}) with T7Δ*0.7* at a Moi of 5. After 2 min, 0.5 ml of the cells was centrifuged for 1 min at 13 000 r.p.m., the supernatant was removed and the pellets were re-suspended in 0.5 ml of fresh medium. After 9 min DMSO or 200 μg ml^{-1} (final concentration) of rifampicin in DMSO was added. Cells were further incubated at 37°C for 1 h and the PFU was measured by plating dilutions of the lysates on a bacterial lawn.

*Construction of T7*Δ0.7*, T7*Δ2 *and T7*Δ0.7Δ2

To delete both gene *0.7* and gene *2* from the T7 genome, we first constructed a plasmid, p*0.7-cmk-0.7*, encoding the *cmk* gene flanked by 60 bp of upstream and 60 bp of the downstream gene *0.7* sequences. Liquid culture of *E. coli* DH5α cells (Invitrogen) transformed with p*0.7-cmk-0.7* plasmid at an OD_{600} ~0.3 were infected with T7 wild-type phage at a Moi of 0.1 and grown until complete lysis. The resulting lysate was plated on hosts lacking the *cmk* gene. This procedure selected for T7Δ*0.7*::*cmk*. We next used either the resulting T7Δ*0.7*::*cmk* phage or wt-T7 phage to infect hosts harbouring p*2-trxA-2*, a plasmid encoding the *trxA* gene flanked by 60 bp of the upstream and 60 bp of the downstream gene *2* sequences. Plating the lysates on a strain encoding gene *2* but lacking both *trxA* and *cmk* or only *trxA* resulted in T7Δ*0.7*::*cmk*/Δ2::*trxA* or T7Δ*2*::*trxA* respectively. Gene *0.7* replacement by *cmk* was confirmed, using direct sequencing, to be between bp 2021 and 3100 (GenBank NC_001604). Gene *2* replacement by *trxA* was confirmed, using direct sequencing, to be between bp 8898 and 9092.

Isolation of suppressor mutants

To isolate suppressor phage that overcome the inhibition of growth on cells overexpressing *udk*, cells overexpressing *udk* were mixed with 10^5 PFU of T7-*Trx*6-ST10 (T7 phage which has a *trxA* insertion in gene *0.7*) in top agar and overlaid on LB plates. After 5 h, 63 plaques were picked and transferred to a 96 well microtiter plate containing 150 μl of cells overexpressing *udk*. The microtiter plate was aerated at 37°C overnight. One microlitre of each lysate was transferred to 150 μl of cells overexpressing *udk* on a fresh plate, and the procedure was repeated. The suppressors were stamped, using a colony replicator, on *E. coli* BR3, and 13 plaques were able to lyse *E. coli* BR3, ranging from clear plaques (2) to turbid plaques (11). The other 50 suppressors did not grow on *E. coli* BR3. Nine suppressors that could grow on *E. coli* BR3, and five suppressors that could grow only on *udk* overexpressing cells were picked for EOP determination on cells overexpressing *udk*, and on *E. coli* BR3. Suppressors were then subjected to genomic sequencing. As we expected to identify mutations in gene *2*, we initially sequenced only the gene *2* region of the DNA of the suppressor mutants. In order to identify the location of the other mutations, DNA from a suppressor phage without a mutation in gene *2* was taken for sequence analysis of all class I and class II DNA region [bp 49–19820 (excluding, because of technical difficulties, 8600–8841; 11580–11621) GenBank NC_001604]. A single nucleotide substitution was located to gene *3.5*. The other three suppressors that did not have mutations in gene *2* were analysed by sequencing gene *3.5* and all carried different substitutions in this gene.

Suppressors overcoming the lack of gene *2* or the lack of both genes *0.7* and *2* were similarly isolated, except that the selection of suppressors was carried out on *E. coli* B. The DNA of these suppressors was analysed by sequencing the left end of T7 DNA (bp approximately 50–2500, GenBank NC_001604), and the sequence of gene *3.5* was also determined in several suppressor mutants (bp 10440–11280, GenBank NC_001604). Sequence analysis was carried out either directly using purified genomic phage DNA or on amplified PCR fragments.

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