doi:10.1016/j.jmb.2004.10.018 J. Mol. Biol. (2004) 344, 1251–1263

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The Heterodimeric Primase of the Hyperthermophilic Archaeon *Sulfolobus solfataricus* Possesses DNA and RNA Primase, Polymerase and 3′-terminal Nucleotidyl Transferase Activities

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A eukaryotic-type primase was identified in the crenarchaeon *Sulfolobus solfataricus*. The two-subunit DNA-dependent primase, termed PriSL, was purified following co-expression of the subunits in *Escherichia coli* and its activity was characterised. PriSL was capable of utilising both ribonucleotides and deoxyribonucleotides for primer synthesis in the presence of natural, or synthetic, single-stranded DNA. A broad distribution of products was detected, ranging from dinucleotides to DNA molecules in excess of 7 kb and RNA up to 1 kb in length. However, PriSL had a significantly higher affinity for ribonucleotides than for deoxyribonucleotides. Using site-directed mutagenesis, two aspartate residues crucial for nucleic acid synthesis and residues important for the binding of free nucleotides were identified. In addition to the primase and polymerase activities, we reveal that the primase possesses a template-independent 3′-terminal nucleotidyl transferase activity.

Keywords: DNA replication; polymerase; terminal transferase; archaea; primase

Introduction

Although the archaea are prokaryotic organisms, they possess information processing pathways more similar to those of eukaryotes than of bacteria. In particular, the archaeal transcription and DNA replication machineries are homologous to their eukaryotic counterparts.[1,2] In all organisms studied to date, the major replicative DNA polymerase is not capable of initiating *de novo* DNA synthesis. Rather, the polymerase acts to extend an oligonucleotide primer that is synthesised by the DNA primase.[3] Cellular primases appear to be classified along two phylogenetic lineages: the bacterial primases, typified by *Escherichia coli* DnaG, and the more complex archaeal and eukaryotic primases.

Bacterial primases are single subunit enzymes that possess a zinc binding motif in the N-terminal domain of the protein and an RNA polymerase domain in the C-terminal region.[4] Structural studies have revealed that the catalytic centre of the bacterial enzymes is related to that of other phosphoryl transfer enzymes such as type IA and type II topoisomerases. This has allowed the definition of this signature motif as the TOPRIM fold.[5]

In contrast to the single subunit bacterial primases, the primases of archaea and eukaryotes appear considerably more complex.[3,6] Eukaryotic primase enzymes have a small (approximately 49 kDa) catalytic subunit that associates tightly with a larger subunit (typically 58 kDa). The catalytic subunit shares no significant sequence or structural homology to the bacterial primase. Although the catalytic subunit has intrinsic primase activity, its association with the large subunit modulates stability, DNA binding and synthetic capability of the enzyme.[7] These two subunits associate with two further proteins, the B subunit and DNA polα, to form the tetrameric polα primosome complex. The polα primosome has therefore the capacity to both synthesise RNA primers and then extend them for a short distance with DNA synthesised by polα. Intriguingly, archaea do not possess homologues of either polα or the B subunit. However, archaeal homologues of

Abbreviations used: DN, deoxynucleotide; dDN, dideoxynucleotide; CS, catalytic site; DB, DNA binding.

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small and, more recently, large subunits of eukaryotic primase have been identified.[8–11] In addition, the crystal structure of the small, catalytic subunit of the primase of the euryarchaeon, *Pyrococcus furiosus*, has recently been determined.[12] Studies of the isolated small subunit of the *Pyrococcus* enzyme revealed that this protein preferentially synthesised DNA and could produce molecules in excess of a kilobase in length.[8] However, recent studies of the reconstituted *Pyrococcus* large and small subunit heterodimer have indicated that the large subunit reduces the processivity of the DNA polymerase activity and confers the ability to synthesise RNA oligomers upon the enzyme.[9]

Here, we describe the first characterisation of a DNA primase from the Crenarchaea. We identify the genes for the two subunits of the primase from the hyperthermophile *Sulfolobus solfataricus* and reconstituted the heterodimeric enzyme. We examine the nucleic acid synthetic capability of the primase and uncover a novel activity of the complex, namely a 3′-terminal nucleotidyl transferase-like activity.

Results

Purification of the PriSL complex

Examination of the annotation of the genome sequence of *Sulfolobus solfataricus* P2[13] revealed the presence of a predicted open reading frame,

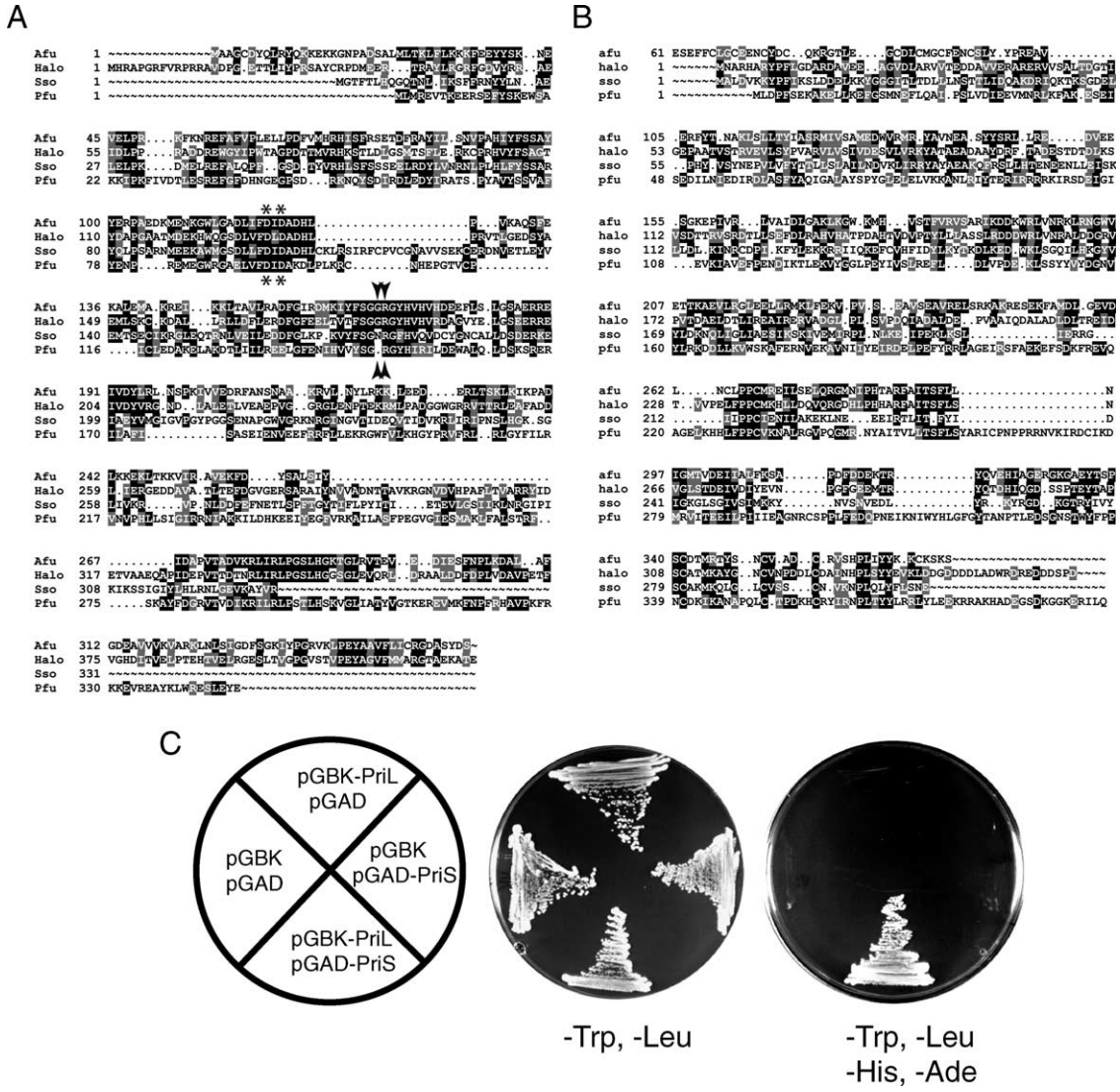

Figure 1. *S. solfataricus* primase small catalytic subunit (PriS) and large subunit (PriL) interact. Amino acid sequence alignments of PriS (A) and PriL (B) with their homologues in *Archeoglobus fulgidus* (Afu), *Halobacterium spNRC1* (Halo) and *Pyrococcus furiosus* (Pfu). Conserved amino acid residues are highlighted in black. The asterisks indicate the predicted catalytic residues (D101 and D103) and the arrowheads indicate the predicted DNA-binding residues (N175 and R176), as described.[12] (C) The genes encoding *S. solfataricus* primase subunits PriS and PriL were inserted into the yeast two-hybrid vectors pGADT7 and pGBKT7, respectively. Yeast cells were transformed with the plasmids, as indicated in the Figure and grown on non-selective (-Trp, -Leu) and selective (-Trp, -Leu, -His, -Ade) media.

Sso1048, encoding a protein homologous to the small, catalytic subunit of the archaeal/eukaryal primases. Hereinafter, this protein will be referred to as PriS (Figure 1(A)). Intriguingly, the gene for PriS overlaps that of PCNA2, one of the subunits of the heterotrimeric sliding clamp of *S. solfataricus*.[14] PsiBlast searching of the *S. solfataricus* genome also revealed the presence of a potential homologue of the primase large subunit (Sso0557), subsequently referred to as PriL (Figure 1(B)). The *priL* gene was located within 2 kb of the gene encoding the B-type DNA polymerase, polB1. It should be noted that in *S. solfataricus*, the predicted protein encoded by *priL* (35.7 kDa) is actually of lower molecular mass than that encoded by *priS* (37.6 kDa). Nevertheless, we felt that it would be simplest to refer to the proteins based on the homology to the differently sized eukaryotic proteins.

To test whether the PriL and PriS proteins could interact, the genes for the two proteins were cloned into pGADT7 and pGBKT7 for yeast two-hybrid analysis. PriL was expressed as a fusion protein with the *GAL4* DNA-binding domain and PriS was fused to the *GAL4* activation domain. When expressed individually, neither PriL nor PriS fusion proteins supported growth on medium lacking histidine and adenine. However, when both fusion proteins were expressed in yeast, growth was supported on selective medium, indicating activation of the *HIS3* and *ADE2* reporter genes (Figure 1(C)). These data, in combination with the co-purification of the two subunits described below, strongly suggest that the two proteins form a heterodimeric complex.

Since attempts to express PriL and PriS individually as recombinant proteins were unsuccessful, and in light of the yeast-two hybrid data, a strategy involving the co-expression of both proteins in *E. coli* was adopted. Using the pETDuet-1 vector and after induction of protein synthesis with IPTG, two bands were detected by SDS-PAGE. The initial expression construct utilised yielded a native length PriS, and PriL fused to a hexa-histidine tag, used for the subsequent purification of the protein on Ni-NTA agarose. Heat-treated cell extract was thus applied over a Ni-NTA agarose column and bound proteins were eluted with imidazole. The proteins were then further purified on a heparin column. Analysis of the eluted fractions by SDS-PAGE and Coomassie staining showed that PriS and PriL co-purified (Figure 2(A)). Further analysis of these two protein bands by mass fingerprinting identified the upper band as PriS and the lower band as PriL, in good agreement with their predicted molecular masses. The co-purification of the non-His tagged PriS with PriL over Ni-NTA and heparin column strongly supports the two-hybrid data which indicated that these two proteins interact.

Since the presence of the His-tag might interfere with protein activity, a construct for the co-expression of native length, non-tagged PriS and PriL was generated. These proteins were purified from *E. coli* extracts using a heat treatment step, followed by fractionation over HiTrap Heparin column (Figure 2(B)). The purified protein was further subjected to analytical gel filtration on a Superdex 200 column and was found to elute with an apparent molecular mass of ~67 kDa, in good agreement with a heterodimer (Figure 2(C)). This conclusion was supported by quantitation of the Coomassie-stained gel, which indicated that PriL and PriS eluted with a 1 : 1 stoichiometry. Trace amounts of a lower molecular mass species can be detected in Figure 2(A), mass spectrometry revealed that this was a breakdown product of PriL (data not shown).

The primary sequence of *S. solfataricus* PriS shows regions of high identity to the *Pyrococcus furiosus* enzyme (Figure 1(A)). The structure of the PriS subunit of *P. furiosus* has been solved and resulted in predictions of active site residues (Figure 2(D), red) and regions of the primase that may be involved in nucleic acid binding[12] (Figure 2(D), green). An intriguing feature of the proposed DNA-binding groove is the presence of a highly conserved arginine residue that extends into the groove, apparently blocking it (Figure 2(D), dark blue). However, a more recent structural study has proposed that this region of the protein may not be involved in DNA binding, but rather in binding the nucleoside triphosphate substrate.[15] To test these predictions, site-directed mutagenesis was used to introduce alanine substitutions at the predicted catalytic aspartates and blocking residues. These mutant enzymes were purified as described above. Their elution profile, obtained after analytical gel filtration, was indistinguishable from the wild-type protein, indicating that the mutant enzymes also form heterodimers (Figure 2(C)). The enzymatic activity of these mutant proteins is analysed below.

Primase activity of PriSL

Primases are DNA-dependent RNA polymerases that synthesise RNA primers up to 14 nt long in eukaryotes and bacteria.[3,16,17] To test whether the PriSL complex possessed primase activity, the enzyme was incubated with single-stranded M13 DNA in the presence of a nucleoside triphosphates (NTPs) containing ^{32}P-labelled NTP (as indicated for each experiment). The reaction products were then separated on a denaturing polyacrylamide gel, as described in Materials and Methods. When the reaction was performed at 20 °C, very little product was synthesised (Figure 3(A)). From 37 °C to 60 °C, primers of variable size (up to 75 nt) were produced, exhibiting a smear-like profile on the gel. At 75 °C, the temperature at which *S. solfataricus* naturally grows, products with an apparent length of 14 nt and 7 nt were mainly synthesised, together with low amounts of products up to ca 30–40 nt in size. Since short RNA products (<6 nt) have been reported to migrate aberrantly slowly in high percentage denaturing polyacrylamide gels,[18] unlabelled ApA was run alongside the primase

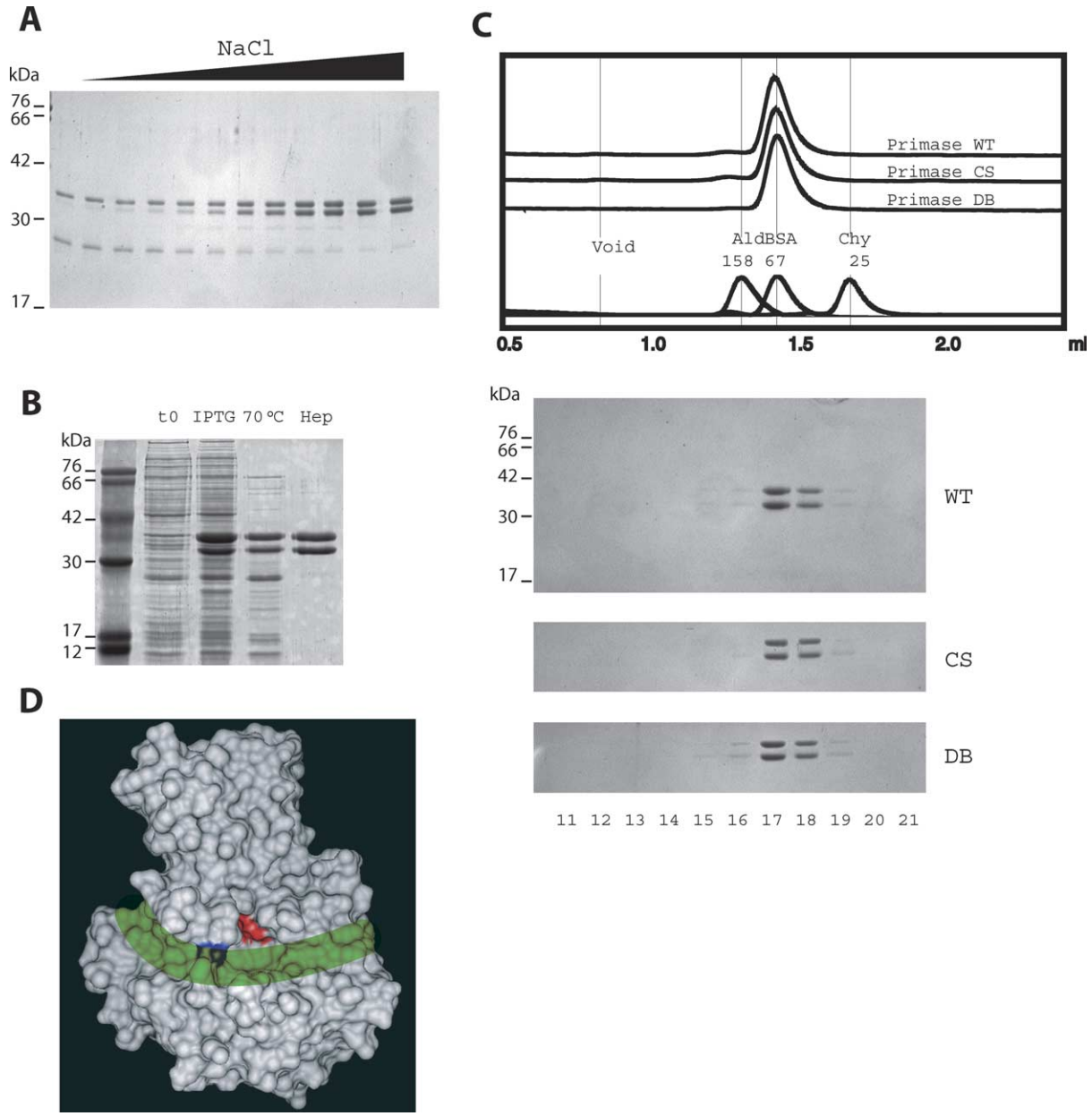

Figure 2. Purification of *S. solfataricus* primase. (A) SDS-PAGE of the elution fractions of native length PriS and His-tagged PriL from a heparin column. (B) SDS-PAGE of protein extracts at various stages of the purification of PriSL: before (t0) and after (IPTG) the induction of protein expression with IPTG, after heat-treatment (70 °C) and after purification on a heparin column (Hep). (C) Elution profile of purified primases (wild-type and mutant proteins) from a Superdex 200 column (top panel). Protein standards used: chymotrypsinogen A (Chy, 25 kDa), bovine serum albumin (BSA, 67 kDa) and aldolase (Ald, 158 kDa). The elution fractions were subsequently analysed by SDS-PAGE and Coomassie staining. (D) Structure of the catalytic subunit PriS of *P. furiosus* (from Augustin *et al.*;[12] PDB acc. no. 1G71). Residues predicted to be involved in enzyme catalysis are shown in red and regions proposed to be involved in nucleic acid binding are shown in green. An intriguing feature of the proposed DNA-binding groove is the presence of a highly conserved arginine residue (in dark blue) that extends into the groove, apparently blocking it.

reaction products to determine whether any of the species might be dinucleotides. By using UV shadowing at 254 nm, we found that the apparent 14 nt length product co-migrated with the dinucleotide (labelled DN in Figure 3(A)). It is apparent from the gel shown in Figure 3(A) that, by increasing the temperature of the primase assay, the quantity of both high and low molecular mass products diminished and the candidate dinucleotide was accumulated. The utilisation of another natural single-stranded DNA, ϕX174, led to the same conclusions (data not shown). These observations suggested that these possible dinucleotides might represent aborted primers not elongated by the primase complex at 75 °C. The nature of this product will be investigated further below.

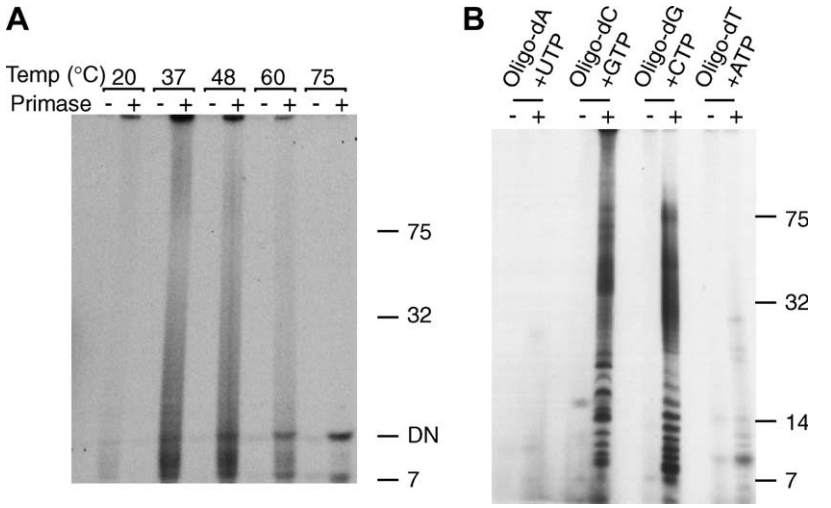

Figure 3. Primase activity with various DNA templates. PriSL was incubated with various single-stranded DNA templates, in the presence of the indicated nucleotides and at the indicated temperatures. The reaction products were separated on a denaturing, 20% acrylamide gel and radioactivity was detected by autoradiography. The approximate size of the bands (in nucleotides) is indicated on the right-hand side of each panel. DN, di-ribonucleotide, as determined by UV shadowing. (A) 1.2 μM PriSL was incubated with M13 and a mixture of unlabelled NTPs and [α-^{32}P]CTP. (B) 0.3 μM PriSL was incubated at 60 °C with homo-oligomers (20-mers) and a mixture of the indicated unlabelled and radiolabelled NTP.

When presented with synthetic DNA templates, eukaryotic primases appear to have a preference for polypyrimidines.[19] To determine whether PriSL from *S. solfataricus* also had DNA substrate preferences, the activity of the enzyme was studied in the presence of each of the four homo-oligomers (20-mer oligo(dA), -(dC), -(dG) and -(dT)). The four different oligonucleotides were used with varying efficiencies, with oligo(d)A being the poorest substrate and oligo(dC) the most preferred one (Figure 3(B)). The latter led to the formation of a ladder of products that differed from one another by one nucleotide length and exhibited a remarkable profile: although the oligonucleotide substrates were 20 nt in length we observed a ladder of products larger than the initial substrate, i.e. extending upwards from the 20 nt in length (Figure 3(B)). As described below, this greater than template length synthetic capability is dependent on the catalytic activity of PriSL.

PriSL synthesises products greater than template length

The ability of the PriSL complex to synthesise greater than template length primers could be explained by a number of mechanisms. First, the primase may slip repeatedly during synthesis, thereby generating extended products. Alternatively, a primer generated in one round of synthesis could anneal to a second template and be extended by the primase. Third, it is conceivable that the primase could possess a terminal nucleotidyl transferase-like activity, that adds nucleotides to the 3′ end of a DNA molecule in a template-independent manner.[20,21] In contrast with this third possibility, the first two mechanisms would be template-dependent and therefore obey Watson–Crick base-pairing rules. Thus, to determine whether PriSL requires Watson–Crick base-pairing to produce these greater than unit length products, the ability of PriSL to add ATP, GTP, CTP or UTP to any of the four DNA homo-oligomers was tested. Each DNA substrate was therefore incubated with PriSL and supplied with each of the four ribonucleotides separately. Strikingly, as shown in Figure 4, any ribonucleotide could be added to any DNA template tested, allowing the formation of variable length products. A closer inspection of these gels led to the following observations. Firstly, in the presence of a 20-mer homo-oligomer and NTPs that are not its Watson–Crick complementary base, the primers synthesised were >20-mer. In addition, there seemed to be a preference for certain NTPs according to the homo-oligomer present. For example, ATP was utilised preferentially over UTP in the presence of oligo(dC). Conversely, the incorporation of UTP was much higher than that of ATP when oligo(dT) was used. Secondly, the strongest signal obtained with PriSL was in the presence of an oligo(dC) template and its complementary base, GTP. In this case, a wide range of RNA products were synthesised, from 12 nt long to very high molecular mass products.

The absence of an absolute requirement for Watson–Crick base-pairing to generate the greater than template length products is reminiscent of a 3′-terminal nucleotidyl transferase activity. To test whether this might be the case, a 20-mer oligonucleotide, called deoxy template, was ^{32}P-labelled on its 5′ end and incubated with primase and CTP only. An oligonucleotide of the same sequence but with a dideoxynucleotide on its 3′ end (dideoxy template), instead of the normal dCMP, was used as a control. Because of the absence of hydroxyl group on the 3′-terminal ddCMP, this oligonucleotide cannot be extended by a terminal transferase. As can be seen in Figure 4(E), we detect a ladder of products of increasing molecular size extending up the gel when the deoxy template was used. These products were not observed in the presence of the di-deoxy template. Taken together these data provide strong evidence for a terminal transferase-like activity of the PriSL complex.

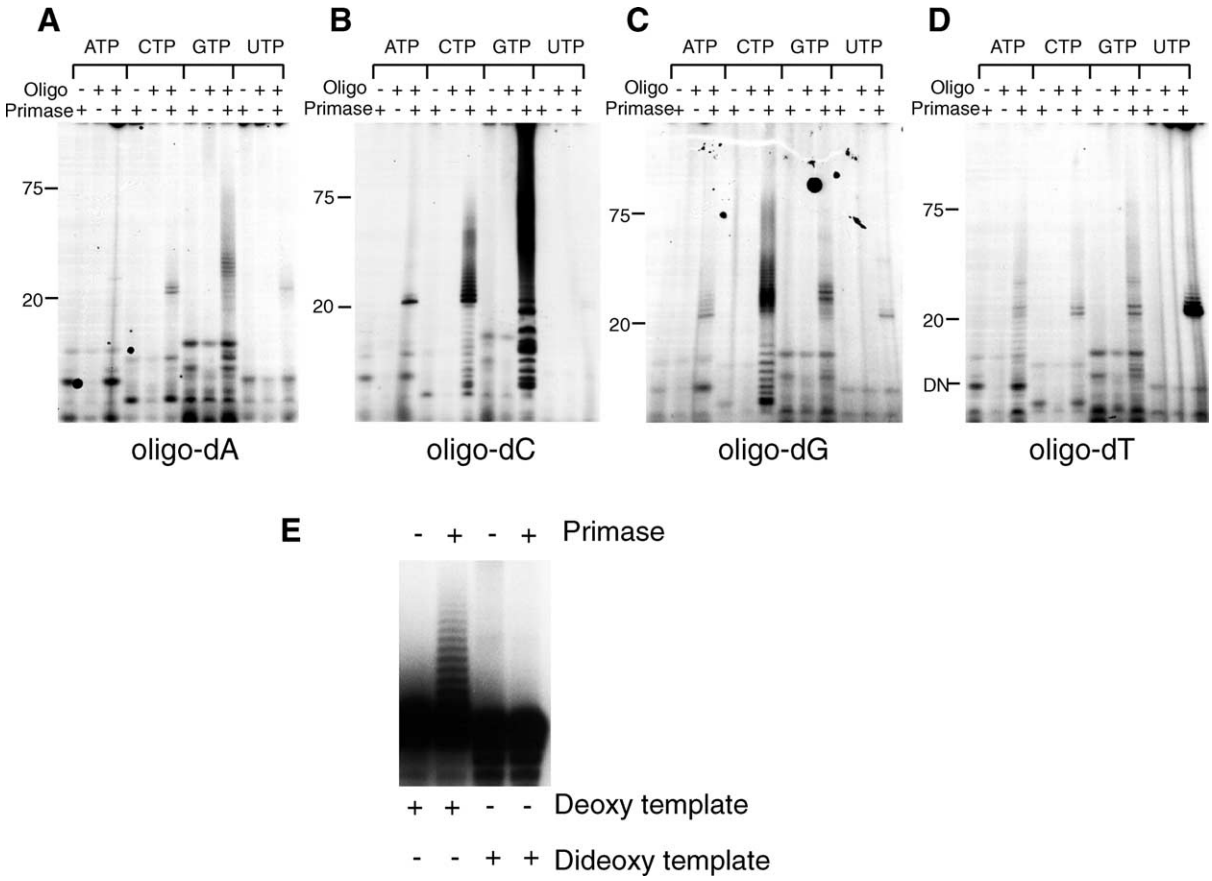

Figure 4. PriSL possesses a terminal transferase-like activity. The reaction products were separated on a denaturing, 20% acrylamide gel and radioactivity was detected by autoradiography. The approximate size of the bands (in nucleotides) is indicated on the left-hand side of each panel. (A)–(D) 0.3 μM PriSL was incubated at 48 °C for 30 minutes, with various 20-mer homo-oligomers (as indicated under each panel) and each of the four NTPs. DN, di-ribonucleotide. Note that the base composition of each dinucleotide affects its migration. Here, only the dinucleotide ApA is indicated by DN. (E) 1.2 μM PriSL was incubated with CTP and two, (5′-^{32}P)-end-labelled random 20-mer oligonucleotides, the deoxy template (TTGTACACGGCCGCATAATC) and a dideoxy template of identical sequence but with dideoxy-CMP instead of dCMP as the 3′ residue. After 40 minutes incubation at 60 °C, the reaction was stopped as described in Materials and Methods and the samples were analysed as described above.

PriSL can synthesise RNA and DNA products

Being RNA polymerases by definition, the major role of primases is to synthesise RNA primers. However, primase from the Euryarchaeon *Pyrococcus* was shown to be capable of incorporating not only ribonucleoside triphosphates but also deoxyribonucleoside triphosphates (dNTPs) into primers.[9,11] Therefore, we wished to determine whether this unusual behaviour was also found in the crenarchaeal *S. solfataricus* primase. PriSL was incubated with M13 and a mixture of either unlabelled NTPs and [α-^{32}P]ATP, or unlabelled dNTPs and [α-^{32}P]dATP. Two negative controls were included, one without any primase and another containing the primase but without M13, in order to compare the background signal with the product profile obtained from the reaction containing both primase and M13 (Figure 5(A)). Significantly, PriSL was indeed capable of accepting dNTPs thereby demonstrating that this capability is not restricted to the euryarchaea but extends to the crenarchaea. As observed above for the RNA products synthesised by PriSL (Figure 3(A)), some DNA products also failed to enter the polyacrylamide gel. We speculated that this may correspond to long products and accordingly, separated the reaction products on agarose gels. RNA and DNA samples were therefore analysed on a formaldehyde–agarose gel (Figure 5(B)) and an alkaline agarose gel (Figure 5(C)), respectively. As shown in Figure 5, RNA products of up to 1 kb (Figure 5(B)) and DNA products of up to 7 kb long (Figure 5(C)) were detected.

Next, we wished to determine whether the primase could utilise dNTPs in the terminal transferase reaction. Accordingly, we incubated the (5′-^{32}P)-radiolabelled deoxy and dideoxy templates used for Figure 4(E) with primase in the presence of dCTP. As can be seen in Figure 5(D) the primase can extend the deoxy, but not the dideoxy, template with dCMP.

Another striking observation is the accumulation of products with an apparent size of 20 nt (labelled

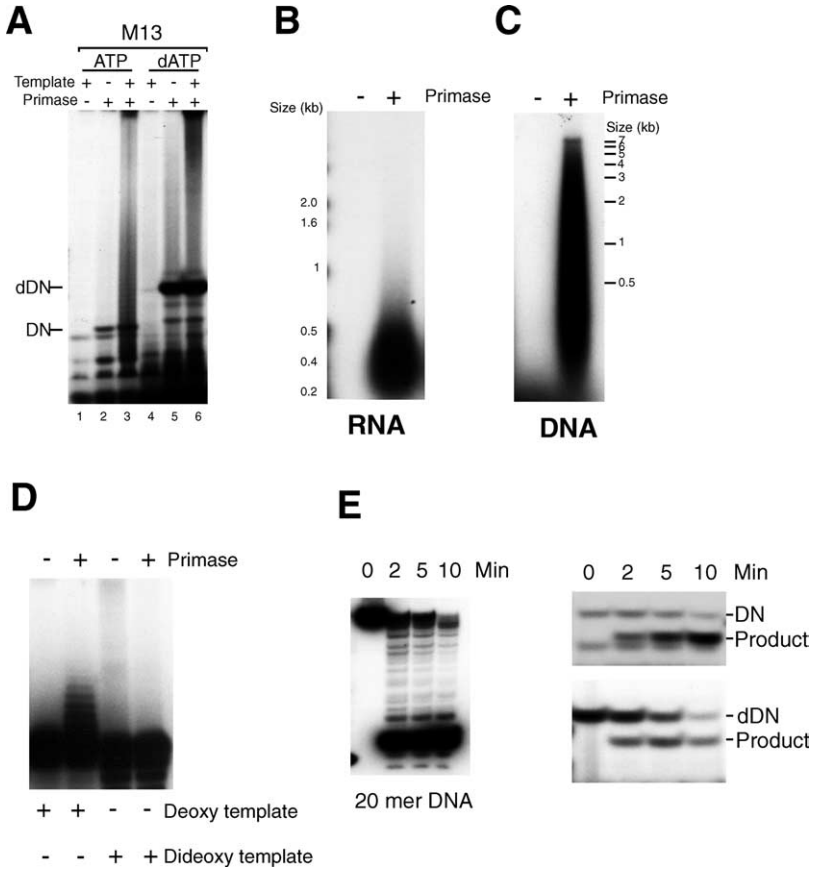

Figure 5. PriSL can utilise both NTPs and dNTPs and synthesise high molecular mass products. Primase assay was performed at 60 °C for 30 minutes, with either M13 and a mixture of all four unlabelled (d)NTPs and [α-^{32}P](d)ATP. (A) The reaction products were separated on a denaturing, 20% acrylamide gel and radioactivity was detected by autoradiography. The approximate size of the bands (in nucleotides) is indicated on the right. DN, di-ribonucleotide; dDN, di-deoxynucleotide; as determined by UV shadowing. (B) RNA products were resolved on a 1% formaldehyde/agarose gel, as described in Materials and Methods. (C) DNA products were resolved on a 1% alkaline agarose gel, as described in Materials and Methods. (D) 1.2 μM PriSL was incubated with dCTP and two, (5′-^{32}P)-end-labelled random 20-mer oligonucleotides, the deoxy template (TTGTACACGGCCGCATAATC) and a dideoxy template of identical sequence but with dideoxy-CMP instead of dCMP as the 3′ residue. After 40 minutes incubation at 60 °C, the reaction was stopped as described in Materials and Methods and the samples were analysed as described above. (E) (d)DN products synthesised by PriSL at 75 °C, in the presence of M13, were phenol/chloroform extracted and the aqueous phase was incubated with phosphodiesterase I for two, five and ten minutes at 37 °C. A 5′ radiolabelled 20-mer single-stranded DNA was used as a control for the activity of the phosphodiesterase.

dDN in Figure 5(A)), both in the negative control lane containing PriSL without M13 template and in the lane containing PriSL and DNA template. As shown previously for RNA products,[18] short oligomers display aberrant mobility in 20% acrylamide gels. Consequently, we speculated that this may be a short (<6 nt) product. The co-electrophoresis of these reactions with unlabelled dApdA followed by UV shadowing allowed the tentative identification of this species as a di-deoxynucleotide (dDN).

To test further the nature of these candidate dinucleotides, we recovered the DN and dDN products from primase reactions and digested them with a range of nucleases. We found that the candidate dinucleotide species were resistant to digestion with micrococcal nuclease under conditions where a 20-mer oligonucleotide was fully digested (data not shown). Previous work has revealed that dinucleotides are highly resistant to digestion by micrococcal nuclease.[22] To confirm that these species possess a normal phosphodiester linkage, we next digested them with snake venom phosphodiesterase. This led to the loss of the band corresponding to dinucleotide (Figure 5(E)). Further, under the conditions employed, we detected processive shortening of a 20-mer oligonucleotide; in contrast, the candidate dinucleotide produced a single product species in one step. Taken together, the co-migration of these species with synthetic dinucleotide, the resistance to cleavage by micrococcal nuclease, the susceptibility to snake venom phosphodiesterase and the resultant digestion pattern strongly support the identification of these species as dinucleotides.

Activity of mutant enzymes

As mentioned above, two mutated derivatives of PriSL were generated by site-directed mutagenesis. Based on the crystal structure of the primase catalytic subunit from *P. furiosus*,[12] residues involved in the putative catalytic and DNA-binding sites of PriS were mutated to introduce alanine substitutions (Figure 1(A)), thus creating mutant primases catalytic site (CS) and DNA binding (DB), respectively. To test the importance of these residues, the primase and terminal transferase-like activities of CS and DB were studied. The ability of the mutant proteins to incorporate dNTPs was also investigated. Using M13 as a template and a mixture of unlabelled and radiolabelled (d)NTPs,

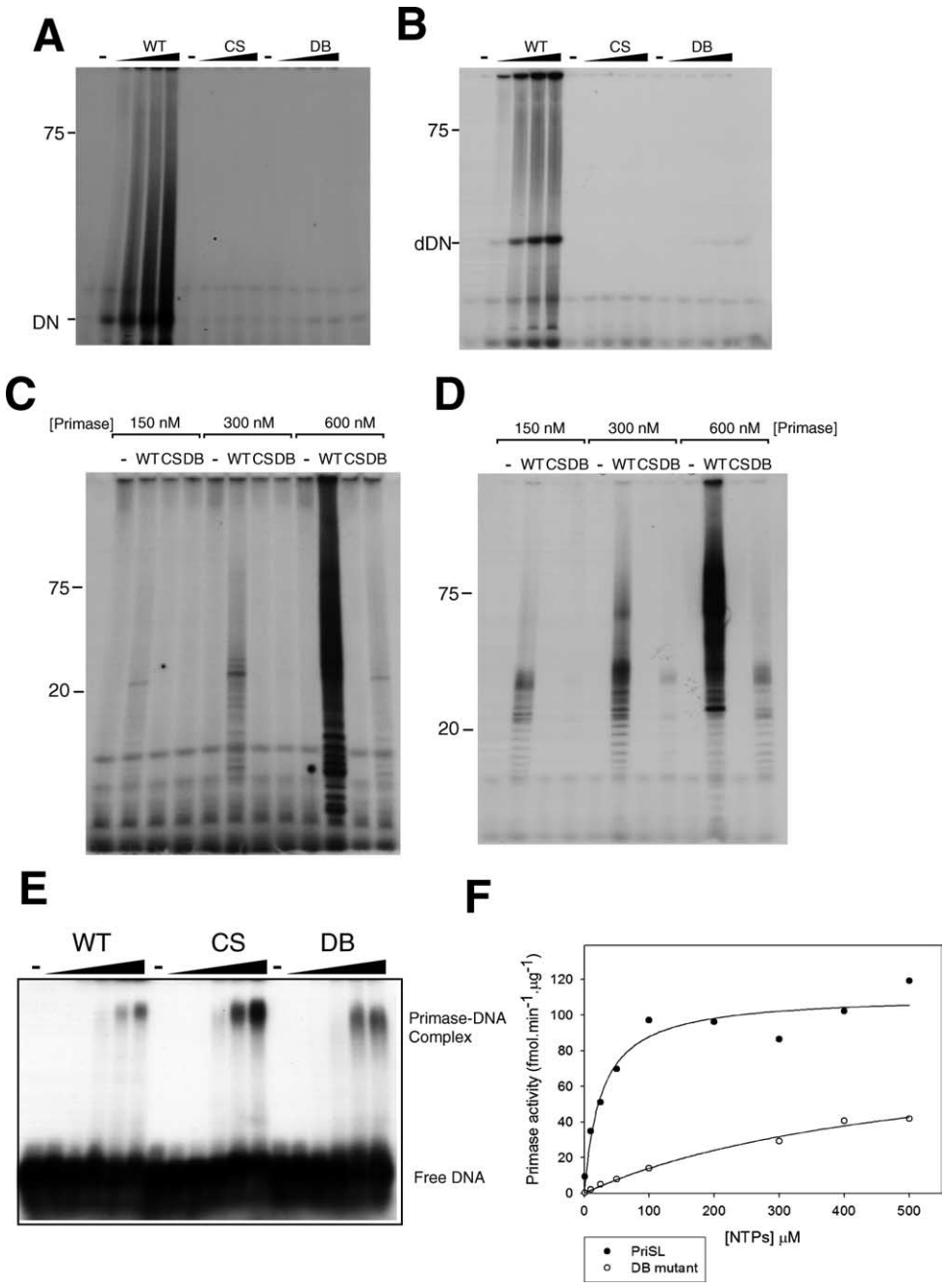

Figure 6. Comparison of PriSL, CS and DB. The primase activity of PriSL (WT), catalytic site mutant (CS) and DNA-binding site mutant (DB) was assessed in the presence of M13 or oligo(dC). The products obtained with ribonucleotides and deoxyribonucleotides were separated on a denaturing, 20% acrylamide gel and radioactivity was detected by autoradiography. The approximate size of the bands (in nucleotides) is indicated on the left-hand side of each panel. DN, di-ribonucleotide; dDN, di-deoxynucleotide. (A) and (B) Single-stranded circular M13 was utilised as a template and the concentration of PriSL, CS and DB was varied (0, 0.6, 1.2, 1.8 and 2.4 μM). The reactions were performed at 60 °C for 30 minutes, with all four unlabelled NTPs and [α-^{32}P]ATP (A) or with all four unlabelled dNTPs and [α-^{32}P]dATP (B). (C) and (D) Oligo(dC)$_{20}$ was utilised as a template and the concentration of PriSL, CS and DB was varied (0, 0.15, 0.3 and 0.6 μM). The reactions were performed at 48 °C for 30 minutes, with unlabelled GTPs and [α-^{32}P]GTP (C) or unlabelled dGTPs and [α-^{32}P]dGTP (D). (E) Electrophoretic mobility shift assay of the primases. Increasing concentrations (0.1, 0.3, 0.6, 1.2 and 1.5 μM) of PriSL (WT), of the catalytic site mutant primase (CS) and of the DNA-binding site mutant primase (DB) were incubated with a 5′ radiolabelled, 30-mer single-stranded DNA (6 μM) at 75 °C for 30 minutes. The reaction mix was subsequently resolved on a 0.5% agarose gel, as described in Materials and Methods. (−) No enzyme reaction. Radioactivity was detected by autoradiography. (F) Primase activity of PriSL and DB mutant protein. The primase activity of PriSL and DB was assessed in the presence of M13 and increasing concentrations of NTPs (10–500 μM), as described in Materials and Methods. Curves were generated by a least-square fit to a Michaelis–Menten hyperbola (calculated by SigmaPlot).

the activity of increasing concentrations of PriSL, CS and DB was compared (Figure 6(A) and (B)). In the presence of ribonucleotides, no evidence of product formation was observed for any of the mutated enzymes (Figure 6(A)). However, when deoxyribonucleotides were utilised (Figure 6(B)), DB accumulated dinucleotides, albeit at a lower rate than the wild-type primase, but no other product was synthesised. CS, on the other hand, appeared to have lost all catalytic activity.

To investigate these proteins further, a 20-mer oligo(dC) was used in place of M13, with the addition of a mixture of unlabelled and radiolabelled (d)GTP, as sole source of nucleotides (Figure 6(C) and (D)). In agreement with the previous results, CS did not exhibit any detectable catalytic activity, even when higher concentrations of enzyme were utilised. This result demonstrates that the terminal transferase-like activity of PriSL is dependent on the catalytic activity of the primase. Therefore, as predicted from the crystal structure, we find that D101 and D103 are necessary for PriSL catalytic activity. In contrast, DB had detectable, but severely diminished activity when compared with the wild-type enzyme (Figure 6(C) and (D); compare lanes wt and DB).

Augustin and colleagues[12] proposed that residues N175 and R176 would be crucial for DNA-binding. To determine whether the reduced activity of the DB enzyme was due to impaired DNA-binding of the enzyme, we utilised an electrophoretic mobility shift assay to compare the DNA-binding properties of PriSL, CS and DB. Each enzyme was incubated, at increasing concentrations, with a radiolabelled 30 nt, single-stranded DNA oligonucleotide. The reaction mixture was subsequently loaded on an agarose gel in order to separate primase–DNA complexes from free DNA. The autoradiograph shows that all three proteins bind DNA (Figure 6(E)). In fact, the yield of protein–DNA complex with the CS mutant is reproducibly higher than with the wild-type. Remarkably, the DB mutant is fully capable of binding DNA and this binding is similar to, if not stronger than, that of the wild-type protein. This result demonstrates therefore, in contrast to the prediction of Augustin and colleagues, that N175 and R176 are not necessary for the DNA binding properties of the enzyme. Interestingly, a very recent structural study has implicated this region of the protein in NTP-binding.[15] Therefore, to investigate whether these mutations affected the binding of free nucleotides, the rate of catalysis of PriSL and DB in the presence of (d)NTPs was studied. To do this, the concentration of the primases and of the DNA template, M13, were kept constant and the (d)NTPs concentration was varied. We plotted the reaction velocity as a function of the substrate concentration and found that both PriSL and DB obey Michaelis–Menten kinetics when NTPs are used (Figure 6(F)). The values of K_m and V_{max} for (d)NTPs were thus calculated and compared for each primase. In the presence of NTPs, the values for K_m and V_{max} were 25 μM and 111 fmol min^{-1} μg^{-1}, respectively, for PriSL. The values for the DB mutant were 250 μM and 60 fmol min^{-1} μg^{-1}, respectively, indicating that the affinity of DB for NTPs is approximately tenfold lower than that of the wild-type primase and that its enzymatic capability is diminished. Therefore, the mutation of N175 and R176 did not alter the DNA binding properties of the primase but modified its affinity for free NTPs. Interestingly, when dNTPs were tested, even with the wild-type protein, the reaction velocity increased linearly with increasing substrate concentration, even at dNTP concentrations as high as 43 mM (data not shown). Because it was technically impossible to study this reaction at higher dNTP concentrations, the K_m and V_{max} values could not be obtained. As discussed below, this finding has important ramifications for substrate selection by the archaeal primase.

Discussion

To date, only the catalytic subunit of the primase from *Methanococcus jannaschii* and two heterodimeric primases from closely related *Pyrococcus* species have been described, all three organisms belonging to the Euryarchaea. The primase catalytic subunit from *M. janaschii* was reported to be very similar to the eukaryotic primase as it has a preference for polypyrimidine templates and synthesises only RNA products.[10] However, these results are in striking contrast with those obtained by Ishino and co-workers who made the startling discovery that the primase catalytic subunit of *P. furiosus* does not synthesise RNA primers but instead utilises dNTPs for the formation of up to 2.4 kb long DNA strands.[8] Subsequently, the same group reconstituted the primase heterodimer and showed that shorter DNA products (<0.7 kb) were obtained in the presence of the large subunit. Most importantly, the primase was now able to synthesise RNA primers.[9] Similarly, the primase from *P. horikoshii* was shown to utilise both NTPs and dNTPs, with DNA synthesis being tenfold more effective than RNA synthesis.[11] We find that, like the *Pyrococcus* primases, the primase from the crenarchaeon *S. solfataricus* can synthesise RNA and DNA products, suggesting this dual specificity is a common feature of archaeal primases. Further, our work reveals that remarkably long products can be synthesised, when PriSL was incubated with 7.2 kb single-stranded circular M13, DNA products up to approximately 7 kb were synthesised. The physiological relevance of the RNA and DNA synthetic capability of this enzyme is currently unclear. However, it is tempting to speculate that the dual activities of the archaeal primases may reflect the absence of homologues of DNA polα in archaeal genomes. It is possible, therefore, that the archaeal primases act to initiate RNA primer synthesis and also extend it with DNA. Further, it is conceivable that *in vivo* other replication factors are required to modulate these activities of PriSL. In

E. coli, for example, the binding of DnaC to the DNA–DnaB–DnaG complex leads to the synthesis of shorter primers.[23]

Our study of the affinity of PriSL for ribo- and deoxyribonucleotides, may shed light on the physiological relevance of the ability of PriSL to synthesise both DNA and RNA. The K_m value for ribonucleotides was found to be 25 μM and we estimated the K_m value for dNTPs at approximately 28 mM (data not shown). No information about archaeal intracellular nucleotide concentration is available but, the average concentrations from about 600 published values in mammalian cells were approximately 0.3–3.1 mM NTPs and 5–37 μM dNTPs.[24] In this context, PriSL is more likely to utilise ribo- than deoxyribonucleotides, firstly because its affinity for NTPs is far greater than that for dNTPs and secondly because the intracellular dNTP concentration is much lower than the K_m value of the primase for dNTPs. The assessment of nucleotide concentration in *S. solfataricus* would nevertheless be extremely useful in understanding the behaviour of PriSL *in vivo*.

Frick & Richardson[3,25] have proposed a model which postulates that the primase possesses two nucleotide-binding sites, namely the initiation and the elongation sites. The primase binds first to single-stranded DNA, and then binds two nucleotides and catalyses the formation of a dinucleotide with the release of pyrophosphate. The dinucleotide is then transferred to the initiation site, allowing the incorporation of a third nucleotide in the now empty elongation site. The authors also suggested that the length of oligonucleotide synthesised could be limited by the length the initiation site can accommodate. The accumulation of dinucleotides by PriSL (Figures 3–5) is consistent with this model as these products can be seen as abortive primers. Our results suggest that the archaeal primase does not require binding to DNA prior to nucleotide binding (see Figures 4(A)–(D) and 5(A)). This observation is supported by the recent crystallisation of the primase catalytic subunit–UTP complex from *Pyrococcus horikoshii*.[15]

In addition to the anticipated primase activity of the enzyme, we have detected a 3′-terminal nucleotidyl transferase activity mediated by the primase. In this light, it is interesting to note that the recent crystal structures of the *Pyrococcus* primase catalytic subunit has revealed that the architecture of the enzyme's active site is closely related to that of the family X DNA polymerases, the family that includes eukaryotic terminal transferase.[12,15,26] Furthermore, two additional members of the pol X family of DNA polymerases, human polλ and human polμ, have also been demonstrated to possess terminal transferase activity.[27,28] It is possible therefore, that the ability to synthesise DNA in a template independent manner is a common property of this family of polymerases. The physiological function of the terminal transferase activity of archaeal primase and of human polλ and polμ is currently unclear; however, it has been speculated that this activity of the human proteins may play a role in DNA repair.

Thus, the archaeal primase and DNA pol X family share a number of features, including a common active site architecture and terminal transferase activity. Recent work has revealed that pol X family members have the ability to initiate DNA synthesis *de novo*.[29] Taken together with the absence of a DNA pol X family DNA polymerase in *S. solfataricus*, these observations may indicate that the PriSL primase plays additional roles in the *Sulfolobus* DNA damage repair pathways.

Materials and Methods

Cloning of Sso1048 and Sso0557 into an expression vector

The two genes were amplified by PCR from *S. solfataricus* genomic DNA using *Pfu* DNA polymerase and two sets of primers: priS-5′ (5′-GGATCCCATATGGGGA CTTTTACATTGCAC) and priS-3′ (5′-GAATTCCTCGAG TCATCTAACATAAGCCTTTACCT), which contained the underlined restriction sites NdeI and XhoI, respectively, for Sso1048 and priL-5′ (5′-GAATTCCATGGCAT TAGACGTTAAAAAG) and priL-3′ (5′-CCCCTGCAG CTATTCGTTACTTAGGAAGTAGAG), which contained the restriction sites NcoI and PstI, respectively, for Sso0557. The genes *priS* and *priL* were then inserted into the corresponding sites of pETDuet-1 vector (Novagen) to obtain the expression vector, pETpri.

Expression and purification of *S. solfataricus* primase

E. coli Rosetta (DE3) cells containing pETpri were grown at 37 °C, in LB medium supplemented with ampicillin and chloramphenicol. When the absorbance at 600 nm reached 0.6, 1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the co-expression of PriS and PriL. After four hours, the cells were harvested by centrifugation and the pellet was resuspended in a solution containing 50 mM Tris (pH 8), 0.3 M NaCl, 14 mM β-mercaptoethanol and a cocktail of protease inhibitors (Complete, Roche) prior to sonication. The extract was then centrifuged and the supernatant incubated at 75 °C for 20 minutes to denature bacterial proteins. After a final centrifugation, the supernatant was diluted with one volume of 50 mM Tris (pH 8) and applied to a heparin column (Amersham Biosciences), pre-equilibrated with buffer A (50 mM Tris (pH 8), 0.15 M NaCl). The proteins were eluted using a 30 ml linear gradient to buffer B (50 mM Tris (pH 8), 0.3 M NaCl). The active fractions were concentrated using a 20 ml Vivaspin spin column (Vivascience).

Site-directed mutagenesis

Based on the crystal structure of *Pfu* primase catalytic subunit,[12] two mutated proteins were synthesised: catalytic site mutant (CS), for which residues crucial for the catalytic activity were modified (D101A and D103A) and DNA binding mutant (DB), for which residues proposed to be involved in DNA binding activity were modified (N175A and R176A). To do so, the single primer site-directed mutagenesis technique was used, with either (5′-GTCCGATTTGTTATTTGCTATAGCTGCTGATCAC

CTATGCAAA) for CS or (5′-CCTAAGGTTTACTTTTC TGGAGCTGCAGGTTTTCACGTACAAGTCG) for DB. Following a PCR reaction in the presence of *Pfu* Turbo and *S. solfataricus* DNA ligase 1, the resulting plasmids were digested with DpnI prior to their transformation into *E. coli*.

Western blot analysis

Total protein extracts from exponentially growing *S. solfataricus* cells were prepared and applied to a Superdex 200 column (Amersham Biosciences). The eluted proteins were separated by 11.5% (w/v) SDS-PAGE and blotted onto a nitrocellulose membrane. Rabbit polyclonal antibodies raised against PriSL were used for the detection of the enzyme. This was in turn detected using horseradish peroxidase-coupled anti-rabbit secondary antibodies. The blot was developed using the ECL Western blotting detection system (Amersham Biosciences).

Two-hybrid analysis

The genes *priS* and *priL*, obtained as described above, were inserted into the Clontech vectors pGADT7 and pGBKT7, respectively. Yeast AH109 were transformed with these plasmids and grown at 30 °C for two to three days, on solid medium lacking leucine and tryptophan. Positive colonies were picked and streaked on a -Leu -Trp plate and on a -Leu -Trp -His -Ade plate. Cells were grown for a further two to three days, at 30 °C. The expression of PriS and PriL, fused to the epitope tags HA and *c*-myc respectively, was confirmed by western blot analysis using antibodies raised against HA and *c*-myc (data not shown).

Primase assay

The primase assay was performed at temperatures and primase concentrations indicated in the text. Radiolabelled chemicals were all obtained from Amersham Biosciences. The quantities of single-stranded DNA utilised were: 250 ng of M13mp18 or 60 ng of homo-oligomers (20-mer). All reactions were carried out in a total volume of 10 μl, in primase buffer (50 mM glycine–NaOH (pH 9), 10 μM $ZnCl_2$, 10 mM $MnSO_4$, 5 mM MgAc, 1 μM (d)NTPs and [α-^{32}P](d)NTP], for 30 minutes. The reaction was stopped with the addition of 10 μl of formamide buffer (80% (w/v) deionised formamide, 10 mM EDTA (pH 8), 1 mg/ml bromophenol blue] and boiled for three minutes. The samples were subsequently resolved on a 20% (w/v) acrylamide/8 M urea gel and radioactivity was detected by autoradiography. Preliminary experiments showed that PriSL requires manganese ions for catalytic activity and that the optimal glycine–NaOH buffer is at pH 9 (pH 8 at 60 °C).

Terminal transferase activity of PriSL

Two random 20-mer oligonucleotides (a deoxy template: TTGTACACGGCCGCATAATC and a dideoxy template which possesses a dideoxy-CMP at its 3′ end: TTGTACACGGCCGCATAATddC), end-labelled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs). The unincorporated radiolabel was removed using a G25 spin column. Then 60 ng (~1 μM) of each oligonucleotide was incubated either with 1.2 μM PriSL and 1 mM non-labelled (d)CTP at 60 °C, in primase buffer, or with 15 units of TdT and 1 mM non-labelled (d)CTP, at 37 °C, in the buffer provided by the manufacturer. The reactions were stopped with the addition of 10 μl of formamide dye and boiled for three minutes. The samples were subsequently resolved on a 20% acrylamide/8 M urea gel and radioactivity was detected by autoradiography.

Formaldehyde agarose gel analysis of primase RNA products

The primase reaction was performed as described above, with 1.2 μM PriSL, M13mp18, NTPs, [α-^{32}P]ATP, at 60 °C except that instead of the formamide dye used above, the reaction was stopped by the addition of RNA loading buffer (5×buffer: 0.25% (w/v) bromophenol blue, 40 mM EDTA (pH 8.0), 0.88 M formaldehyde, 20% (v/v) glycerol, 31% (v/v) formamide, 40% (v/v) 10×gel buffer (0.2 M Mops, 50 mM sodium acetate, 10 mM EDTA (pH 7.0)). The samples were analysed on a 1% agarose gel made up in 1×gel buffer with 18% (v/v) 37% formaldehyde. The gel was run at 5 V/cm for three hours, rinsed in DEPC-treated water, incubated in ten gel volumes of 50 mM NaOH, 1.5 M NaCl for 30 minutes and neutralised in 0.5 M Tris–HCl (pH 7.4), 1.5 M NaCl for 20 minutes before being transferred to 10×SSC (SSC is 0.15 M NaCl, 0.015 M trisodium citrate (pH 7.0)). After 45 minutes incubation, the RNAs were transferred to a nylon membrane by capillarity. RNA products were detected by autoradiography. A 1 kb ladder (Invitrogen) was radiolabelled with T4 polynucleotide kinase following the manufacture's protocol and used as molecular mass marker.

Alkaline agarose gel analysis of primase DNA products

The primase reaction was performed as described above, with 1.2 μM PriSL, M13mp18, dNTPs, [α-^{32}P]dATP, at 60 °C except that instead of the formamide dye used above, the reaction was stopped by the addition of one volume of 20 mM EDTA and mixed with loading buffer (0.3 M NaOH, 6 mM EDTA, 18% (w/v) Ficoll type 400, 0.25% (w/v) xylene cyanol for a 6× buffer) before being analysed on a 1% (w/v) agarose gel made up in 50 mM NaOH, 1 mM EDTA. The gel was run at 30 V (2 V/cm) for 1.5 hours and at 20 V for 16 hours, dried and DNA products were detected by autoradiography. A 1 kb ladder (InVitrogen) was radiolabelled with T4 polynucleotide kinase following the manufacture's protocol and used as molecular mass marker.

Phosphodiesterase I treatment of primase products

The primase reaction was performed as described above, with 1.2 μM PriSL, M13mp18, (d)NTPs, [α-^{32}P](d)ATP. The reaction was incubated at 75 °C for 40 minutes to promote the formation of dinucleotides (DN and dDN). One volume of phenol/chloroform was added to the samples to stop the reaction. Each tube was vortexed thoroughly and centrifuged at 6500 rpm for 30 seconds.[29] The aqueous phase was transferred to fresh tubes and used to study the effect of snake venom phosphodiesterase I (Amersham Biosciences). A 20-mer single-stranded DNA was radiolabelled with T4 polynucleotide kinase following the manufacture's protocol and used as a control for degradation. This ssDNA and both DN and dDN were incubated with 0.24 unit of phosphodiesterase for up to ten minutes. The reaction

was stopped with one volume of formamide buffer and samples were boiled three minutes before being loaded on a 20% acrylamide gel and run until the blue dye reached the middle of the gel. Radioactivity was detected by autoradiography.

Electrophoretic mobility shift assay

A 30-mer single-stranded deoxyribonucleotide primer was labelled at the 5′ terminus with [γ-^{32}P]ATP (6000 Ci/mmol) using T4 polynucleotide kinase (New England Biolabs). The unincorporated radiolabel was removed using a G25 spin column. This DNA probe (6 μM) was incubated with increasing concentrations of PriSL, CS and DB (0.1–1.5 μM), in NEBuffer 4 (New England Biolabs). After 30 minutes incubation at 75 °C, the samples were loaded onto a 0.5% agarose gel (20 cm × 20 cm) and run at 100 V for three hours in 5 mM Caps (pH 10) (protocol modified from Liu *et al.*[9]). The gel was then transferred to a 7% (w/v) trichloroacetic acid solution and left shaking for 30 minutes before being dried under vacuum and exposed to an X-ray film.

Kinetic experiments

All reactions were carried out in a total volume of 10 μl, with 1.2 μM primase (PriSL or DB), in 50 mM glycine–NaOH (pH 9), 10 μM $ZnCl_2$, 10 mM $MnSO_4$, 5 mM MgAc and 67 nM [α-^{32}P](d)ATP. The amount of DNA template was kept constant (250 ng M13mp18) and the concentration of nucleotides was varied (1–500 μM NTP or 1 μM–43 mM dNTP). The reactions were performed at 60 °C for 30 minutes. A 5 μl volume was spotted onto Hybond-N$^+$ (Amersham Biosciences) and the membrane was washed in 2×SSC (30 minutes, twice) and in 70% (v/v) ethanol to removed unincorporated radiolabel before the radioactivity was quantified using a scintillation counter. A standard curve for [α-^{32}P](d)ATP was used to convert the counts per minute to moles of product synthesised. The data distribution was analysed using the Hanes plot and the values for K_m and V_{max} were obtained from Direct Linear plots.

Acknowledgements

Work in the laboratory of S.D.B. is funded by the Medical Research Council. We thank Jessica Downs and members of S.D.B.'s laboratory for useful comments on the manuscript.

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Edited by J. Karn

(Received 16 July 2004; received in revised form 5 October 2004; accepted 11 October 2004)