Molecular Microbiology (2010) 77(2), 492–504 ■
doi:10.1111/j.1365-2958.2010.07221.x
First published online 9 June 2010

A novel nucleotide kinase encoded by gene *1.7* of bacteriophage T7

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Summary

Gene *1.7* of bacteriophage T7 confers sensitivity of both phage T7 and its host *Escherichia coli* to dideoxythymidine (ddT). We have purified the product of gene *1.7*, gp1.7. It exists in two forms of molecular weight 22 181 and 17 782. Only the C-terminal half of the protein is required to confer ddT sensitivity. We show that gp1.7 catalyses the phosphorylation of dGMP and dTMP to dGDP and dTDP, respectively, by using either GTP, dGTP or dTTP as the phosphate donor. Either form of gp1.7 exhibit identical kinase activity as compared with wild-type gp1.7 that contains a mixture of both forms. The K_m of 70 μM and Kcat of 4.3 s^{-1} for dTMP are similar to those found for *E. coli* thymidylate kinase. However, unlike the host enzyme, gp1.7 efficiently catalyses the conversion of the chain-terminating dideoxythymidylate (ddTMP) to ddTDP. This finding explains the sensitivity of phage T7 but not *E. coli* to exogenous ddT. Gp1.7 is unusual in that it has no sequence homology to any known nucleotide kinase, it has no identifiable nucleotide-binding motif and its activity is independent of added metal ions. When coupled with nucleoside diphosphate kinase, gp1.7 exponentially converts dTMP to dTTP.

Introduction

In recent years the replisome has been dissected and reassembled in a number of systems. Often neglected in these studies are proteins that provide the immediate precursors of DNA synthesis, the deoxynucleoside 5′-triphophates (dNTPs). The plethora of information on the myriad enzymes of nucleotide metabolism in *Escherichia coli* would suggest that little new information could be gained. Yet little is known about the assembly of these proteins into a functional complex, much less their association with the replisome. The channelling of dNTPs directly to the replisome has long been an appealing model in which the newly synthesized precursors are delivered directly to the DNA polymerase (Mathews, 1993). Bacteriophage takes advantage of these pathways to reproduce successfully in the host. For example, the T-even bacteriophage do not break down the host DNA as extensively as in T7-infected cells, but the T-even phage do encode their own nucleotide kinase in order to phosphorylate deoxyhydroxymethylcytosinemonophosphate (Duckworth and Bessman, 1967). The incorporation of this unusual nucleotide into T4 DNA enables its glucosylation and subsequent protection from host enzymes. This kinase also phosphorylates dTMP and dGMP.

The highly efficient and economical manner by which bacteriophage T7 replicates its genome makes it an attractive system to study DNA replication. The structures of the four proteins that constitute the basic replisome of phage T7 have been determined and the interactions that coordinate events at the replication fork have been dissected (Hamdan and Richardson, 2009). A reconstituted replisome mediates fork movement in a manner that mimics that *in vivo* as judged by ensemble and single-molecule techniques (Hamdan *et al.*, 2009). The extensive DNA synthesis that occurs over a 10 min period after phage infection to produce over 100 copies of the T7 chromosome necessitates an abundant supply of deoxyribonucleotide precursors, both for DNA synthesis and for fuelling the T7 helicase, an enzyme that preferentially uses dTTP. T7 derives most of the nucleotides found in its DNA from the breakdown of host DNA (Labaw, 1951; 1953). The host DNA is degraded to 2′deoxynucleoside 5′-monophophates (dNMPs) by the joint action of the gene *3* endonuclease and gene *6* exonuclease (Center and Richardson, 1970; Kerr and Sadowski, 1972). How these dNMPs are eventually converted to dNTPs is not known. *E. coli* encodes four different dNMP kinases, each specific for one dNMP (Neuhard and Kelln, 1996; Zalkin and Nygaard, 1996). At least one of these kinases, CMK, is essential for T7 growth (Qimron *et al.*, 2006). It is generally assumed that either the nucleoside diphosphate kinase (NDK) or the adenylate kinase of the host converts the dNDPs to dNTPs (Lu and Inouye, 1996) but the

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question remains as to whether the activity of these kinases is sufficient to meet the demand of T7 DNA replication.

We recently reported a serendipitous finding that has led to new insight into nucleotide metabolism in T7-infected cells (Tran *et al*., 2008). These studies were based on the observation that phage T7 growth and T7 DNA synthesis are inhibited by the presence of dideoxythymidine (ddT) at concentrations that are not toxic to *E. coli*. The inhibition of DNA synthesis suggested that the ddT was converted to ddTTP and the chain-terminating ddTMP was incorporated into T7 DNA by the T7 DNA polymerase. T7 DNA polymerase incorporates ddNMPs with essentially the same efficiency as it does dNMPs (Tabor and Richardson, 1995). Therefore, we originally sought to screen for mutations in gene *5*, the structural gene for T7 DNA polymerase (Doublié *et al*., 1998). Surprisingly, when we isolated phage T7 that could grow in the presence of ddT, all contained a mutation in gene *1.7*, a non-essential gene of unknown function, while there were no mutations in the T7 gene *5*. However, a T7 phage that encodes an altered DNA polymerase in which tyrosine 526 has been replaced by phenylalanine bypasses the function of gene *1.7* and is resistant to ddT. This single alteration in T7 DNA polymerase leads to discrimination against the incorporation of ddTMP by 8000-fold as compared with dTMP (Tabor and Richardson, 1995). Thus, defects in gene *1.7* most likely lead to a defect in the conversion of ddT to ddTTP. Overproduction of gp1.7 from a plasmid also renders *E. coli* cells sensitive to ddT, indicating that no other T7 proteins are required for conferring sensitivity to ddT. The uptake of thymidine requires phosphorylation of dT to dTMP in order to trap the phosphorylated compound inside the *E. coli* cell (Dube *et al*., 1991). Inhibition by ddT of phage T7 and *E. coli* overproducing gp1.7 requires *E. coli* thymidine kinase (Tran *et al*., 2008), suggesting that gp1.7 exerts its role after the formation of dTMP.

In the present study we have purified gp1.7 and shown that it is a nucleoside monophosphate kinase. The properties of gp1.7 distinguish it from all other known nucleotide kinases. In conjunction with NDK it provides a novel mechanism for the rapid conversion of dTMP to dTTP.

Results

Two forms of gp1.7

Gene *1.7* was originally characterized as a single open reading frame (ORF) of 591 bp corresponding to nt 8166 to 8756 of the T7 genome (Dunn and Studier, 1983), preceded by a ribosome-binding site (RBS) (Fig. 1A). We previously reported that gene *1.7* was responsible for conferring sensitivity of phage T7 growth to ddT (Tran *et al*., 2008). We cloned a DNA fragment of ORF of gene *1.7* into the vector pET28a to overexpress gene *1.7* in *E. coli* BL21 (DE3). The lysate contained two induced proteins at approximately equal intensities (Fig. 1B, lane 3). The larger product had an apparent molecular mass of ~22 000 Da, the expected size of the gene *1.7* ORF. The smaller product had an apparent molecular mass of ~18 000 Da. When gp1.7 was purified, the same two bands were obtained at an equal ratio in each step of the purification (Fig. 1B, lanes 5–7). Mass spectrometric analysis of the slower migrating band showed that it consisted of gp1.7 missing the 41 N-terminal residues. The DNA sequence of this region of the gene reveals an internal RBS, AAGGAG, eight nucleotides upstream of an ATG start codon (Fig. 1A), an arrangement found in other T7 genes (Dunn and Studier, 1983).

To confirm that this internal start was responsible for the lower molecular weight product, we analysed the products produced by three different gene *1.7* mutants (see *Experimental procedures*). When either the internal RBS was mutated without changing the coding sequence of the larger gene *1.7*, or the internal initiation codon was changed from ATG (methionine) to CTG (leucine), production of the smaller gene *1.7* product could no longer be detected (Fig. 1C, lanes 3–5). On the other hand, when the gene *1.7* expressed had a deletion of the first 41 codons, only the smaller gene *1.7* product was detected (Fig. 1C, lane 6).

We carried out complementation analysis to determine the ability of each of the two forms of gp1.7 to render phage T7 sensitive to ddT (Fig. 2). The greatest sensitivity occurred when both forms were produced, either when wild-type T7 (wt T7) was used to infect *E. coli* HMS89, or T7Δ1.7 was used to infect *E. coli* HMS89/pGP1.7 that produced both forms of gp1.7 (wt gp1.7). When either only the large form (22 kDa gp1.7) or only the small form (18 kDa gp1.7 or Δ41N) was produced from a plasmid, phage T7Δ1.7 was able to produce plaques in the presence of ddT that were tiny yet larger than when both forms of gp1.7 were produced together. In comparison, when either 22 kDa gp1.7 or 18 kDa gp1.7 were produced alone it resulted in the same sensitivity to ddT as the mutants that deleted the first 94 residues (gpΔ94N) or 124 residues (gpΔ124N) at the N terminus of gp1.7. In contrast, deletion of the nine C-terminal residues of gp1.7 (gpΔ9C) completely eliminated its ability to render phage T7 sensitive to ddT, resulting in plaques of the same size as those produced in the absence of gene *1.7* (T7Δ1.7).

Purification of gp1.7

Overproduced gp1.7 is soluble in lysis buffer lacking NaCl (Fig. 1B, lane 3). However, when the lysis buffer contained 100 mM NaCl, no gp1.7 could be detected in the

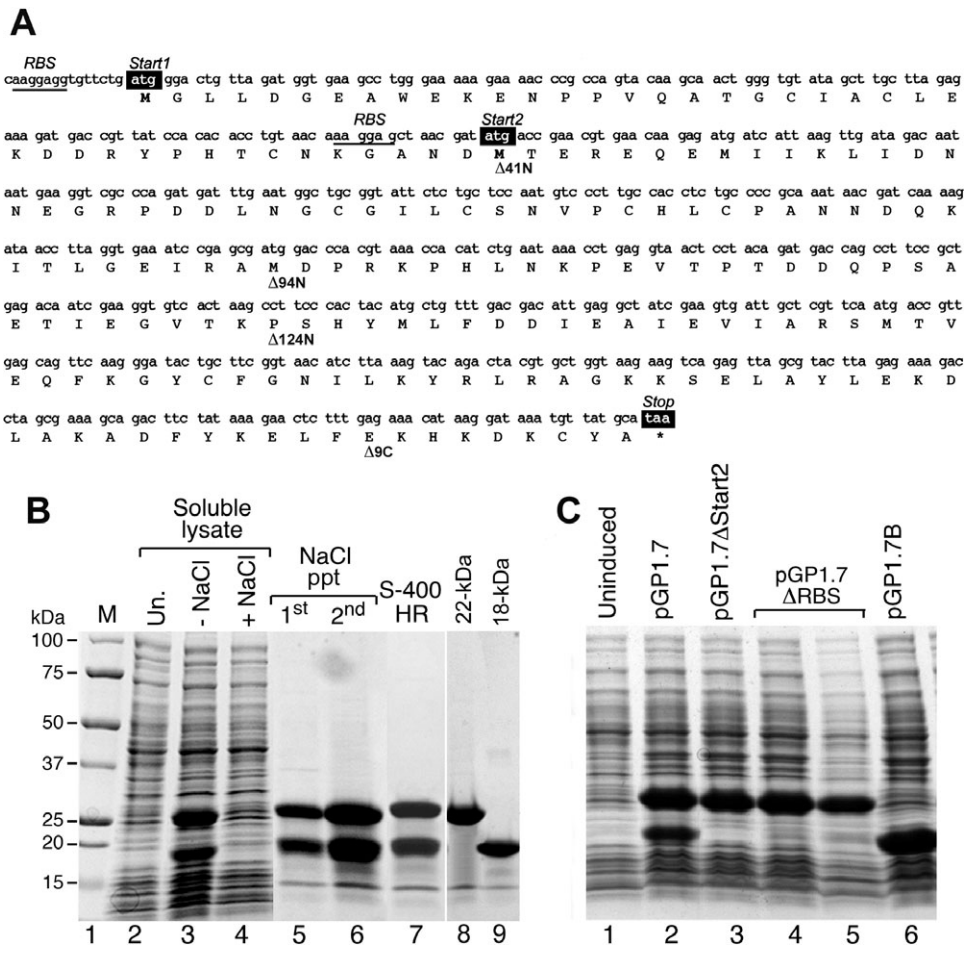

Fig. 1. Overproduction and purification of gp1.7.

A. Coding sequence of gene *1.7*. The coding sequence corresponds to nt 8166 to 8756 of the T7 genome (Dunn and Studier, 1983). *Start1*: the first initiation site that is used for initiation of the large form of gp1.7 (22 181 Da); *Start2*: an internal start site that is used for initiation of the small form of gp1.7 (17 782 Da); *Stop*: termination site for the gene products produced by both *Start1* and *Start2*; Δ41N, Δ94, Δ124N and Δ9C: relative end points of the different N-terminal and C-terminal deletion mutants described in Fig. 2.

B. Overexpression and purification of gp1.7. Gp1.7 was overproduced from *E. coli* BL21(DE3) harbouring the expression vector pGP1.7. Samples were analysed on a 4–20% gradient SDS-PAGE. Lane 1, markers; lane 2, lysate from uninduced cells; lane 3, soluble lysate from induced cells prepared in the absence of NaCl; lane 4, soluble lysate from induced cells prepared in the presence of 100 mM NaCl; lane 5, gp1.7 after the first NaCl precipitation; lane 6, gp1.7 after the second NaCl precipitation; lane 7, purified gp1.7 after gel filtration through Sephacryl S-400 HR; lane 8 and lane 9, large (22 kDa) and small (18 kDa) forms of gp1.7 were independently purified using the same procedure.

C. Effect of mutations in the internal *Start2* and *RBS* on production of the two forms of gp1.7. The internal *Start2* site and *RBS* were modified as described in *Experimental procedures*. Induced cell lysates were analysed on a 4–20% gradient SDS-PAGE. Lane 1, uninduced lysate; lane 2, pGP1.7 wt; lane 3, pGP1.7ΔStart2, in which the internal *Start2* ATG (methionine) was changed to CTG (leucine); Lanes 4 and 5, pGP1.7ΔRBS, in which the original *RBS* AAGGAG was changed to AGGGTG (lane 4) and AGGGCG (lane 5); lane 6, pGP1.7B that expresses only the small form (18 kDa) of gp1.7.

soluble lysate (Fig. 1B, lane 4); rather, it was all located in the pellet after centrifugation (data not shown). This insolubility of gp1.7 in NaCl prevents standard approaches to purification by ion exchange chromatography. However, as the precipitated protein became soluble when the NaCl was removed, we used multiple steps of salting in/out to purify gp1.7 (see *Experimental procedures*). Most contaminants are eliminated by the first NaCl precipitation (Fig. 1B, lane 5). Further purification consisted of a second NaCl precipitation step (Fig. 1B, lane 6), followed by size exclusion chromatography using Sephacryl S-400 HR (Fig. 1B, lane 7). A summary of this procedure is shown in Table 1. Using this procedure we have also successfully purified the large and small forms of gp1.7 from *E. coli* harbouring the plasmids described expressing either the 22 kDa form or the 18 kDa form. The plasmid expressing only the 22 kDa gp1.7 contained gene *1.7* in which the internal initiation codon was changed from ATG to CTG (leucine). The smaller form was expressed from a plasmid in which a

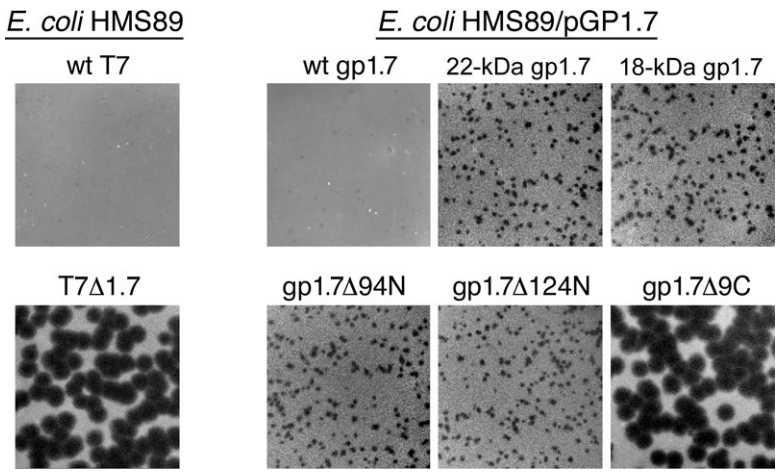

Fig. 2. Complementation assays were carried out to study the effect of large and small forms of gp1.7, and deletion mutants of gp1.7 on sensitivity of T7 growth to ddT. Wild-type T7 (wt T7) and a T7Δ1.7 that deletes the entire gene *1.7* (T7Δ1.7) were used to infect *E. coli* HMS89 in the control experiments on the left panel. For the complementation assays shown on the right, T7Δ1.7 was used to infect *E. coli* HMS89 that contained variations of the plasmid pGP1.7 that expressed different forms of gp1.7. In the top row, the plasmids examined expressed both forms of gp1.7 (wt gp1.7), only the large form of gp1.7 (22 kDa gp1.7), and only the small form of gp1.7 (18 kDa gp1.7). In the bottom row, the plasmids examined expressed three different truncations of gp1.7 that missing 94 (gpΔ94N), 124 (gpΔ124N) residues at N terminus (see Fig. 1A), respectively, and 9 residues (gpΔ9C) at C terminus. T7-infected *E. coli* were grown on plates containing 1 mM ddT in LB media. The plates were incubated at 37°C for 6 h and then photographed.

truncated gene *1.7* (first 41 codons deleted) was present. Each form was apparently homogeneous as judged by electrophoresis on SDS-gels (Fig. 1B, lanes 8 and 9).

Gp1.7 does not have thymidine kinase (TDK) activity

Our earlier report suggested that gp1.7 played a role in thymidine metabolism (Tran *et al*., 2008). Possible steps involved include the uptake of thymidine and the addition of each of the three phosphates to synthesize dTTP. It has been previously shown that TDK-dependent phosphorylation of thymidine (dT) to thymidylate (dTMP) is essential to trap exogenous thymidine inside *E. coli* cells (Dube *et al*., 1991). However, we presented genetic evidence that gp1.7 was not a thymidine kinase, as phage T7 was not sensitive to ddT in *E. coli tdk*, a strain that is defective in the host thymidine kinase (Tran *et al*., 2008). On the track towards our further understanding of the function of gene *1.7*, we compare here the ability of wild-type *E. coli*, *E. coli tdk* and *E. coli tdk* containing the pGP1.7 plasmid to accumulate [^{3}H]dT inside the cells (Fig. 3). Only *E. coli* containing the *tdk* gene could accumulate [^{3}H]dT, regardless of the presence of gp1.7. Because the *tdk* gene encodes the TDK, which adds the first phosphate onto thymidine, this result, along with our previous genetic evidence, strongly suggest that phage T7 does not encode a TDK.

Gp1.7 is a novel nucleotide kinase

The availability of purified gp1.7 enables a direct assay of thymidylate kinase (TMK) activity, which adds the second phosphate to dTMP to yield dTDP on the pathway to dTTP. We tested each of the rNTPs and dNTPs for their ability to serve as the phosphate donor in a dTMP kinase reaction (Fig. 4A). In the presence of dGTP, dTTP or GTP, gp1.7 catalyses the conversion of dTMP to dTDP; the other dNTPs and NTPs (including ATP) could not serve as phosphate donors. Thus, the first unusual property of the T7 kinase is the nucleotide specificity of the phosphate donor, as other nucleotide kinases preferentially use ATP as the donor (Nelson and Carter, 1969; Jong and Camp-

Table 1. Summary of purification of gp1.7 by sodium chloride precipitation (see *Experimental procedures* for details).

Step	Protein (mg)	Activity (unit)	Units per mg	Gp1.7 recovered (%)
Crude lysate	380	74 000	190	100
First NaCl precipitation	41	44 000	1100	60
Second NaCl precipitation	27	33 000	1100	45
S-400 HR	16	20 000	1300	27

One unit of enzyme is defined as the amount that catalyses the conversion of 1 μmole of dTMP to dTDP in 5 min, with dGTP as the phosphate donor in the standard assay.

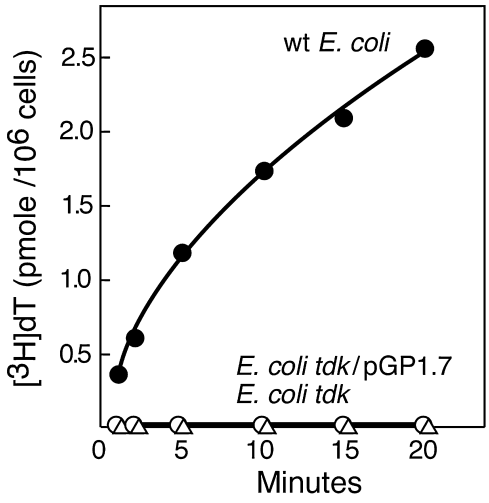

Fig. 3. *Escherichia coli* thymidine kinase is essential for the accumulation of dT in *E. coli* cells. *E. coli* strain HMS89 (DE3) (●), HMS89(DE3) *tdk* (○), and HMS89(DE3) *tdk*/pGP1.7 (△) were grown at 30°C to A_{600} = 0.4 in LB medium. Cells were induced with 1 mM IPTG and then DNA synthesis was inhibited by the addition of 250 μg ml^{-1} nalixidic acid. The uptake of [^{3}H]dT was measured as described in *Experimental procedures*.

bell, 1984). The formation of dTDP from dTMP is proportional to the amount of gp1.7 present (Fig. 4B), and the formation of dTDP is linear with time at limiting gp1.7 concentrations (Fig. 4C). Gp1.7 shows optimum activity between pH 7 and 7.5 (data not shown). The substrate specificity of gp1.7 was also examined. In addition to dTMP, dGMP is also a substrate for gp1.7, whereas dAMP, dCMP and dUMP are not substrates (data not shown).

We have shown in the previous section that the presence of both large (22 kDa) and small (18 kDa) forms of gp1.7 resulted in the greatest sensitivity of T7Δ1.7 to ddT *in vivo*. We compared the kinase activity of the large and small forms of gp1.7 with that of the mixture of two forms that were co-purified from cells expressing wild-type gene *1.7*. Results in Table 2 show that the large and small forms of gp1.7 exhibit identical kinase activity as compared with the mixture regardless of the substrates. In all cases, dGMP was a better phosphate receptor with an apparent Km approximately 50% of that obtained with dTMP (Table 2). We have used the mixture of two forms for subsequent studies as the mixture is normally present *in vivo*.

Escherichia coli also encodes a TMK that converts dTMP to dTDP. We purified this enzyme and compared its activity with that of gp1.7. ATP is the most efficient donor for *E. coli* TMK (Nelson and Carter, 1969), so ATP was used as the donor in assays with *E. coli* TMK, whereas dGTP was used as the donor in assays with gp1.7. The two enzymes have comparable levels of dTMP kinase activity (Fig. 4D). The K_m for dTMP is 70 μM for gp1.7 and 27 μM for *E. coli* TMK. The Kcat for dTMP is nearly identical for the two enzymes, 4.3 s^{-1} for T7 gp1.7 and 5.7 s^{-1} for *E. coli* TMK.

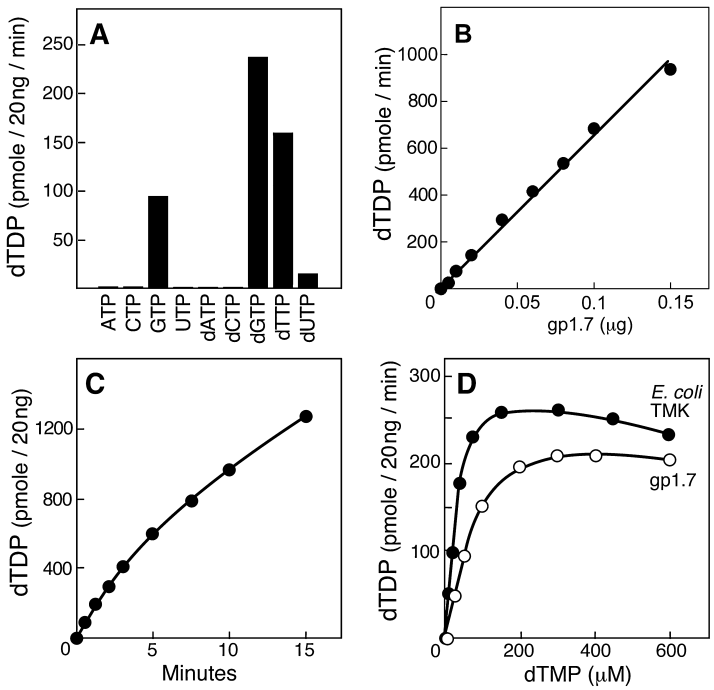

Fig. 4. Gp1.7 has dTMP kinase activity.
A. Various nucleoside triphosphates were examined for their ability to serve as phosphate donor in the kinase reaction. Standard reaction mixtures (20 μl) contained 100 μM [^{3}H]dTMP (60 cpm pmol^{-1}) and 2.5 mM of each nucleoside triphosphate. Reactions were initiated by the addition of 20 ng of purified gp1.7, and were allowed to proceed for 2.5 min at 37°C. Reactions were terminated by heating at 98°C for 3 min and the amount of [^{3}H]dTDP formed was determined by thin layer chromatography as described in *Experimental procedures*.
B. Effect of gp1.7 concentration on dTMP kinase activity. Reaction mixtures (20 μl) containing increasing amount of gp1.7 and dTTP as phosphate donor were incubated at 37°C for 2.5 min. The reactions were terminated and the amount of [^{3}H]dTDP formed was determined as described above.
C. The effect of time on the rate of the dTMP kinase reaction. Standard reaction mixtures (200 μl) containing 400 ng of gp1.7 and dTTP as phosphate donor were incubated at 37°C. At the indicated times 20 μl aliquots were removed and the amount of [^{3}H]dTDP formed was determined as described above.
D. Comparison of the dTMP kinase activity of gp1.7 (○) and *E. coli* TMK (●). The standard reaction mixtures (20 μl) contained 20 ng of either gp1.7 or *E. coli* TMK and the indicated amount of [^{3}H]dTMP. 2.5 mM dGTP was used as the phosphate donor for gp1.7 and 2.5 mM ATP was used as the phosphate donor for *E. coli* TMK. Reactions were incubated for 2.5 min at 37°C. The amount of [^{3}H]dTDP formed was determined as described above.

Table 2. Kinetic constants for dTMP and dGMP substrates for 18 kDa gp1.7, 22 kDa gp1.7 and the mixture of two molecular weight forms.

		Mixture of two forms		22 kDa gp1.7		18 kDa gp1.7	
Receptor ↓	Donor ↓	Km (μM)	Kcat (s^{-1})	Km (μM)	Kcat (s^{-1})	Km (μM)	Kcat (s^{-1})
dGMP	GTP	20 ± 4	1.2 ± 0.2	23 ± 4	1.3 ± 0.3	20 ± 6	1.2 ± 0.1
	dGTP	35 ± 7	5.6 ± 0.3	37 ± 8	5.7 ± 0.2	34 ± 7	5.6 ± 0.3
	dTTP	39 ± 6	5.0 ± 0.2	40 ± 7	5.1 ± 0.2	37 ± 7	4.9 ± 0.2
dTMP	GTP	173 ± 26	1.7 ± 0.5	168 ± 30	1.5 ± 0.3	171 ± 27	1.7 ± 0.2
	dGTP	70 ± 11	4.3 ± 0.1	73 ± 9	4.5 ± 0.3	68 ± 13	4.1 ± 0.1
	dTTP	77 ± 13	3.5 ± 0.3	73 ± 10	3.3 ± 0.2	74 ± 11	3.6 ± 0.2

The standard reactions contained 20 ng of enzyme and increasing concentration (5–2000 μM) of either dGMP or dTMP as the receptor and an excess (2.5 mM) of either GTP, dGTP or dTTP as the phosphate donor. After 2 min incubation at 37°C, the reaction was stopped and kinase activity was determined as described in *Experimental procedures*. The apparent Michaelis constant Km and Kcat for each receptor/donor combination was calculated using PRISM 4.0 (GraphPad Software).

Gp1.7 is a metal-independent nucleotide kinase

In the absence of a divalent cation, gp1.7 retains approximately 70% of the activity observed in the presence of Mg^{2+} (Table 3). Omission of Mg^{2+} and the addition of EDTA does not further decrease the activity of gp1.7. In contrast, as is typical of most nucleotide kinases, *E. coli* TMK has no detectable activity in the absence of Mg^{2+}. This lack of an exogenous metal requirement distinguishes gp1.7 from other known nucleotide kinases (Nelson and Carter, 1969; Knowles, 1980; Jong and Campbell, 1984) as does its lack of a typical nucleotide-binding motif (see *Discussion*).

Table 3. Effect of Mg^{2+}on dTMP kinase activity.

	[^{3}H]dTDP (pmol)	
	E. coli TMK	Gp1.7
$+Mg^{2+}$	600	440
$-Mg^{2+}$	< 5	370
$-Mg^{2+}$ + EDTA	< 1	377

Reaction mixtures for *E. coli* TMK (20 μl) contained 100 μM [^{3}H]dTMP, 2.5 mM ATP and 20 ng *E. coli* TMK. Reaction mixtures for gp1.7 (20 μl) contained 100 μM [^{3}H]dTMP, 2.5 mM dTTP and 20 ng of gp1.7. Where present, 10 mM Mg^{2+} or 5 mM EDTA was added. Reactions were incubated at 37°C for 2 min. dTMP kinase activity was determined by the formation of [^{3}H]dTDP as described in the *Experimental procedures*.

ddTMP is a substrate for gp1.7

In contrast to T7 DNA polymerase, *E. coli* DNA polymerases I (Tabor and Richardson, 1995) and III (Johanson and McHenry, 1982) use ddNTPs inefficiently compared with dNTPs. We previously reported that overexpression of gene *1.7* in *E. coli* renders the cells sensitive to 0.1 mM ddT, whereas in the absence of gene *1.7* the growth of *E. coli* is not affected by 5 mM ddT (Tran *et al.*, 2008). We showed that the inhibition of growth was accompanied by a cessation of DNA synthesis. In this report we measure the pool size of ddTMP, ddTDP and ddTTP in cells growing in the presence of ddT with and without gp1.7 (Fig. 5A). In the absence of gp1.7 there is no detectable ddTDP or ddTTP, whereas in its presence there are abundant ddTDP and ddTTP pools. Gp1.7 has little effect on ddTMP pools.

The *in vivo* results were further confirmed when we compared the phosphorylation of dTMP and ddTMP in crude lysates either in the presence or absence of overproduced gp1.7 (Table 4). In the absence of gp1.7, 21% of dTMP was converted to dTDP and dTTP, but only 0.1% of ddTMP was converted to ddTDP and ddTTP. In contrast, in the presence of gp1.7, 37% of dTMP was converted to dTDP and dTTP and 24% of ddTMP was converted to ddTDP and ddTTP.

The above results suggest that ddTMP is a substrate for gp1.7 but not for the *E. coli* enzyme. This is indeed the case as shown using purified gp1.7 and *E. coli* TMK. *E. coli* TMK efficiently phosphorylates dTMP but has no detectable activity with ddTMP (Fig. 5B). In contrast, gp1.7 phosphorylates ddTMP at essentially the same rate as it does dTMP (Fig. 5C). The initial rates of the reaction in this experiment for dTMP and ddTMP are 0.15 and 0.145 pmol s^{-1}, respectively, in comparison with 0.165 pmol s^{-1} obtained for dTMP with *E. coli* TMK (Fig. 5D). These results confirm that ddT inhibits phage T7 growth selectively via its conversion to ddTMP by *E. coli* thymidine kinase, which is then converted to ddTDP by the T7 gp1.7, which is finally converted to ddTTP where it is incorporated as a chain-terminating nucleotide into replicating T7 DNA.

Exponential increase in dTTP from dTMP by coupled reaction of gp1.7 and E. coli *NDK*

The finding that dTTP serves as the phosphate donor for the phosphorylation of dTMP by gp1.7 is at first consideration puzzling. This reaction, taken alone, would eventually result in a decrease of dTTP with a portion of the

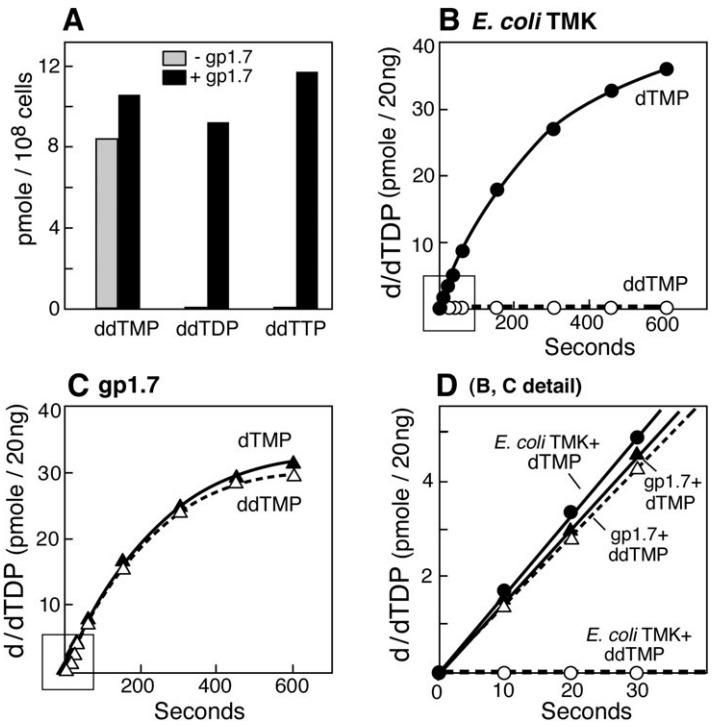

Fig. 5. ddTMP kinase activity of gp1.7 and *E. coli* TMK.
A. Effect of gp1.7 on dideoxythymidine pools *in vivo*. *E. coli* HMS89 (DE3) and HMS89 (DE3)/pGP1.7 were grown to A_{600} = 0.5. Gene *1.7* was induced by the addition of 1 mM IPTG for 30 min prior to the addition of 100 μM [^{3}H]ddT (50 μCi ml^{-1}). After incubation at 30°C for 30 min, intracellular nucleotides were extracted and the amounts of [^{3}H]ddTMP, [^{3}H]ddTDP and [^{3}H]ddTTP were determined as described in *Experimental procedures*. Grey bars: *E. coli* HMS89(DE3); Black bars: *E. coli* HMS89(DE3)/pGP1.7.
B. Comparison of dTMP and ddTMP kinase activity of *E. coli* TMK. Standard reaction mixtures (200 μl) contained 500 pmol (25 μCi) of either [^{3}H]dTMP (●) or [^{3}H]ddTMP (○) and 2.5 mM ATP. The mixtures were incubated at 37°C and 20 μl aliquots were removed at the indicated times. The amount of [^{3}H]dTDP and [^{3}H]ddTDP synthesized was determined as described in the *Experimental procedures*.
C. Comparison of dTMP and ddTMP kinase activity of T7 gp1.7. Standard reaction mixtures (200 μl) contained 500 pmol (25 μCi) of either [^{3}H]dTMP (▲) or [^{3}H]ddTMP (△) and 2.5 mM dTTP. Reactions were carried out as for (B) above.
D. Comparison of initial rate at linear range of dTMP and ddTMP kinase activity catalysed by *E. coli* TMK and gp1.7. These data are taken from the initial time points shown within the boxes in (B) and (C) above.

Table 4. Effect of gp1.7 on dTMP kinase activity in *E. coli* lysates.

Substrate →	dTMP			ddTMP		
Product formed (pmol) →	dTDP	dTTP	%[a]	ddTDP	ddTTP	%[a]
HMS89(DE3) lysate	7.3	14	21	0.02	0.08	0.1
HMS89(DE3)/pGP1.7 lysate	16	21	37	14	9.8	24

Lysates from *E. coli* HMS89(DE3) and HMS89(DE3)/pGP1.7 that had been induced with IPTG were prepared as described in *Experimental procedures*. Reaction mixtures (50 μl) contained 100 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2.5 mM ATP, 100 pmol [^{3}H]dTMP or [^{3}H]ddTMP and 1 μg of crude lysate. Reaction mixtures were incubated at 37°C for 5 min followed by heat inactivation at 98°C for 3 min. The products were analysed by TLC as described in *Experimental procedures*. Kinase activity was determined by measuring the formation of [^{3}H]dTDP or [^{3}H]ddTDP.
a. Percentage of total dTMP or ddTMP phosphorylated.

dTMP also being converted to dTDP. This is precisely the result observed in the experiment shown in Fig. 6A where dTDP accumulates at the expense of dTMP and dTTP. As expected, for every mole of dTTP consumed two moles of dTDP are formed.

However, *in vivo* the dTDP formed in the reaction would be immediately converted to dTTP by *E. coli* NDK (Parks and Agarwal, 1973) or adenylate kinase (Lu and Inouye, 1996). In the reaction catalysed by NDK, all NTPs and dNTPs can serve as the phosphate donor; however, because the *E. coli* intracellular concentration of ATP is the highest, it is primarily responsible for the conversion of dNDPs to dNTPs. When NDK and ATP are added to the reaction catalysed by gp1.7, there is an exponential increase in the amount of dTTP formed (Fig. 6B). In this reaction, a small amount (50 pmol) of dTDP was added to prime the formation of dTTP by NDK. The dTTP then serves as the phosphate donor for the dTMP kinase reaction by gp1.7 to double the amount of dTDP. Nearly 97% of the dTMP is phosphorylated in this reaction, with 75% converted to dTTP and 25% to dTDP. One possible explanation of why the conversion to dTTP was not complete could be competitive inhibition of NDK by the ADP that accumulates in the reaction (Sedmak and Ramaley, 1971). In this coupled reaction catalysed by NDK and gp1.7, ATP is used indirectly as a phosphate donor to convert dTMP to dTDP, which is then converted to dTTP by NDK.

Discussion

Phage T7 growth is inhibited by the presence of ddT in the medium at concentrations that do not inhibit the growth of its host, *E. coli* (Tran *et al.*, 2008). As we predicted, the

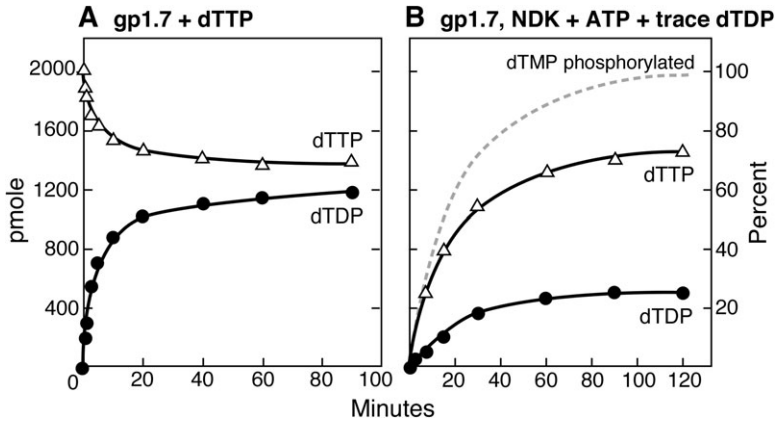

Fig. 6. dTTP synthesis mediated by gp1.7 and *E. coli* NDK.
A. Two dTDPs are formed by gp1.7 for each dTTP hydrolyzed. Reaction mixtures (200 μl) contained either 100 μM [^{3}H]dTMP and 100 μM dTTP, or 100 μM dTMP and 100 μM [^{3}H]dTTP. Reactions were initiated by the addition of gp1.7 to a final concentration of 100 nM. 20 μl aliquots were removed at the indicated times and the amount of dTDP formed (●) was determined by measuring the amount of [^{3}H]dTDP derived from [^{3}H]dTMP in one reaction, plus the amount of [^{3}H]dTDP derived from [^{3}H]dTTP in the other reaction. The lost of dTTP (△) was determined in the reaction containing [^{3}H]dTTP as the donor.
B. dTTP synthesis mediated by gp1.7 and *E. coli* NDK. Reaction mixtures (200 μl) containing 100 μM [^{3}H]dTMP, 5 μM dTDP and 1 mM ATP were incubated with 100 nM gp1.7 and 50 nM *E. coli* NDK under the same reaction conditions as in (A). The amount of dTTP (△) and dTDP (●) formed from [^{3}H]dTMP was determined as described in the *Experimental procedures*. The percentage of total dTMP phosphorylated was calculated from the sum of [^{3}H]dTDP and [^{3}H]dTTP formed and plotted as the grey broken line.

inhibition is a result of ddTMP being incorporated by T7 DNA polymerase, a DNA polymerase that incorporates these chain-terminating nucleotides almost as well as dNMPs (Tabor and Richardson, 1995). Therefore it seemed likely that the ability of T7 DNA polymerase, in contrast to *E. coli* DNA polymerase I (Tabor and Richardson, 1995) or DNA polymerase III (Johanson and McHenry, 1982), to readily incorporate ddTMP into replicating DNA could explain this difference in the sensitivity of T7 and *E. coli* to ddT. We initially screened for T7 phage that were resistant to ddT thinking that they would have mutations in gene *5*, the gene encoding the T7 DNA polymerase. However, nearly all mutations obtained were in T7 gene *1.7*, a non-essential gene of unknown function. In an earlier study, we found that a single mutation in gene *5* that changes the tyrosine at residue 526 to phenylalanine can increase the ability of T7 DNA polymerase to discriminate against ddNMPs by a factor of several thousand (Tabor and Richardson, 1995). We did not identify this mutation in our initial screen for ddT-resistant phage because it requires a transversion of a single base, a mutation that will occur much less frequently than mutations in gene *1.7* that simply reduce its activity (Tran *et al.*, 2008).

Clearly, ddT must be taken up by the cell and converted to ddTTP in order to be incorporated as ddTMP by DNA polymerase. Our *in vivo* results implicated gp1.7 in this process. Furthermore, since when gp1.7 is overproduced in *E. coli* the cells become sensitive to ddT, gp1.7 must be active in the absence of any other T7 proteins. Uptake of dT or ddT into *E. coli* cells relies on the coupled action of a permease and thymidine kinase (Fig. 7B). dT is transported into the cells by the *nup* gene product, a non-specific nucleoside permease (McKeown *et al.*, 1976). Thymidine kinase is essential for trapping dT inside the cells by phosphorylating it to dTMP (Dube *et al.*, 1991). We found that the *tdk* gene of *E. coli* is essential for sensitivity to ddT (Tran *et al.*, 2008) and that gp1.7 cannot substitute for the host thymidine kinase to accumulate dT within the cell. Thus the transport of ddT and its conversion to ddTMP are mediated by host functions. In this study we obtained *in vivo* data showing that the presence of gp1.7 drastically increases the ddTTP pool in *E. coli*, whereas it has little effect on the ddTMP pool. Our data suggest that a greater amount of ddT is converted to ddTMP in cells overproducing gp1.7 relative to those cells lacking gp1.7. Although gp1.7 does not have thymidine kinase activity, its presence could stimulate host thymidine kinase activity by favouring the forward reaction to form ddTMP. In the absence of gp1.7 there is an equilibrium between ddT and ddTMP catalysed by thymidine kinase. These results together suggest that gp1.7 must exert its role after the formation of dTMP or ddTMP.

We have now purified gp1.7 and shown that it is a nucleotide kinase that converts dTMP to dTDP and dGMP to dGDP. Its activity on dTMP is comparable with that of *E. coli* thymidylate kinase. In addition to its unusual substrate specificity, gp1.7 uses dTTP and dGTP but not ATP as the phosphate donor. In contrast, most other nucleotide kinases require ATP as the donor. Most interesting is the ability of gp1.7 to phosphorylate ddTMP at a rate compa-

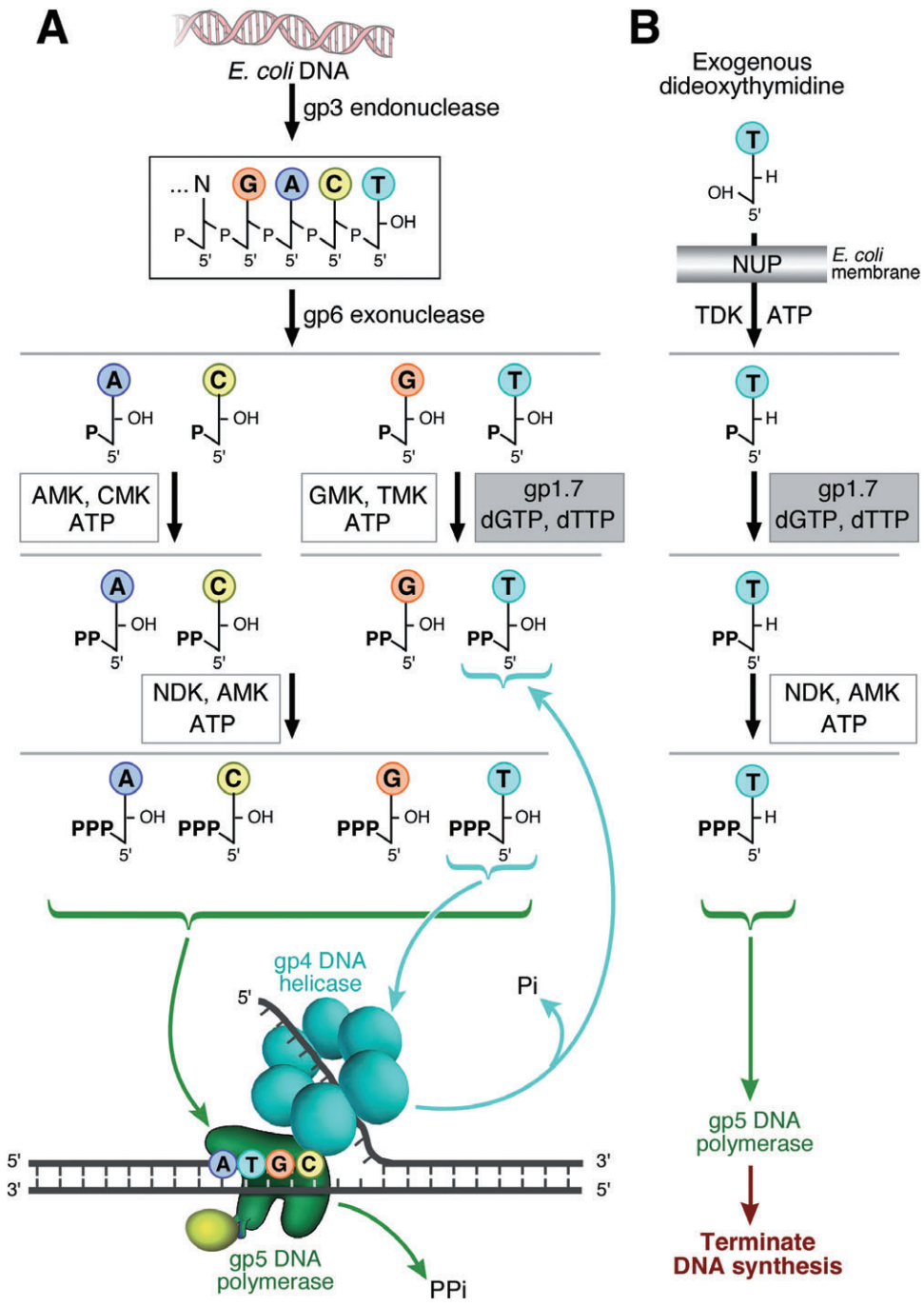

Fig. 7. Pathways for the synthesis of dNTPs and ddTTP in T7-infected cells.
A. T7 derives most of the phosphorous incorporated into its DNA from the breakdown of *E. coli* DNA by the joint action of gp3 endonuclease and gp6 exonuclease. Resulting dNMPs are converted to dNTPs by host nucleotide kinases. Gp1.7 is the only known nucleotide kinase encoded by phage T7 involved in the synthesis of dNTPs. dNTPs are used as precursors for DNA synthesis by T7 gp5 DNA polymerase, and dTTP hydrolysis is the energy source to drive translocation by the gp4 DNA helicase. For detailed description of these pathways, see the *Discussion*.
B. Pathway for inhibition of phage T7 growth by exogenous ddT.

rable with that observed with dTMP. In contrast, we show that the *E. coli* thymidylate kinase inefficiently uses ddTMP. Thus the sensitivity to ddT shown by T7 and by *E. coli* overproducing gp1.7 is due to the ability of gp1.7 to convert ddTMP to ddTDP, which is then converted to ddTTP. This is confirmed *in vivo* as significant pools of ddTDP and ddTTP were detected only in *E. coli* cells overproducing gp1.7.

The discovery of this novel thymidylate kinase encoded by phage T7 gene *1.7* with its unique specificities has resolved the question of the selective inhibition of phage T7 growth by ddT. The mechanism for this is summarized as in Fig. 7B. However, the role of gp1.7 in nucleotide metabolism in T7-infected cells is not entirely clear. Previous studies have shown that inactivation of gene *1.7* results in a delay in the onset of maximal DNA synthesis, and a longer eclipse period and time required for maximum progeny production (Endy *et al*., 2000; Tran *et al*., 2008). Moreover, we have noted that *E. coli* overexpressing gene *1.7* grows at a slightly faster rate. DNA synthesis in phage T7-infected cells must occur at a rapid rate in order to amplify its genome over 100-fold in a 10 min period (Kruger and Schroeder, 1981). A rate-limiting step in the phage life cycle is the accumulation of sufficient pools of dNTP precursors for incorporation into newly synthesized DNA by the T7 DNA polymerase. Phage T7 has partially solved this problem by using the preformed nucleotides found in the host DNA, bypassing the requirement for the *de novo* synthesis of nucleotides (Fig. 7A). T7 derives most of the phosphorus incorporated into its DNA from the breakdown of *E. coli* DNA (Labaw, 1951; 1953). The host DNA is degraded to dNMPs by the joint action of the T7 gene *3* endonuclease (Center and Richardson, 1970) and T7 gene *6* exonuclease (Kerr and Sadowski, 1972). In *E. coli*, dNMPs are converted to dNDPs by four distinct dNMP kinases, each specific for a different dNMP (Neuhard and Kelln, 1996; Zalkin and Nygaard, 1996). *E. coli* adenylate kinase, which phosphorylates both AMP and dAMP (Lu and Inouye, 1996), is likely sufficient to provide for dADP synthesis in T7-infected cells. We have shown that the *cmk* gene of *E. coli*, which encodes the CMP kinase responsible for synthesizing dCDP, is essential for T7 growth (Qimron *et al*., 2006). T7 gp1.7 provides for the synthesis of dTDP and dGDP. dNDPs are converted to dNTPs by the *E. coli* enzymes NDK (Parks and Agarwal, 1973) and adenylate kinase (Lu and Inouye, 1996). We have examined the combined action of gp1.7 and NDK in the conversion of dTMP to dTTP. In this reaction, the dTDP formed by gp1.7 is converted to dTTP by NDK using ATP as the phosphate donor. The resulting dTTP is then used as the phosphate donor by gp1.7 to phosphorylate another dTMP. This reaction produces two dTDPs, one from phosphorylation of dTMP and one from the phosphate donor. Because in each cycle two dTDPs are converted to two dTTPs, this doubles the amount of dTTP available to gp1.7 and rapidly exhausts the pool of dTMP. The conversion of dGMP to dGTP by gp1.7 and nucleotide diphosphate kinase presumably proceeds in a similar manner. Interestingly, *in vivo* a significant portion of the dTTP formed is used for the translocation and unwinding of the DNA by the T7 gene 4 helicase, reactions that require the hydrolysis of dTTP (Matson and Richardson, 1983). The product of this reaction is likewise dTDP (Fig. 7A).

Gene *1.7* is located in a congested area of the class II genes of phage T7. It was originally characterized as a single ORF of 591 bp (nt 8166 to 8756), sharing one overlapping nucleotide with gene *1.6* at its 5′ and five overlapping nucleotides with gene *1.8* at its 3′ end (Dunn and Studier, 1983). We show that gp1.7 exists as two molecular weight forms, with molecular masses of 22 181 and 17 782. The large form is the full-length gp1.7 while the smaller form is missing 41 residues at its N-terminus. The small form arises from an internal RBS and initiation site that is in frame with the first initiation site. Removal of either the internal RBS or the internal start codon eliminates the smaller form. Overlapping genes are not unusual in the T7 genome. For example, gene *4*, which encodes the phage helicase and primase, also specifies two overlapping proteins (Dunn and Studier, 1983). Our complementation assays using plasmids that express each of the two forms of gp1.7 suggest that the internal RBS is necessary for rapid inhibition of phage T7 growth by ddT. Perhaps by simultaneously using two RBSs, T7 is able to more rapidly synthesize gp1.7. *In vitro* the large and small forms of gp1.7 have the same kinase activity and nucleotide specificity as the wild-type enzyme (Table 2). Gp1.7 stands unique among the known nucleotide kinases. Unlike other thymidylate kinases, all of which use ATP as the phosphate donor, gp1.7 efficiently uses dTTP and dGTP. Likewise, the substrate specificity for dTMP and dGMP is puzzling as there are few structural similarities between these two nucleotides. Interestingly, bacteriophage T4 also encodes a dNMP kinase that functions with dTMP and dGMP, as well as deoxyhydroxymethylcytosine monophosphate (Brush *et al*., 1990). Finally, the lack of a requirement for an exogenous metal ion by gp1.7 is unique among nucleotide kinases. The possibility that the protein harbours a tightly bound metal is possible although, if so, it is not removed by EDTA. Perhaps these unusual properties are not too surprising considering that gp1.7 does not share sequence homology to any known nucleotide kinase and does not contain obvious motifs identified with nucleotide-binding sites.

The physical properties of gp1.7 are equally interesting. Our initial attempts to purify gp1.7 were met with frustration due to an inability to fractionate it by conventional column chromatography. The presence of even small amounts of NaCl precipitates the protein, which then returns to solution by removing the NaCl. We have taken advantage of this property to purify the protein by repeated precipitation with NaCl followed by resolubilization. A closer study of this curious property of gp1.7 is necessary in order to understand its molecular basis. The gel filtration profile of gp1.7 indicates that it has an extremely high stokes radius suggesting the formation of

a large complex (data not shown). It is possible that the nucleotide-binding motifs are created at the interface of these protein–protein interactions or that a protein-based compartment (Kang and Douglas, 2010) is created wherein unique phosphorylation steps can occur. We have not yet examined the possibility of physical interactions between gp1.7 and other proteins involved in T7 DNA replication.

Experimental procedures

Bacterial strains, bacteriophage and materials

Phage T7 was from our laboratory stock. Phage T7Δ1.7 has a deletion in gene *1.7* and corresponds to T7Δ1.7-2 previously described (Tran *et al*., 2008). *E. coli* HMS89 (*xth-1 thi argE mtl xyl str-R ara his galK lacy proA leu thr tsx supE*), a derivative of *E. coli* K12 AB1157 (White *et al*., 1976), was used for *in vivo* assays with ddT. *E. coli* HMS89 (DE3) was obtained by lyzogenizing *E. coli* HMS89 with lambda phage DE3 using the λDE3 Lysogenization Kit (Novagen). *E. coli* DH5α (Invitrogen) was used for all plasmid manipulations. BL21 (DE3) was from Novagen. Radioactive materials were from Moravek Biochemicals. TLC PEI Cellulose F plates were from Merck KGaA.

Purification of gp1.7

Gene *1.7* was cloned into the expression vector pET28a to produce pGP1.7. *E. coli* BL21 (DE3)/pGP1.7 was grown in 2 l of LB medium at 30°C. At A_{600} = 1 gene *1.7* was induced by the addition of IPTG to final concentration of 1 mM. After incubation for 4 h at 30°C, the cells were harvested and suspended in lysis buffer containing 20 mM Tris-HCl pH 7.4 and 10 mM β-mercaptoethanol. Cells were sonicated using a 15% pulse for 10 × 5 s in a Branson Digital Sonifier. The lysate was centrifuged at 15 000 r.p.m. in a 45Ti rotor (Beckman) to remove the insoluble material. 5 M NaCl was added slowly to the soluble fraction to a final concentration of 200 mM, followed by incubation on ice for 2 h. Gp1.7 was collected by centrifugation at 30 000 r.p.m. for 45 min in a 45Ti rotor. The pellet was resuspended in 20 ml lysis buffer in the absence of NaCl. The gp1.7 was precipitated a second time by the addition of 5 M NaCl to a final concentration of 200 mM. After centrifugation, the pellet was suspended overnight in 10 ml lysis buffer in the absence of NaCl.

The resuspended protein was further purified by gel filtration chromatography using a Sephacryl S-400 HR column (1.5 × 100 cm). The column was equilibrated with 20 mM Tris-HCl, pH 7.4 and 10 mM β-mercaptoethanol at a flow rate of 1.5 ml min^{-1}. The sample was applied and 3 ml fractions was collected. The location of the eluted proteins was determined by Bradford assays on 15 µl aliquots. The peak fractions were pooled. Samples were analysed by SDS-PAGE on a 4–20% gradient gel.

Preparation of E. coli *extracts for thymidylate kinase assay*

Escherichia coli HMS89(DE3) and HMS89(DE3)/pGP1.7 were grown in 5 ml LB to A_{600} = 0.8. Gene *1.7* was induced by the addition of 1 mM IPTG for 30 min. Cells were harvested by centrifugation and then suspended in 500 µl of lysis buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, and 1 mM PMSF. Cells were sonicated using a Branson Digital Sonifier at a setting of 15% pulse for 10 × 5 s, followed by centrifugation at 14 000 r.p.m. for 30 min in a micro centrifuge. The supernatant was collected. Total protein was determined using a Bradford assay (Bio-Rad).

Purification of E. coli *TMK and NDK*

Thymidylate kinase and NDK were each purified using a 6-His tag at their amino terminus. Strains used to overproduce each of these proteins were obtained from the ASKA library (Kitagawa *et al*., 2005); *E. coli* AG1 containing the vector pCA24N-6His-tmk for overproducing TMK and *E. coli* AG1 containing the vector pCA24N-6His-ndk for overproducing NDK. In each case, 2 l of cells was grown to A_{600} = 1 and induced by 1 mM IPTG for 4 h. The cells were then harvested and suspended in 30 ml of Buffer A (20 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 50 mM NaCl and 1 mM PMSF). The cells were lysed by sonication as described above and the insoluble fraction was removed by centrifugation. The supernatant was loaded onto a 10 ml DEAE DE52 column (Whatman), pre-equilibrated with Buffer A. The resin was washed with 100 ml of Buffer A followed by a 150 ml linear gradient of 0.05 to 500 mM NaCl in Buffer A. TMK and NDK each was eluted at approximately 130 mM NaCl. The fractions containing either TMK or NDK were pooled and then mixed with 2 ml of Ni-NTA superflow resin (Qiagen) in the presence of 1 mM PMSF, 0.1% Triton X-100, 0.05% Tween 20, 10 mM β-mercaptoethanol, 10% glycerol and 10 mM imidazole (Buffer B). After gentle rocking at 4°C for 2 h, the mixture was loaded onto a 15 ml Biorad Exposure Column. The resin was washed with 300 ml of Buffer B containing 10 mM imidazole, then 150 ml of Buffer B containing 20 mM imidazole, and then 75 ml of Buffer B containing 40 mM imidazole. The bound proteins were then eluted by 20 ml steps of 100, 150, 200 and 300 mM imidazole in Buffer B. The purity of the proteins was determined by SDS-PAGE using a 4–20% gradient gel. After the Ni-NTA column, both proteins appeared homogeneous.

Measurement of thymidine uptake by E. coli

Escherichia coli HMS89(DE3), HMS89(DE3) *tdk* and HMS89(DE3) *tdk*/pGP1.7 were grown at 30°C to A_{600} = 0.3 in LB medium. Gene *1.7* was induced by the addition of 1 mM IPTG for 20 min. Nalidixic acid was added to a final concentration of 200 µg ml^{-1} and the cells were incubated for an additional 10 min (this pretreatment resulted in inhibiting the incorporation of [^{3}H]thymidine into trichloroacetic acid insoluble fractions in the wild-type cells by 95% but had very little effect on dT uptake). At time zero, [^{3}H]dT was added to a final concentration of 25 µM (50 µCi ml^{-1}). At the indicated times, 200 µl was removed, filtered by vacuum through nylon Millipore filters (pore size 0.45 µM), and then washed with 2 ml of cold LB medium. The filters were then dried and the ^{3}H retained on the filter was measured using a liquid scintillation counter.

Measurement of dideoxythymidine pool size in E. coli

Escherichia coli HMS89(DE3) and HMS89(DE3)/pGP1.7 were grown and induced with IPTG as described above. At time zero, [^{3}H]ddT was added to the culture at a final concentration of 100 μM (50 μCi ml^{-1}). After an additional 30 min, the cells were harvested by centrifugation. Nucleotides were extracted by suspending the cell pellets in 0.5 N perchloric acid for 30 min on ice, followed by centrifugation to remove the cell debris. The supernatants were then neutralized by slowly adding 10 N KOH. Precipitated $KClO_4$ was removed by centrifugation at 13 000 r.p.m. for 30 min at 4°C. 100 μl of the supernatants was applied to TLC PEI Cellulose F plates (Merck KGaA), together with nucleotide markers (10 μg each of dTMP, dTDP and dTTP). The plates were chromatographed using 0.5 N LiCl and 2 N acetic acid until the front had moved a distance of 13 cm. Regions corresponding to the dTMP, dTDP and dTTP markers were excised from the plates, and the radioactivity was determined in a scintillation counter.

Gp1.7 thymidylate kinase assay

The thymidylate kinase activity of gp1.7 was determined by measuring the conversion of [^{3}H]dTMP to [^{3}H]dTDP. In this study dTTP was routinely used as phosphate donor, otherwise indicated. Standard reaction mixtures (20 μl) contained 0.1 mM [^{3}H]dTMP (65 cpm pmol^{-1}), 2.5 mM dTTP, 100 mM Tris-HCl, pH 7.5, 10 mM $MgC1_2$, 5 mM DTT and the indicated amounts of gp1.7. The reaction mixture was incubated at 37°C for the indicated times. The reaction was stopped by heating at 98°C for 2 min and an aliquot was applied to a TLC PEI Cellulose F plate (Merck KGaA), together with unlabelled nucleotide markers (10 μg each of dTMP, dTDP and dTTP). The plate was chromatographed in 0.5 N LiCl and 2 N acetic acid until the solvent front advanced 17 cm. Regions corresponding to the positions of dTMP, dTDP and dTTP were excised from the plate, and the radioactivity in each region was determined using a liquid scintillation counter. Similar method was applied to examine the substrate specificity of gp1.7. In some instances the enzyme activity was determined by measurement of the conversion of dTTP to dTDP. For these assays, [^{3}H]dTTP was used as the phosphate donor. Thymidylate kinase activity of *E. coli* TMK was performed using essentially the same reaction mixture described above except that 2.5 mM ATP was present in place of dTTP.

Construction of gene 1.7 lacking its internal RBS and start codon

Expression plasmids harbouring gene *1.7* that contains either a mutated internal RBS or a mutated internal initiation codon were constructed by mutagenesis of the plasmid pGP1.7 using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cat #200523). In pGP1.7 ΔStart2, the internal initiation codon ATG (methionine; see Fig. 2A) was changed to CTG (leucine). In the two pGP1.7 ΔRBS mutants, the internal RBS sequence AAGGAG was changed to AGGGTG and AGGGCG (see Fig. 2A); neither change altered the coding sequence of the full-length gp1.7.

Gp1.7 complementation assay

ddT-resistant phage T7Δ1.7 (Tran *et al*., 2008) was used to test for gene *1.7* complementation by wild-type gp1.7, either form of gp1.7 alone, and various deletion mutants introduced into the plasmid pGP1.7. *E. coli* HMS89/pGP1.7 expresses gene *1.7* upon infection by T7. Therefore, phage T7Δ1.7 is sensitive to ddT in *E. coli* HMS89/pGP1.7 cells that express gp1.7 that is able to complement wt gp1.7. Plasmids carrying mutant gene *1.7* used in the complementation assay include pGP1.7ΔRBS and pGP1.7B, which expresses only large (22 kDa gp1.7) and only small (18 kDa gp1.7 or gp1.7Δ41N) form of gp1.7 respectively; pGP1.7Δ94N and pGP1.7 Δ124N, which express gp1.7 truncated 94 and 124 residues at N-terminus respectively; and pGP1.79ΔC, that deletes 9 residues at C-terminus.

Acknowledgements

This work was funded by United States Public Health Service Grant # GM54397 and DOE Grant # DE-FG02-96ER 62251. We are grateful for helpful discussions with Bernard Weiss, Ian Molineux and Christopher Walsh.

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