ACADEMIC PRESS

Temperature requirements for initiation of RNA-dependent RNA polymerization

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Abstract

To continue the molecular characterization of RNA-dependent RNA polymerases of dsRNA bacteriophages (*Cystoviridae*), we purified and biochemically characterized the wild-type (wt) and a temperature-sensitive (*ts*) point mutant of the polymerase subunit (Pol) from bacteriophage ϕ12. Interestingly, initiation by both wt and the *ts* ϕ12 Pol was notably more sensitive to increased temperatures than the elongation step, the absolute value of the nonpermissive temperature being lower for the *ts* enzyme. Experiments with the Pol subunit of related cystovirus ϕ6 revealed a similar differential sensitivity of the initiation and elongation steps. This is consistent with the previous result showing that de novo initiation by RdRp from dengue virus is inhibited at elevated temperatures, whereas the elongation phase is relatively thermostable. Overall, these data suggest that de novo RNA-dependent RNA synthesis in many viral systems includes a specialized thermolabile state of the RdRp initiation complex.
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Introduction

Genomes of related RNA viruses often share relatively low homology, which is a likely consequence of the high mutation rate (Holland et al., 1982). In this respect, one important question is to what extent can differences in protein sequence be tolerated while preserving the 3D structure and function. We are studying bacteriophage ϕ6 and related double-stranded RNA (dsRNA) bacteriophages from the *Cystoviridae* family, focusing primarily on protein P2, the RNA-dependent RNA polymerase (RdRp, or Pol) used by these viruses for replication and transcription of their genomes.

Bacteriophage ϕ6, an enveloped virus with a tripartite dsRNA genome, was isolated from bean straw infested with

Pseudomonas syringae pv. *phaseolicola* (Mindich, 1988; Vidaver et al., 1973). Until recently, ϕ6 has been alone in the genus *Cystovirus,* family *Cystoviridae* (van Regenmortel et al., 2000). The isolation of eight additional cystoviruses, ϕ7 to ϕ14, all containing three segments of dsRNA, revealed some to be very similar to ϕ6 and others only distantly related (Mindich et al., 1999).

The close relatives of ϕ6 include ϕ7, ϕ9, ϕ10, and ϕ11, while bacteriophages ϕ8, ϕ12, and ϕ13 are more distantly related to ϕ6. Bacteriophages from the latter group are unable to infect the normal host of ϕ6, *P. syringae* pv. *phaseolicola* HB10Y. However, they can infect a mutant strain, LM2333, and it is likely that these phages attach directly to the LPS (Mindich et al., 1999). The nucleotide sequences of the ϕ8, ϕ12, and ϕ13 genomes have been determined (Gottlieb et al., 2002a, 2002b; Hoogstraten et al., 2000; Qiao et al., 2000). Their overall genetic organization is similar to ϕ6, although they differ in nucleotide and amino acid sequences, except that the amino acid motifs characteristic of viral RdRps are present

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in the P2 (Pol) protein sequences (Gottlieb et al., 2002a; Hoogstraten et al., 2000; Qiao et al., 2000). φ12 Pol displays low but statistically significant identity to the Pol proteins of bacteriophages φ6, φ8, and φ13, at 21, 24, and 20%, respectively (Gottlieb et al., 2002a).

The GDD signature of the conserved span III of viral RNA-dependent RNA polymerases (Koonin et al., 1989; equivalent to the C motif by Poch et al., 1989) was also present in the φ12 P2 at amino acid positions 468–470 (Gottlieb et al., 2002a). Interestingly, φ8 P2 also contains the GDD sequence (Hoogstraten et al., 2000), whereas the comparable motif in φ6 and φ13 is SDD (Mindich, 1988; Qiao et al., 2000).

Recently, crystal-soaking experiments of the φ6 P2 with NTPs revealed a binding site in the substrate pore that orders triphosphate moieties by attachment to the key basic residues lysine 223 and arginines 225, 268, and 270 (Butcher et al., 2001). The amino acid sequences of the polymerases from φ8 and φ13 also show conservation of these basic residues (Hoogstraten et al., 2000; Qiao et al., 2000). In φ12 Pol, the corresponding residues are Lys256 and arginines 258, 295, and 297 (Gottlieb et al., 2002a). Although this entire region would be expected to be highly conserved, the φ12 Pol sequence near Arg295 and Arg297 is most distant in comparison to the analogous regions of the other cystoviral polymerases. Yet, the φ12 Pol Lys256-Arg258 subregion resembles that of φ8 Pol (Gottlieb et al., 2002a).

In previous work, we compared enzymatic properties of the φ6 Pol with those of the Pol subunits from φ8 and φ13 (Yang et al., 2001, 2003). φ12, the most recently characterized member of the *Cystoviridae* family, offers an additional opportunity to examine functional properties of the RdRp subunit. Here, we cloned and expressed genes of wild-type and a *ts* mutant of φ12 Pol in *Escherichia coli*. Both proteins were purified and shown to catalyze RNA-dependent RNA synthesis using the de novo initiation mechanism. The temperature optima of the two proteins were found to be different. Interestingly, while characterizing biochemical properties of these enzymes, we found that the initiation step is significantly more thermolabile than the elongation of the RNA synthesis. This was also the case for the φ6 Pol (this study) and for the dengue polymerase subunit (Ackermann and Padmanabhan, 2001). Taken together, these observations suggest that a thermolability of de novo initiation complex may be a universal feature of RNA-dependent RNA synthesis.

Results

Cloning of the recombinant φ12 Pol gene

Two φ12 Pol expression constructs were generated, one encoding the wild-type (wt) protein and the other one containing a spontaneous missense mutation in the *Pol* gene that changes the naturally occurring Thr425 to Ile (T425I). To make both constructions, the *Pol* encoding sequence was PCR-amplified from the L segment cDNA carried on a plasmid template. This was cloned into the pET-21d vector (Novagen) downstream of the T7 promoter and a strong ribosome-binding site.

The entire sequence of the *Pol* gene in recombinant plasmid pPG14 was determined and found to contain the single amino acid change T425I as compared to the published sequence (Gottlieb et al., 2002a; GenBank Accession No. AF408636). To correct the mutation, the *Pol* gene was again PCR-amplified and a fragment of it encompassing the region of the amino acid change was used to replace the comparable DNA fragment carried on pPG14. The *Pol* gene carried by the resultant plasmid pPG24 was sequenced to ensure that the codon for the amino acid Thr425 was restored. *E. coli* strains BL21(DE3/pPG14) and BL21(DE3/pPG24) produced the mutant and wild-type Pol proteins, respectively.

Predicting 3D structure of φ12 Pol

To predict the 3D structure of the wt φ12 Pol, we used program 3D-PSSM to search the database of known protein structures (Kelley et al., 2000; accessed at http://www.sbg-.bio.ic.ac.uk/~3dpssm/). The two best structural homologs of φ12 Pol returned by the program were φ6 Pol (PDB ID 1hil; PSSM E value 2.32e − 84) and rabbit hemorrhagic disease calicivirus RdRp (PDB ID 1khv; PSSM E value 0.0126). The φ6 Pol structure was used as a template to build 3D model for φ12 Pol (Fig. 1). The model correctly positions the putative catalytic aspartates (Asp469 and Asp470) and the NTP binding arginines (Arg295 and Arg297) in the interior of the φ12 Pol and predicts the "priming" platform to contain His622 residue, as compared with Tyr630 of φ6 Pol (Butcher et al., 2001). Interestingly, the Thr425 residue, which is mutated in one of the two φ12 Pol variants described above, resides in the palm domain facing the protein interior. Because the palm is a catalytically important part of RdRp, structurally conserved across many nucleic acid polymerases (Hansen et al., 1997), we used the mutated φ12 Pol for further analyses along with its wt counterpart.

Expression and purification of recombinant φ12 Pols

The production of soluble φ12 Pols was achieved by incubating corresponding *E. coli* strains at 18°C in the presence of 10 μM IPTG. Expression at higher concentrations of IPTG (20 μM to 1 mM) led to a substantial increase in the overall polymerase production, but the solubility of the recombinant protein decreased dramatically.

A combination of Reactive Brown 10 agarose and heparin agarose was used to purify φ12 Pol wt. Cleared bacterial lysate containing the recombinant Pol was loaded onto
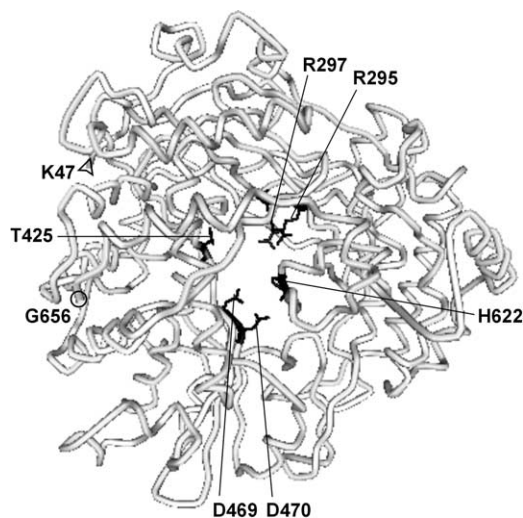
Fig. 1. Structural model for the wt φ12 Pol. Database of known protein structures was searched for wt φ12 Pol structural homologs using the program 3D-PSSM (Kelley et al., 2000). The best hit was φ6 Pol structure (PDB ID 1hi1), which was used as a template to build the φ12 Pol model shown here. Residues involved in RNA synthesis reaction are shown in black. Thr425 is also indicated. The arrowhead and circle show the positions of Lys47 and Gly656, the N- and C-terminal boundaries of the 3D model.

brown agarose, which was followed by the elution with a linear 0–1 M NaCl gradient (buffered with 25 mM Tris–HCl, pH 8.0, 1 mM EDTA). φ12 Pol containing fractions were diluted with 25 mM Tris–HCl, pH 8.0, 1 mM EDTA to bring the NaCl concentration to 50 mM, and the sample was loaded onto a heparin agarose column. The bound φ12 Pol was eluted with a linear 0–1 M NaCl gradient, as above. The T425I mutant was purified similarly, except it had to be concentrated after the heparin agarose step using Centrex (S&S; membrane cutoff 30 kDa), because of a reduced protein yield. The concentration step also helped increase the T425I purity by removing low-molecular protein contaminants. The purified φ12 P2 proteins migrated on SDS–PAGE according to their predicted molecular mass of ~75.4 kDa (Fig. 2A). Typical yields of purified T425I and wt proteins were approximately 2 and 4 mg per liter of bacterial culture, respectively.

φ12 Pol wt and T425I catalyze RNA replication in vitro

RNA replication activity of the purified polymerases was assayed as described earlier for the φ6 enzyme (Makeyev and Bamford, 2000b; Yang et al., 2001). The mixtures containing 50 mM Tris–HCl, pH 8.9, 20 mM ammonium acetate (NH₄OAc), 5 mM MgCl₂, 2 mM MnCl₂, 6% PEG4000, 0.1 mM EDTA, and ssRNA template pEM16 were incubated for 1 h at 23°C. Both wt and T425I were enzymatically active, producing full-length dsRNA forms, indistinguishable from the φ6 Pol dsRNA product (Fig. 2B). The φ12 Pol wt was nearly as active as the φ6 Pol, whereas

the specific activity of the φ12 Pol T425I was lower under the conditions employed. No radioactive bands were detected when polymerase or the RNA substrate was omitted from the reaction mixture (lane 1 and not shown). These results demonstrate that φ12 Pol possesses RdRp activity in vitro and that the T425I mutation noticeably affects the enzyme performance. Analysis of the reaction products using strand-separating conditions showed that most of the newly produced chains migrated as template-sized RNA, although dimer-length species were also detected (not shown). This suggests that φ12 Pol can initiate RNA synthesis de novo as it has been shown for φ6 Pol (Laurila et al., 2002).

Effect of the reaction conditions on the polymerase activity

The above assay was utilized to study the effects of several parameters on the activity of φ12 Pol wt and T425I. The wt enzyme synthesizes dsRNA over a wide pH range, while T425I mutant pH optimum appears to be relatively sharp. However, the pH optima for the two enzymes in a Tris–HCl buffer are similar, pH 8.9. Interestingly, both φ12 Pol variants show very low specific activity in a HEPES–KOH buffer (Figs. 3A and B). This HEPES intolerance distinguishes φ12 Pol from the earlier described Pols of cystoviruses φ6, φ8, and φ13 (Yang et al., 2001).

To determine the monovalent cation requirements, reactions were supplemented with different concentrations of NH₄OAc. Low salt concentrations, 40 mM for the T425I and 80 mM for the wt, stimulated the RNA synthesis noticeably. Further increase in the concentration was inhibitory, particularly for the T425I enzyme (Figs. 3C and D).

Manganese (Mn²⁺) is known to stimulate the isolated Pol subunits of φ6, φ8, and φ13 (Yang et al., 2001). We studied the effect of this ion on the two variants of the φ12
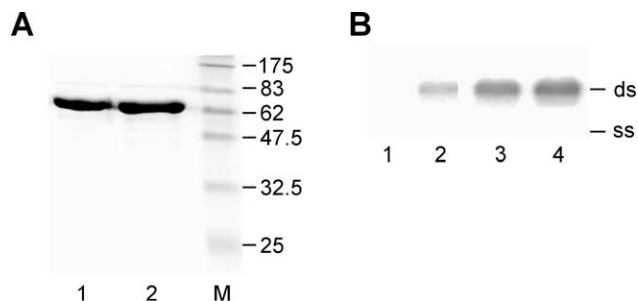

Fig. 2. Purified polymerase subunits catalyze RNA replication in vitro. (A) Purified recombinant φ12 Pol subunits analyzed by SDS–PAGE, followed by Coomassie G-250 staining. Lanes: 1, T425I; 2, wt; M, protein molecular mass standards. (B) Purified φ12 Pol subunits catalyze in vitro replication of sΔ⁻/(ssRNA produced by T7 transcription of pEM16 linearized with BpiI). Reaction mixtures were incubated at 23°C for 1 h and analyzed by standard agarose gel electrophoresis. Lanes: 1, negative control with the enzyme storage buffer; 2, φ12 T425I Pol; 3, φ12 wt Pol; 4, φ6 Pol positive control. Positions of the ssRNA template and dsRNA product are shown on the right.
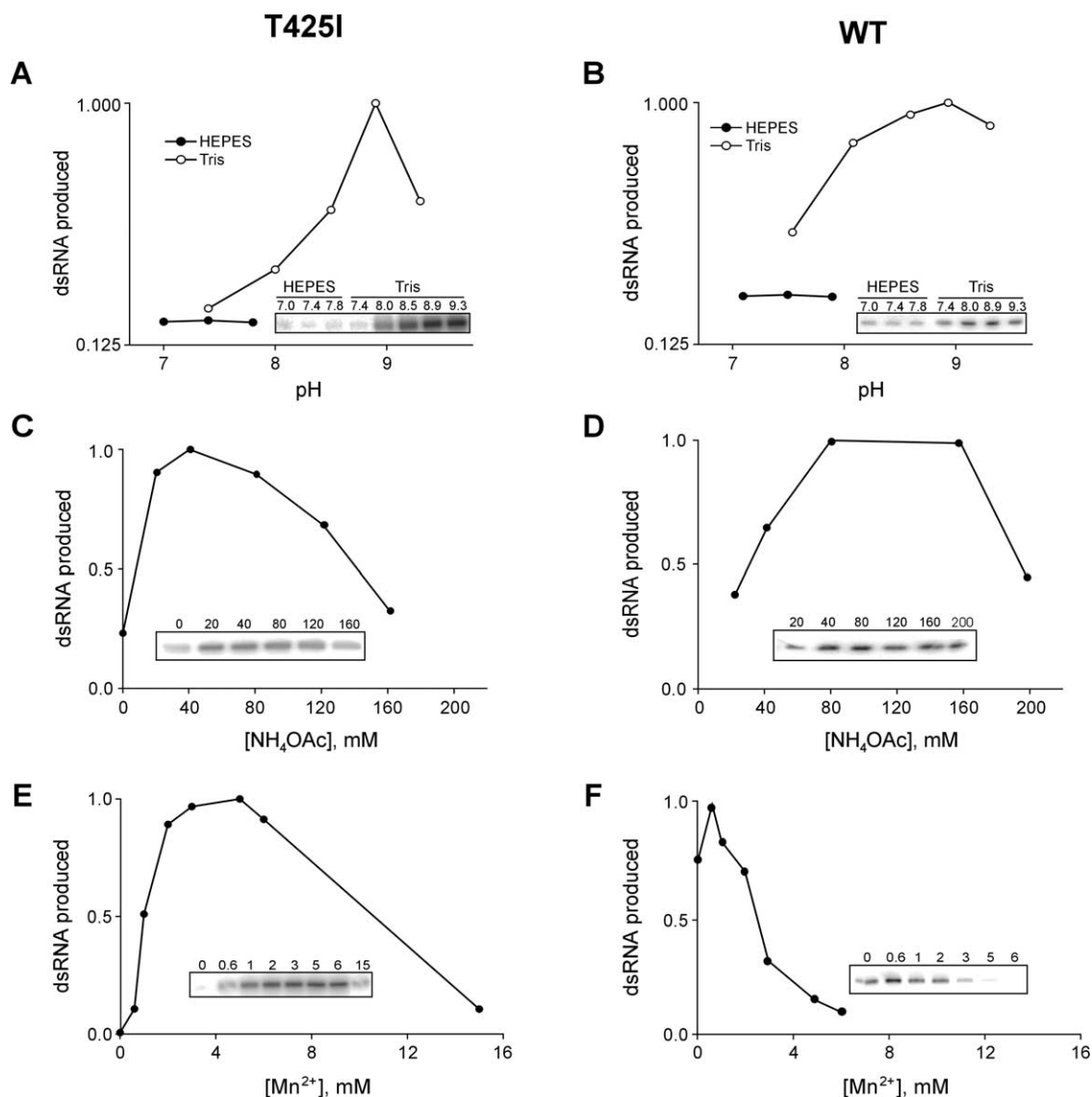
Fig. 3. Effects of the reaction conditions on the activity of the ϕ12 Pol subunits. Reactions containing sΔ^- ssRNA and either wt or T425I Pol were incubated at 30 or 15°C, respectively, for 1 h and analyzed by agarose gel electrophoresis. Radioactivity in the bands of the newly produced dsRNA was quantified by phosphoimaging. The graphs are normalized so that the highest value within each panel is set to 1. Apart from the varying conditions, the other components of reaction mixtures were as described under Materials and methods. (A–B) pH effect; (C–D) NH$_4$OAc concentration effect; (E–F) Mn^{2+} concentration effect.

Pol. The optimal Mn^{2+} concentration was 2–6 mM for T425I (Fig. 3E) and 0.6 mM for the wt enzyme (Fig. 3F). At about 1–2 mM Mn^{2+} the polymerase activity of T425I was increased by more than 10-fold, whereas the stimulatory effect of 0.6 mM Mn^{2+} was relatively modest in the case of the wt (~1.3-fold). Furthermore, higher concentrations of Mn^{2+} (5–6 mM) inhibited the wt enzyme, while being well tolerated by the T425I Pol.

ϕ12 Pol template preferences

To examine the ϕ12 Pol template preferences, we used five variants of sΔ^+ ssRNA. One of the variants, s$\Delta^+(\phi)$, had the natural ϕ6 s$^+$ terminus ($\cdots$CUCUCUCUCU3$'$),

while the other four sΔ^+ templates contained different one-nucleotide additions (A3$'$, C3$'$, G3$'$, and U3$'$). In both wt and T425I reactions, replication efficiency increased considerably, when C3$'$ was used as a terminal template nucleotide (Fig. 4). G3$'$ was the second best addition. The effects of the other two terminal bases were either neutral or somewhat inhibitory.

We also tested ϕ12 Pol replicase activity with a set of firefly luciferase mRNAs, luc(ϕ), ending with $\cdots$ CCCAAGCUUA3$'$, the other four variants containing single nucleotide additions at the 3$'$ end. The addition of C3$'$ and G3$'$ was stimulatory, such as for the sΔ^+ template series (Fig. 4). The A3$'$ and U3$'$ additions stimulated both ϕ12 Pol variants, although moderately. The wt favored U3$'$
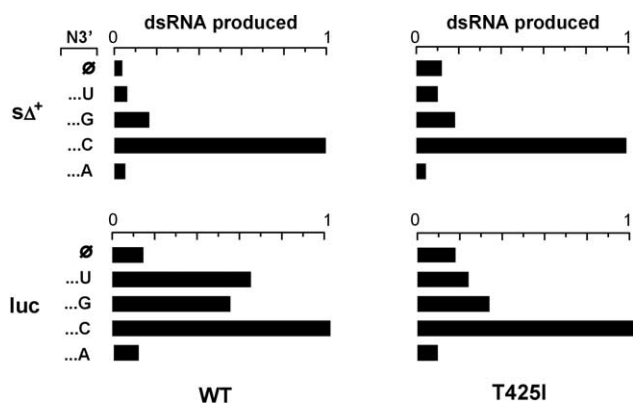
Fig. 4. Effects of the template 3'-terminal nucleotide on replication efficiency. Two sets of ssRNA templates, luc and $s\Delta^+$, were assayed with the ϕ12 Pols wt or T425I at 30 and 15°C, respectively. Each set contained five RNA variants with different 3' ends (N3'), either without modifications (Φ) or extended with one additional 3'-terminal nucleotide (U3', G3', C3', or A3'). Reaction products were separated by standard agarose gel electrophoresis followed by the phosphoimager analysis of the dsRNA band intensities. The graphs are normalized so that the highest value within each panel is set to 1.

terminated luc more than the T425I mutant. This increased affinity toward 3'-terminal C and U is consistent with the fact that transcription of ϕ12 genome segments is initiated with either G (for S and M) or A (for L) (Gottlieb et al., 2002a, 2002b).

The effects of the incubation temperature on the replication efficiency

To test the effects of the incubation temperature, RNA synthesis was initially carried out in the optimized buffer at pH 8.9 as described above with the $s\Delta^-$ ssRNA template (T7 transcript of pEM16 cut with *BpiI*) (Makeyev and Bamford, 2000a). Reactions were incubated for 1 h at different temperatures, from 4 to 50°C, and the reaction products were analyzed by agarose gel electrophoresis. For the wt, the highest dsRNA synthesis was observed at 30°C, while T425I was inactivated at ≥30°C almost completely, showing the maximal activity at 15°C (not shown). However, because ssRNAs templates were partially degraded at pH 8.9 at 50°C, we repeated the experiment for both wt and T425I at pH 8.4, where ssRNA was stable at 50°C. The results were similar to those obtained at pH 8.9 as shown in Fig. 5. However, specific activity of the wt exceeded that of T425I more dramatically at pH 8.4 than at pH 8.9, because T425I has a more narrow pH optimum (Figs. 3A and B and not shown). We conclude from the above experiments that the T425I mutant exhibits temperature-sensitive (*ts*) properties.

Temperature sensitivity of T425I is expressed at the initiation but not elongation stage

To determine which stage of the polymerization reaction limits the T425I thermostability, we used the heparin trap

assay. Heparin is a competitive inhibitor of RNA synthesis acting on the initiation step through its interaction with RdRp apoenzyme. However, heparin apparently cannot bind to the already assembled initiation complex and is therefore benign for the elongation step (Ackermann and Padmanabhan, 2001).

T425I was preincubated at different temperatures in buffer containing Tris–HCl pH 8.4 with ssRNA template $s\Delta^+$13 and three unlabeled NTPs (ATP, CTP, GTP) to form the initiation complex. After this, heparin and [α-^{32}P]UTP were added and the incubation was continued at the same or a different temperature (Fig. 6A). When the T425I mixture was preincubated at 30°C, very little dsRNA was produced, regardless of the temperature used for the elongation step. However, when 15°C was used as the initiation temperature, high yields of the dsRNA product were obtained during elongation at either 15, 30, or 50°C (Fig. 6B). This strongly suggests that the initiation step is thermosensitive, whereas elongation is relatively stable.

Temperature optimum of the wild-type Pols is also limited by initiation

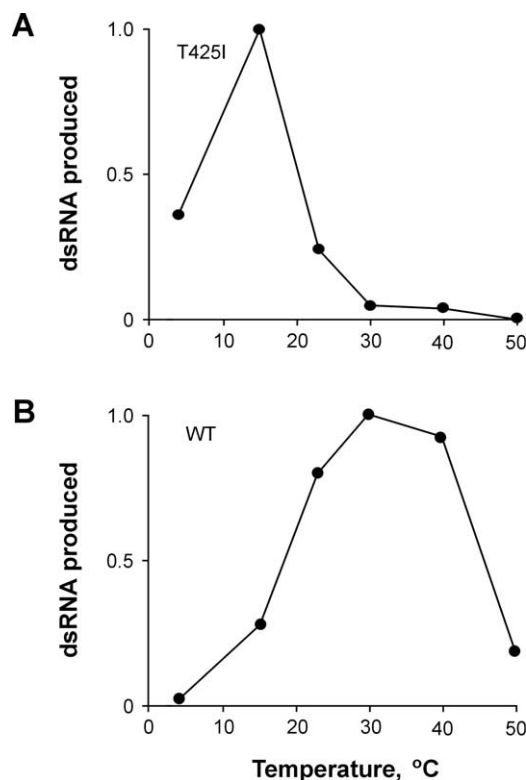We further tested whether the temperature optimum of ϕ12 Pol wt is also due to a higher thermosensitivity of the

Fig. 5. Temperature effect on replication. Reaction mixtures containing the $s\Delta^-$ template and either T425I (A) or wt (B) ϕ12 Pols in the presence of Tris–HCl pH 8.4 were incubated at different temperatures from 4 to 50°C. Reaction products were analyzed by 1% agarose gel-electrophoresis followed by phosphorimaging. The highest polymerization activity value within each panel is set to 1.
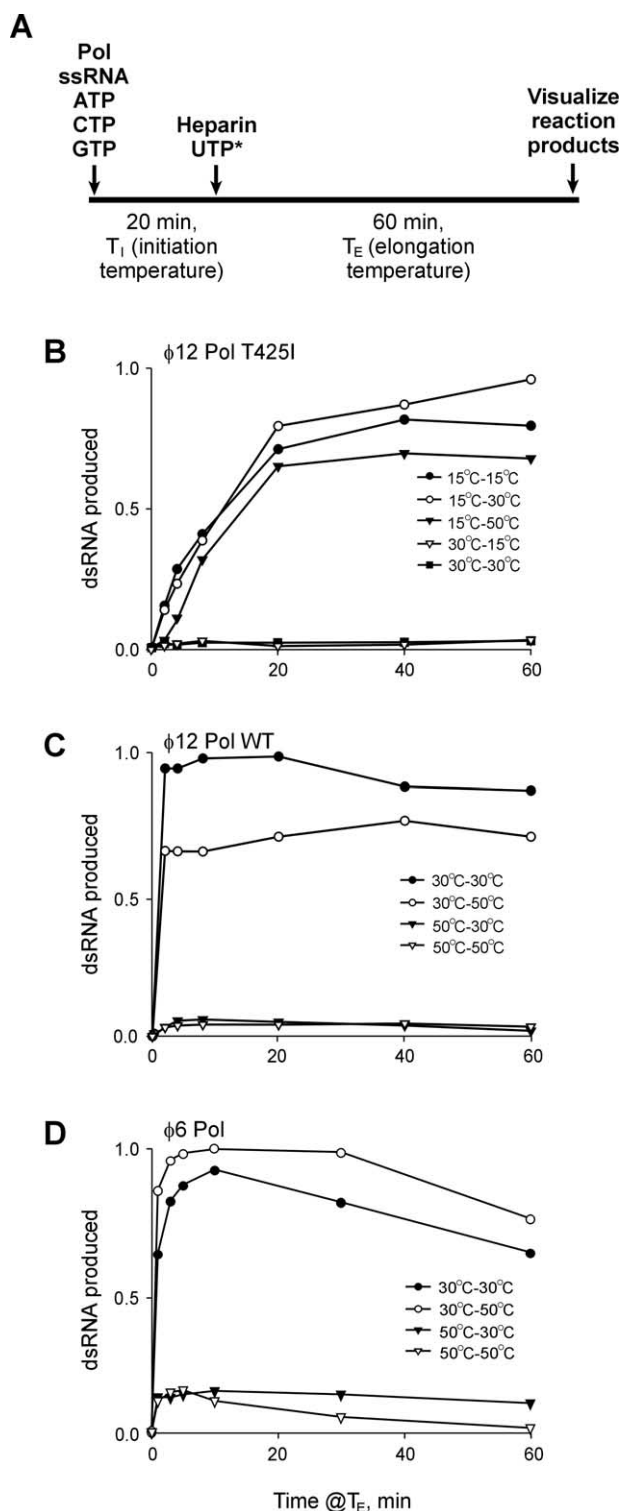
Fig. 6. The upper temperature limit of RNA synthesis is due to thermo-sensitive initiation. (A) Schematic of the heparin trap experiment. (B–D) Purified Pols were preincubated at pH 8.4 with either $s\Delta^+13$ (T425I) or s^+13 template (wt $\phi12$ Pol and $\phi6$ Pol) and three nucleotides (ATP, GTP, and CTP) for 20 min at different temperatures (T_I). After the preincubation, heparin (100 $\mu g/\mu l$ for $\phi6$ Pol and 50 $\mu g/\mu l$ for $\phi12$ Pols), and subsequently [α-^{32}P]UTP, were added and the incubation was continued for additional 1 h at the same or different temperature (T_E). Reaction products were analyzed by agarose gel electrophoresis followed by phosphoimaging. The highest value within each panel is set to 1. The four curves in each panel correspond to the four reactions shifted from T_I to T_E (T_I–T_E).

initiation step. A heparin trap experiment was carried out as described above for the T425I mutant except that 30 and 50°C temperature points and ssRNA template s^+13 were utilized (Fig. 6C). The results show that the wt initiation is the temperature-limiting step, as in the case of the *ts* mutant. Similar results were also observed when the $\phi6$ Pol was used in the heparin trap assay with the s^+13 template. In this case, the temperature points were 30 and 50°C. The results again showed that initiation is the temperature-limiting step in that the shift to 50°C after initiation at 30°C allowed elongation to continue (Fig. 6D).

Discussion

The RdRps of the dsRNA viruses perform both replication and transcription reactions as part of a complex with other proteins. However, RdRp subunits (Pols) from cystoviruses $\phi6$, $\phi8$, and $\phi13$ have been demonstrated to catalyze RNA synthesis in isolation (Makeyev and Bamford, 2000a, 2000b; Yang et al., 2001, 2003). In the present study, we purified a recombinant Pol subunit of cystovirus $\phi12$ and showed that, despite its low sequence similarity to the $\phi6$, $\phi8$, and $\phi13$ Pols, this protein also efficiently catalyzes RNA-dependent RNA polymerization in vitro. This once more demonstrates that proteins with clearly divergent amino acid sequences often perform similar biological functions.

In agreement with this notion, the recently determined $\phi6$ Pol structure resembles those of hepatitis C virus (HCV) and calicivirus polymerases (Butcher et al., 2001; E.V. Makeyev and J.M. Grimes, unpublished data), while the sequences of the three proteins are dissimilar. $\phi6$ Pol contains 25 α-helices and 21 β-strands producing hand-like features: fingers, palm, and thumb. Additional structural elaborations, the priming domain and strands interconnecting the fingers and thumb, create a "cupped-hand" architecture with an encircled active site, which is characteristic for other RdRps as well (Butcher et al., 2001).

The $\phi12$ Pol amino acid sequence, when subjected to the Basic Local Alignment Search Tool (BLAST), was found to have 21, 24, and 20% identities to the comparable proteins of $\phi6$, $\phi8$, and $\phi13$, respectively (Gottlieb et al., 2002a). Similar to other cystovirus polymerases, $\phi12$ Pol contains the (G/S)DD motif as identified between $\beta15$ and $\beta16$ of the $\phi6$ Pol (Butcher et al., 2001; Yang et al., 2001). In one notable deviation from the other Pols, the RRRTA sequence that is found to be strictly conserved in the $\beta11$ region of the three other proteins is RTRLV in $\phi12$ Pol (Gottlieb et al., 2002a). This motif has been found to interact with the phosphate groups of the incoming NTP substrate in $\phi6$ (Butcher et al., 2001). In our structural simulation, both GDD motif and the NTP-binding arginines of $\phi12$ Pol are correctly positioned facing the internal cavity of the enzyme (Fig. 1).

The structure of the $\phi6$ Pol complex with the RNA

template and NTPs established a model for de novo initiation of the RNA-dependent RNA polymerization (Butcher et al., 2001). A key aspect of this model is a polymerase–template complex, in which a tyrosine at the C-terminal domain provides a platform on which an initiation complex could be constructed. The tyrosine base stacks with incoming nucleotides that at the same time are pairing with the complementary nucleotide on the template RNA. It has been suggested that in ϕ13, this tyrosine can be functionally substituted by tryptophan (Butcher et al., 2001). We predict that in the ϕ12 Pol the His622 residue, located at the tip of the "initiation" loop of the priming domain, is functionally equivalent to the Tyr630 of ϕ6 Pol (Fig 1). The aromatic nature of histidine side chain makes the stacking interaction with nucleotide bases theoretically possible. In future studies, the "priming" role of the His side chain can be approached by site-directed mutagenesis, as it has been done for ϕ6 Pol (Laurila et al., 2002).

The biochemical properties of ϕ6, ϕ8, and ϕ13 Pols were compared in previous studies (Yang et al., 2001, 2003). While all three enzymes could function over a wide pH range, in Tris–HCl buffer the optima was highest for ϕ8 Pol followed by ϕ13 Pol and then ϕ6 (Yang et al., 2001). The current study with the two ϕ12 Pol variants demonstrated that the pH optima for both of them were about the same, pH 8.9. The T425I substitution in the mutant rendered its activity very sensitive to altered pH conditions (Figs. 3A and B). In addition, both ϕ12 Pol variants exhibited low activity in HEPES buffer, in contrast to the previous observation made upon the ϕ6, ϕ8, and ϕ13 Pols, showing that their enzymatic activity in HEPES–KOH was always higher than that in Tris–HCl at the same pH value.

In regard to the monovalent cation optimum of the ϕ12 Pol variants, the wild-type displayed its largest relative activity from 80 to 160 mM ammonium acetate (NH$_4$OAc) and then its activity diminished with higher concentrations. In contrast, the T425I mutant's activity peaked at 40 mM NH$_4$OAc and dropped considerably at higher concentrations. Pols of the other three cystoviruses ϕ6, ϕ8, and ϕ13, are known to have considerable activity without any NH$_4$OAc (Yang et al., 2001).

Reactions performed upon the two ϕ12 Pol variants with varying amounts of manganese (Mn^{2+}) showed significant differences between them in that the optimal concentration was much lower for the wild-type than the mutant (0.6 vs 2–6 mM, respectively). Furthermore, it was evident that the wild-type enzyme showed significant activity without any Mn^{2+}, while the mutant required this divalent cation for its activity. When these same reaction conditions were tried with the ϕ6, ϕ8, and ϕ13 Pols, all three had an optima of 2 mM, although the concentration dependence was the greatest with the ϕ6 Pol (Yang et al., 2001). This effect was previously seen in the context of the entire ϕ6 polymerase complex, where both the replication and the transcription activities were enhanced in the presence of Mn^{2+} (Gottlieb et al., 1991, 1992; van Dijk et al., 1995).

An important observation is that ϕ12 Pol containing the point mutation T425I is a *ts* enzyme (Fig 5). Together with the narrower pH optimum of the mutated ϕ12 Pol, its *ts* phenotype suggests that the substitution of Thr425 to more bulky Ile affects the stability of the protein fold. In line with this idea the elongation rate of the T425I mutant was lower than that of the wt even at permissive temperature, 15°C (not shown).

Importantly, in both ϕ12 Pol variants, as well as ϕ6 Pol, de novo initiation of RNA synthesis is more thermosensitive than the elongation step (Fig. 6). We further notice that the *ts* phenotype of the T425I ϕ12 Pol is likely to be manifested predominantly at the initiation stage, since the mutant can elongate at 50°C (Fig. 6B). However, T425I-catalyzed elongation is also somewhat affected at higher temperatures, as obvious from the detectable lag period at 50°C (compare the 15–50°C and 15–15°C curves in Fig. 6B).

These results extend the earlier data on the recombinant RdRp subunit of dengue virus (Ackermann and Padmanabhan, 2001). In in vitro assays, dengue polymerase can either initiate RNA polymerization de novo or extend the template 3' terminus folded back to prime the synthesis of a hairpin-like dimer ("back-priming"). At moderate temperatures, the enzyme predominantly uses de novo initiation, whereas back-priming dominates at elevated temperatures (Ackermann and Padmanabhan, 2001). Based on these observations, the authors suggested a model where dengue RdRp can exist in either "closed" or "open" form, and the latter is favored at higher temperatures. When open, the enzyme binds to a fold-back structure located at the template's 3' end with the subsequent elongation producing a dimerized product. Conversely, the closed enzyme, favored at lower temperatures, recognizes the single-stranded 3' terminus of the template and initiates de novo synthesis.

In the case of wild-type ϕ6 Pol and the two ϕ12 Pol variants, both de novo initiation and back-priming were detected, but de novo initiation prevails (Laurila et al., 2002; and data not shown). Based on our temperature-dependent results, it is reasonable to suggest that de novo initiation may require a specific conformation of the Pol subunit (or entire initiation complex) that cannot be attained at increased temperatures. This explanation is consistent with our previous data on de novo initiation (Laurila et al., 2002), and it might be an interesting update to the dengue polymerase model. Indeed, the closed conformation of the polymerase may actively favor de novo initiation, in addition to impeding back-priming through a steric mechanism proposed earlier (Ackermann and Padmanabhan, 2001). Future studies will show whether the temperature resistance of RdRps from other RNA viruses is also limited at the initiation stage and if this property can be useful for developing new approaches to treatment and prevention of viral infections.

From a theoretical standpoint, if the temperature sensitivity is indeed a hallmark of de novo initiation by the RdRp, we wonder whether RNA viruses can infect thermo-

philic organisms. Thus far, no such viruses have been found in thermophilic archaea and eubacteria (Mark J. Young, Thermal Biology Institute, Montana State University, personal communication). Should RNA viruses be discovered, it would be interesting to see how the initiation complex is stabilized at elevated temperatures.

The data presented in this work demonstrate that the thermostability of cystoviral RdRp can be manipulated to some extent through protein engineering. One of the goals of this development would be to create a thermostable RdRp suitable for RNA amplification via a chain reaction process ("RNA-PCR"). The ability of RdRp to initiate nucleic acid synthesis in the absence of defined primers would be an advantage in that RNA samples lacking sequence information could still be amplified. This, coupled with the direct RNA sequencing of the amplified product also utilizing a cystovirus Pol (Makeyev and Bamford, 2001), would provide a powerful diagnostic tool in clinical samples carrying suspected RNA viral pathogens.

Materials and methods

Bacterial strains and plasmids

E. coli strain XL1-Blue Supercompetent Cells (*recA1, end1, gyrA96, thi-1, hsdR17, supE44, relA1,* [F′, *proAB, lacl^q, Z∆M15,* Tn*10* (Tet^r)]) (Stratagene, La Jolla, CA) were used as hosts for both cDNA cloning and plasmid propagation. To construct a plasmid for the ϕ12 P2 protein expression, the P2 encoding sequence was PCR-amplified from the pP12LB template using the Roche High-Fidelity PCR Kit (Roche Molecular Biochemicals, Indianapolis, IN) with oligonucleotides 5′-CGGAGCGAT <u>CCATGG</u>CACCGAC-GAAT-3′, (p2phi12up), and 5′-GTACA<u>GGATCC</u>TTAT-ACGTAATTACCTTTGTAATGTTCT-3′, (p2phi12down), as the upstream and downstream primers, respectively. The PCR fragment was then digested with *Nco*I and *Bam*HI (underlined sites in the primer sequences), gel purified, and ligated with the *Nco*I-*Bam*HI cut large fragment of vector pET-21d (Novagen). *E. coli* strain XL1-Blue was transformed with the ligation mixture and plasmid pPG14 was isolated from the transformants. This plasmid was subsequently transformed into *E. coli* strain BL21(DE3) and cell lysates of these transformants were analyzed by SDS–PAGE for protein P2 production. DNA sequence analysis of the inserted DNA carried by pPG14 demonstrated a P2 mutant, T425I, was being produced. The P2 encoding sequence was again PCR-amplified from the pP12LB template. The resulting PCR fragment was digested with *Nco*I and *Aat*II, gel purified, and ligated into the large fragment of *Nco*I and *Aat*II cut pPG14. *E. coli* strain XL1-Blue was transformed with the ligation mixture and plasmid pPG24 was isolated from the transformants. DNA sequence analysis of this plasmid showed that the wild-type T amino acid residue had been restored to the P2 protein. Plasmid pPG24

was then transformed into *E. coli* strain BL21(DE3) for protein expression.

Expression and purification of recombinant Pol subunits

The wt and the T425I mutant of ϕ12 Pol were produced and purified as described for ϕ6 Pol, with some modifications (Butcher et al., 2000; Makeyev and Bamford, 2000b). Briefly, starter cultures were grown in the Luria-Bertani medium containing 150 mg/ml ampicillin at 37°C to OD_{540} 0.6. The cultures were then diluted 50-fold, and the incubation was continued until OD_{540} reached 1.0. Expression of the recombinant polymerase was induced with 10 μM isopropy-ᴅ-thiogalactopyranoside (IPTG). After the addition of IPTG, bacterial cultures were shaken for 15 h at 18°C. Protein purification was carried out at 4°C. Throughout the purification, the pH value was 8.0. Bacterial pellets were resuspended in buffer A (35 ml of 50 mM NaCl–25 mM Tris–HCl–0.5 mM EDTA) and the suspensions were passed three times through a precooled French pressure cell at ~105 MPa. Phenylmethylsulfonyl fluoride was added to 1 mM after the first passage. The lysate was cleared at 120,000 *g* for 2.5 h. Two successive chromatography steps using Reactive Brown 10 agarose (Sigma) and heparin agarose (Sigma) were employed. In some cases, protein was diluted with buffer C (100 mM NaCl; 10 mM Tris–HCl, pH 8.0; 0.1 mM EDTA) after the heparin agarose step and concentrated using Centrex UF-2 (30-kDa cutoff; Schleicher & Schuell). Protein concentration was determined by the absorbance at 280 nm in 6 M guanidine hydrochloride based on 1.37 OU for 1 mg/ml of ϕ12 Pol (Edelhoch, 1967). Purified polymerases were stored at 4°C for further activity assays.

Preparation of RNA substrates

Single-stranded RNAs (ssRNAs) were produced by in vitro transcription with T7 RNA polymerase as described (Makeyev et al., 1996). Templates for the transcription were prepared by either cutting recombinant plasmid DNA with restriction endonucleases or PCR amplification with *Pfu* DNA polymerase. For PCR, oligonucleotide T7-1 containing the T7 polymerase promoter sequence was always used as an upstream primer. Oligonucleotides 3′ end to 3′end4 were downstream primers for the amplification of the s∆^+ fragment from pEM15 (Makeyev and Bamford, 2000a), whereas pT7-3′end to pT7-3′end4 served as downstream primers to amplify the luciferase gene from pT7luc (Makeyev et al., 1996).

Polymerase activity assay

ϕ6 Pol was assayed in 10 μl reaction mixtures as described earlier (Yang et al., 2001). ϕ12 Pol T4251 performed optimally in mixtures containing 50 mM Tris–HCl pH 8.9, 40 mM ammonium acetate (NH_4OAc), 6% (w/v)

Table 1
Oligonucleotides used in this study

Name	Sequence
p2phi12up	5′CGGAGCGATCCATGGCACCGACGAAT
p2phi12down	5′GTACA GGATCCTTATACGTAATTACCTTTGTAATGTTCT
T7-1	5′CGCGTAATACGACTCACTATAG
pT7-3′end	5′TAAGCTTGGGCTGCAGGT
pT7-3′ end_1	5′ATAAGCTTGGGCTGCAGGT
pT7-3′ end_2	5′CTAAGCTTGGGCTGCAGGT
pT7-3′ end_3	5′GTAAGCTTGGGCTGCAGGT
pT7-3′ end_4	5′TTAAGCTTGGGCTGCAGGT
3′end	5′AGAGAGAGAGCCCCCGA
3′end_1	5′AAGAGAGAGAGCCCCCGA
3′end_2	5′CAGAGAGAGAGCCCCCGA
3′end_3	5′GAGAGAGAGAGCCCCCGA
3′end_4	5′TAGAGAGAGAGCCCCCGA

PEG4000, 5 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM EDTA, 0.1% Triton X-100, 1 mM each of ATP and GTP, 0.2 mM each of CTP and UTP, 0.8 U/μl RNasin, and 0.025–0.25 mCi/ml [α-^{32}P]UTP (Amersham; 3000 Ci/mmol). Optimal conditions for the ϕ12 Pol wt were similar except for 80 mM NH_4OAc and 0.6 mM $MnCl_2$. In all assays, the final concentration of RNA templates was 50–200 μg/ml. Reactions were started by adding Pol up to 8–40 μg/ml, which was followed by 1 h incubation. Typical incubation temperatures were 15°C for T425I and 30°C for the wt ϕ12 Pol. Reaction products were separated by standard agarose gel electrophoresis (Makeyev and Bamford, 2000b). Gels were dried and exposed with Fuji Super RX film or analyzed with a Fuji BAS1500 PhosphoImager.

Heparin trap assays

We modified the protocol described for the dengue virus RdRp, which allows one to separate the initiation and elongation steps of RNA polymerization (Ackermann and Padmanabhan, 2001). ϕ12 Pols were preincubated with ssRNA templates sΔ^+13 (T7 transcript of pEM15 cut with *Sma*I) or s$^+$13 (T7 transcript of pLM659 cut with *Sma*I)(Gottlieb et al., 1992) in the mixtures lacking both labeled and unlabeled UTP for 20 min at different temperatures (initiation step). The other components of the preincubation mixtures were exactly as described above for the ϕ12 Pol polymerase activity assays. Tris–HCl pH 8.4 was used in the reaction buffer instead of pH 8.9 to avoid ssRNA degradation. ϕ6 Pol was incubated with s$^+$13 in the reaction buffer containing 50 mM HEPES–KOH pH 7.8 and 1 mM $MnCl_2$. Heparin was then added to 100 μg/μl for ϕ6 Pol and to 50 μg/μl for ϕ12 Pols and the incubation was continued for another 5 min, after which the reactions were supplemented with 0.2 mM UTP and 0.25 mCi/ml [α-^{32}P]UTP. This was followed by 1 h incubation at appropriate temperatures (elongation step) and aliquots were withdrawn at different time points. Reaction products were separated by standard agarose gel electrophoresis and analyzed with a Fuji BAS1500 PhosphoImager.

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