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Properties of an unusual DNA primase from an archaeal plasmid

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ABSTRACT

Primases are specialized DNA-dependent RNA polymerases that synthesize a short oligoribonucleotide complementary to single-stranded template DNA. In the context of cellular DNA replication, primases are indispensable since DNA polymerases are not able to start DNA polymerization *de novo*.

The primase activity of the replication protein from the archaeal plasmid pRN1 synthesizes a rather unusual mixed primer consisting of a single ribonucleotide at the 5′ end followed by seven deoxynucleotides. Ribonucleotides and deoxynucleotides are strictly required at the respective positions within the primer. Furthermore, in contrast to other archaeo-eukaryotic primases, the primase activity is highly sequence-specific and requires the trinucleotide motif GTG in the template. Primer synthesis starts outside of the recognition motif, immediately 5′ to the recognition motif. The fidelity of the primase synthesis is high, as non-complementary bases are not incorporated into the primer.

INTRODUCTION

Primases of bacteria and eukarya differ in sequence and structure suggesting that these enzymes are not evolutionary related although they perform the same function. Bacteria and most phages have a primase with the catalytic 'toprim' domain which is also found in topoisomerases IA and II (1). In contrast the heterodimeric primases of the eukarya and archaea form a distinct group. The catalytic small subunits of these primases have similar amino acid sequences and adopt a different fold that is related to the RNA recognition motif (RRM). Currently there is no structure available for eukaryotic primases. However, the structures of three archaeal primases (2–4) have been determined and they possess the RRM fold consisting of four β-strands and two α-helices. Additionally a further β-strand (flange), which runs perpendicular to the strands of the RRM, is found in these enzymes. The catalytic residues, namely three acidic residues involved in chelating the catalytic metal ions and a catalytic histidine, are found in three neighbouring strands of the RRM and form the active site of these enzymes (5).

The replication protein of the archaeal plasmid pRN1 is a multifunctional enzyme with primase, DNA polymerase and helicase activities (6). By deletion mutagenesis the N-terminal domain responsible for primase and DNA polymerase activity could be identified and has been named the 'prim/pol' domain as suggested by its two enzymatic functions. The amino acid sequence of the prim/pol domain has no detectable sequence similarity with characterized primases or DNA polymerases. Nevertheless the structure of this domain revealed a high structural similarity to the archaeal primases. In fact, the catalytic core of the enzyme with the RRM fold and the flange is very well conserved and the essential catalytic residues occupy the homologous positions. Point mutants of the active site residues further confirmed that the inferred active site residues are in fact directly involved in catalysis (7). Based on the structure of the prim/pol domain, the inclusion of this domain into a superfamily of archaeo-eukaryotic primases was proposed by Iyer and coworkers (5). The prim/pol domain (pfam PF09250) is found in ~100 proteins. About one-third of these proteins are encoded by viruses, plasmids and bacteriophages. In these genomes, the prim/pol domain could be directly involved in the replication of the respective elements. The remaining instances are found in the genomes of bacteria and archaea and are likely to stem from integrated mobile elements which may no longer be functional.

It is currently unknown how the plasmid pRN1, the prototypical member of a group of crenarchaeal plasmids, is replicated. The plasmid pRN1 shares three conserved open reading frames with the other members of the plasmid family pRN. The encoded proteins, two rather small DNA-binding proteins and the large replication protein with ~900 amino acids, probably constitute the essential core for plasmid maintenance and replication. Our current model is that the helicase activity of the replication protein might unwind the origin and primers are synthesized subsequently. After that either

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the replication protein or the host replication machinery replicates the whole plasmid.

In order to understand initiation of plasmid replication in more detail, we analysed the primase activity of the replication protein. We found that the primase is highly sequence specific and only primes DNA synthesis on templates that possess the trinucleotide GTG. The resulting primer begins with a ribonucleotide but is extended with seven deoxynucleotides.

MATERIALS AND METHODS

Materials and reagents

All oligodeoxynucleotides were purchased from metabion (Germany). α-^{32}P labelled deoxyadenosine 5′-triphosphate (3000 Ci/mmol), α-^{32}P adenosine 5′-triphosphate (3000 Ci/mmol) and γ-^{32}P adenosine 5′-triphosphate (5000 Ci/mmol) were obtained from Hartmann Analytic (Germany).

Purification of the proteins

The full-length protein and the deletion mutants were expressed from the plasmid pET28c (Novagen). The construction of the full-length protein (6) and the deletion mutant C255 (7) were described previously. The deletion mutants C370 and C526 were constructed by amplifying the part of the wild-type gene with primers, which contained restriction sites for NheI and HindIII, and cloning the fragments into pET28c. The expressed proteins possess an N-terminal hexahistidine tag. The deletion mutant N40-C370 was constructed similarly with primers with NcoI and XhoI restriction sites. This protein has a C-terminal hexahistidine tag and starts at amino acid 40.

The purification was done for all proteins essentially as described before (6) with the following differences: the plasmids were transformed into *Escherichia coli* Rosetta (pLys) (Novagen) and grown in 2 × YT at 22°C. The proteins were purified to a purity of >90%, dialysed against 25 mM sodium phosphate pH 7.0, 40% glycerol, 100 mM NaCl, 0.01% 2-mercaptoethanol and stored in aliquots at −20°C. The protein concentrations were determined by UV spectroscopy using their respective theoretical extinction coefficients.

Primase assays

The primase assays were performed in a reaction buffer of 25 mM Tris–HCl pH 7.5, 1 mM DTT and 10 mM $MgCl_2$. In a typical primase reaction of 10 μl, 0.4 μM of protein was incubated with 4 μM of oligodeoxynucleotide and 1 mM ATP in the presence of 10 μM dNTPs supplemented with 0.6 nM [α-^{32}P] dATP for 10 min at 50°C. For the reactions that required a radioactive ribonucleotide, cold ATP was omitted and therefore the concentration of ATP was only between 6 and 10 nM.

The reactions were stopped by adding 4 μl of gel loading buffer containing 80% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.1% bromphenol blue and by heating at 95°C for 5 min. Six microlitres of each reaction were loaded onto 20% denaturing polyacrylamide/urea gels and analysed with an InstantImager. The incorporated radioactivity was quantified with the supplied software.

Kinetic studies

We determined the apparent Michaelis–Menten constant (K_m) and the maximal velocity (V_{max}) by varying the concentration of dATP, ATP and template in a primase assay with 40 nM enzyme. The reactions were incubated at 50°C for 45 min. In these assays, saturating concentrations of 100 μM dNTPs, 1 mM ATP and 6 μM template were used. As DNA templates we used oligodeoxynucleotides C and G (Table 1). To determine the kinetic parameters, varying concentrations of one of the components were added to the reaction mixes, while the other two components were kept constant at the saturating concentration. The reactions were stopped on ice, loading buffer was added and the samples were separated on 20% polyacrylamide/urea gels. After quantification using an InstantImager, the velocity was calculated and the apparent K_m and V_{max} were obtained from least-square curve-fitting. The apparent K_m and V_{max} values were derived from a minimum of three measurements.

DNA-binding assays

The DNA-binding activity of the protein was determined by fluorescence anisotropy measurements. The experiments were performed with a Perkin Elmer LS50B spectrofluorometer. The fluorescence was measured in a 120 μl cuvette (2 × 10 mm) at 25°C.

The oligodeoxynucleotides D and E, (Table 1) were labelled with fluorescein at the 3′ end and were used at a concentration of 40 nM in the assays. The binding buffer consisted of 12.5 mM Tris–HCl pH 8.0, 1 mM $MgCl_2$, 100 mM KCl and 0.01% Tween. The binding isotherms were obtained by reverse titrations. To the mixture of binding buffer and DNA a small aliquot (6 μl) of protein, which had been dialysed against binding buffer was added. Then the protein concentration was gradually decreased by replacing 30% of the mix in the cuvette with a solution of DNA in binding buffer. The anisotropy was measured with excitation at 495 nm and emission was monitored at 526 nm with a cutoff filter at 515 nm. The integration time was 10 s. For each data point at least three measurements were collected. The dissociation constant K_d was obtained by fitting the data with a single-site binding model.

Table 1. Primase templates

30 nt	5′-CCGGATCCTCATAGCAAAACACTAACTGAG
32 nt	5′-CCGCTAGCCCATGGTTGATCAAGCCTTGCGCT
39 nt	5′-TCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCT
A	5′-AATTCTCCCTCTTCTTCT**GTG**CACTCTTCTTCTCCC
B	5′- CTTCTTCT**GTG**C
C	5′- TTTTTTTT**GTG**CACTC
D	5′- TCTTCT**GTG**CACTCTTC-fluorescein
E	5′- TCTTCT**GAG**CACTCTTC-fluorescein
F	5′- AGCTTAGCTT**GTG**TTTTT
G	5′- ACTGCATT**GTG**CACTTT

RESULTS

The primase activity is sequence specific

Our initial characterization of the primase activity of the replication protein ORF904 from *Sulfolobus islandicus* plasmid pRN1 revealed that this enzyme synthesizes short (8 nt-long) primers in the presence of single-stranded M13 DNA. Primer synthesis requires the presence of ATP. In addition, dNTPs are much better incorporated than rNTPs (6) which is unusual for a primase. In order to understand the molecular mechanism of primer synthesis by this plasmid-encoded primase, we investigated in more detail the substrate requirements and the kinetics of primer synthesis.

Initial studies used single-stranded M13 DNA to study the primase activity of ORF904. We wished to use better-defined single-stranded templates and tried all four homopolymers as substrates. Homopolymers did not yield detectable primase activity (data not shown). Next we tested various unrelated oligodeoxynucleotides. Surprisingly the products synthesized by ORF904 differed considerably. As can be seen in Figure 1, a specific primer of 8 nt was synthesized only in the presence of single-stranded plasmid DNA or of substrate A (Table 1). The primer synthesis was only observed when 1 mM ATP is included in the reaction. In contrast, in the presence of two other oligonucleotides (30 and 32 nt, Table 1) a rather long product is synthesized independently of ATP. The lack of ATP dependence and the product length suggest that this product does not stem from the primase activity of ORF904. Instead this activity appears to reflect a somewhat sequence-specific terminal deoxynucleotide transferase activity of the protein which is not considered further in this study. Some oligodeoxynucleotides, e.g. 39 nt (Table 1), did not serve as template for either activity. In control reactions without template DNA, no products are observed further underscoring that ORF904 requires a template for primer synthesis.

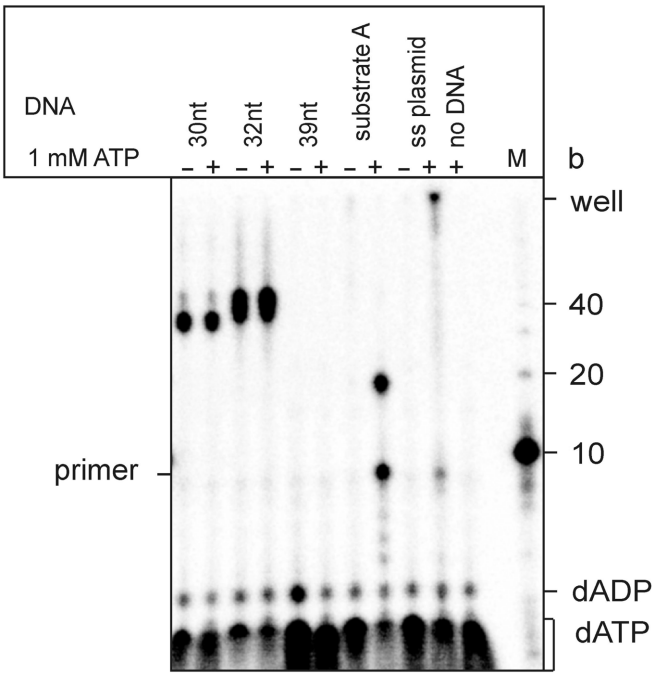

Figure 1. ORF904 is able to synthesize a specific primer on a short oligodeoxynucleotide as template. Primase reaction in the presence of 10 μM dNTPs and [α-^{32}P]dATP were assembled with different templates in the presence (+) or absence (−) of 1 mM ATP. As templates four different oligodeoxynucleotides (30 nt, 32 nt, 39 nt and A) were used at 4 μM or single-stranded plasmid pBluescriptIISK(+)-pRN1 (~40 nM). In lane M, a 10b ladder was loaded. A specific ATP-dependent primer synthesis is only observed with the single-stranded plasmid DNA and with substrate A.

Since ORF904 also has DNA polymerase activity it is able to extend a previously synthesized primer. With M13 as template the extension proceeds to long products that do not enter into this high percentage polyacrylamide gel. For substrate A, the extension of the primer yields a distinct product of about 20 bases, which could result from extending the primer up to the end of the template ('run-off product'). This experiment immediately suggested that the primase activity of ORF904 has certain substrates requirements, which are fulfilled by the long 'complex' single-stranded plasmid DNA and by the 42-nt-long substrate A. In order to better define the sequence requirements we therefore used substrate A as starting point for further investigations.

Identification of the minimal primase substrate

We first defined the minimal substrate that supports primer synthesis. The run-off product of 20 bases (Figure 1) suggested that primer synthesis could start in the middle of substrate A close to the central GTG motif (Table 1). We therefore used oligodeoxynucleotides with deletions at both ends of substrate A. The length of the run-off products shortened, whereas the primer synthesis remained essentially unchanged (Figure 2, lanes 1–3). Further shortening the 5′ end lead to primers with reduced lengths, but the primase activity was still high with these shorter templates (Figure 2, lanes 3–8). Additionally, deletions at the 3′ end were prepared. When the 3′ end was further reduced relative to substrate B, the amount of primer synthesized decreased sharply (compare lane 12 with lanes 13 and 14). Our analysis therefore showed that the 12-bases-long substrate B with the sequence 5′-CTTCTTCTGTGC-3′ represents the minimal substrate which supports synthesis of the full-length primer. The experiment also demonstrated that the base 3′ to the central GTG motif is critical for primer synthesis and that the bases 5′ of the GTG determine the length of the primer or run-off product but do not appear to be important for the efficiency of primer synthesis.

The trinucleotide motif GTG is a central recognition sequence

We next investigated which bases of substrate B make up the recognition site of the primase activity. As a starting point, we used an oligodeoxynucleotide similar to the minimal substrate (substrate B) where we exchanged the cytidines of the 5′ half with thymidines. This substrate has the advantage that the label ([α-^{32}P]dATP) is maximally incorporated during primer synthesis. On the basis of this substrate, we tested the importance of six base positions

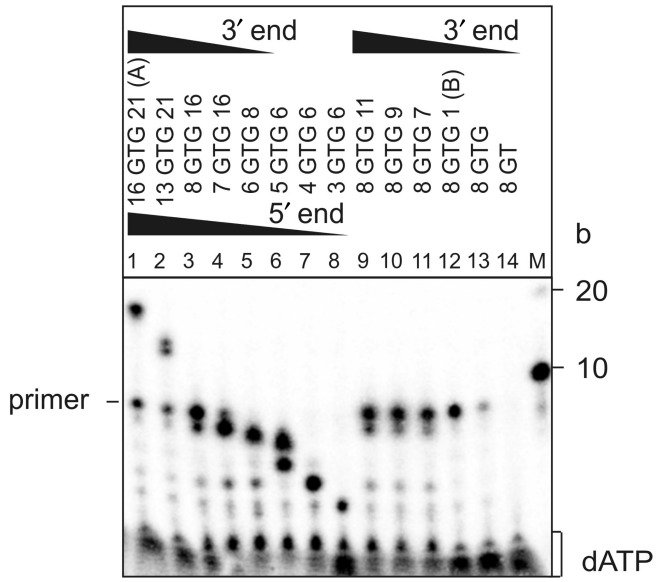

Figure 2. A 12-bp-long oligodeoxynucleotide is the minimal substrate for efficient full-length primer synthesis. Primase reactions in the presence of 10 μM dNTPs and [α-^{32}P]dATP were assembled with different templates in the presence of 1 mM ATP. Lanes 1–8: progressively shortened oligodeoxynucleotides at the 5′ and 3′ end. In the first lane the template was substrate A with the structure 16 nt followed by the motif GTG and then further 21 nt (abbreviation: 16 GTG 21). The shortening of the primer at the 5′ end first reduces the length of the run-off product (lanes 1–3). Further reduction leads to a truncated primer (lanes 4–8). Lanes 9–14: progressively shortened oligodeoxynucleotides at the 3′ end. The minimal primase substrate for full-length primer synthesis has the structure 8 nt followed by GTG and a single nucleotide and was named substrate B. Further reduction of the template at the 3′ end leads to dramatically reduced primer synthesis (compare lanes 12, 13 and 14).

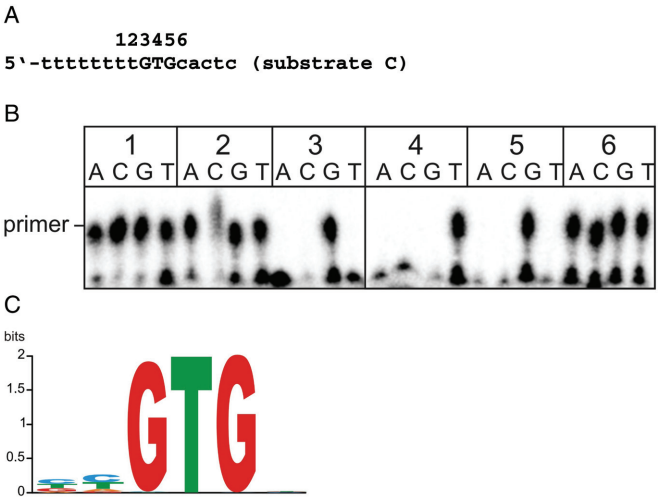

Figure 3. Dissection of the primase recognition site. (**A**) Templates used in the experiment. The numbers above the sequence of substrate C indicate the positions, which were varied in the experiment. (**B**) Primase reactions were assembled in the presence of 10 μM dNTPs, [α-^{32}P]dATP and 1 mM rNTPs with template C differing at positions 1–6. (**C**) Summary and quantification of our results. An efficient primer synthesis required an intact GTG motif.

around the GTG motif bases by replacing each position with all four possible bases (Figure 3B). For position 1 and position 2, all bases supported a high primase activity. In contrast, at position 3 only a guanosine was able to initiate primer synthesis. Likewise at positions 4 and 5 only a thymidine and a guanosine, respectively, were good primase substrates. The templates with the three other bases at these positions did not serve as substrates. At position 6, we could not detect a preference for any base.

Replicate measurements of these primase assays allowed us to define a motif (Figure 3C and Figure S1). The height at each position was calculated from the information content at each position (8). This analysis shows that the trinucleotide GTG in positions 3–5 is an important recognition site for the primase activity of ORF904. Modifications of this motif were not tolerated, demonstrating highly specific sequence recognition by ORF904, and explaining why we did not observe primase activity with homopolymers and most oligodeoxynucleotides. It is possible that there are other recognition sites besides the GTG motif discovered by us. However, given the large variation in sequences tested in our laboratory we do not believe that this is very likely.

Which part of the protein is responsible for the sequence specificity of the primase?

The replication protein ORF904 consists at least of two domains, a prim/pol domain and a superfamily 3 helicase domain (Figure 4A). The prim/pol domain is situated in the N-terminal part of the protein (amino acids 40–255) with the active site of DNA polymerization. The active site is responsible for primase and DNA polymerase activity and bears structural resemblance with the archaeal-eukaryal primases (see before). In order to determine which part of the protein could be responsible for the site-specific recognition of templates with a GTG motif, we tested various deletion mutants with substrates containing the recognition motif GTG. Full primase activity was observed with a deletion mutant from amino acid 1–370. In agreement with prior experiments, no primase activity was detected with shorter deletion mutants. Although the deletion mutant C370 was highly active in primase activity, it had no detectable primer extension activity under these experimental conditions. In contrast, the deletion mutant C526 (spanning amino acids 1–526) and the full-length protein were more active in elongating the primer (Figure 4). Possibly the longer proteins bind better to the DNA substrate and primer elongation occurs more often. Significantly, all deletion mutants retained their strict ATP dependence for primer synthesis (data not shown), ruling out the possibility that ATP binding to the helicase domain is crucial for primer synthesis.

ORF904 binds preferentially to a template with the recognition motif

We wanted to determine whether the preference for the GTG motif in primer initiation is linked to a higher affinity for substrates with this sequence. We determined the dissociation constants (K_d) by fluorescence anisotropy

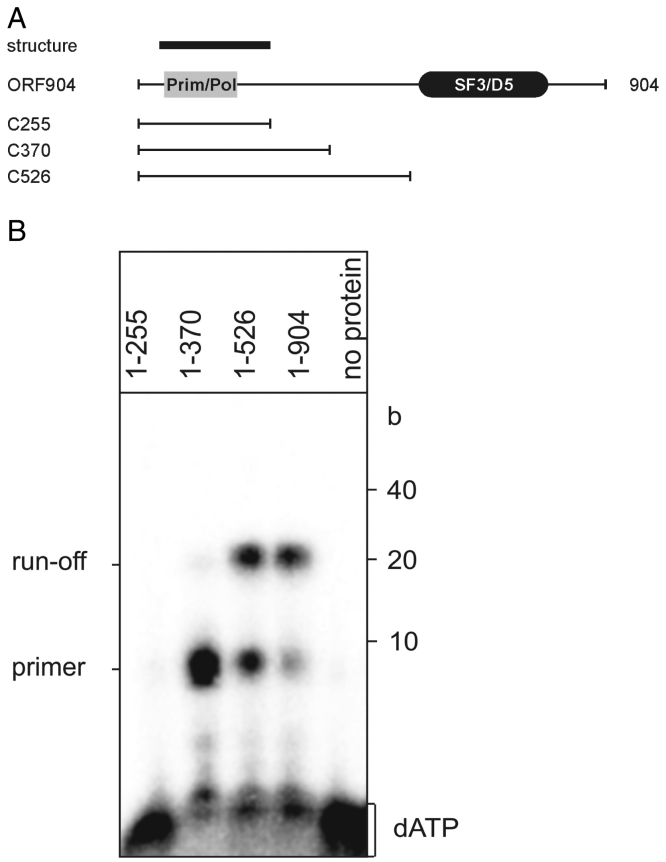

Figure 4. Primase activity of the deletion mutants. (**A**) Domain organization of ORF904 with the prim/pol domain (pfam PF09250) and the helicase superfamily 3 domain shown. (**B**) Primase reaction in the presence of 10 μM dNTPs and [α-^{32}P]dATP were assembled with different proteins in the presence of 1 mM ATP and substrate A. Lane 1: deletion mutant C255 (amino acid 1–255), lane 2: deletion mutant C370, lane 3: deletion mutant C526 and lane 4: full-length protein.

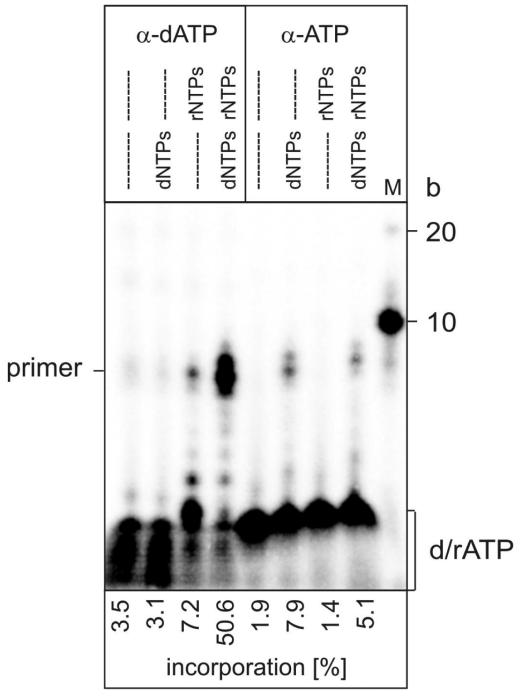

Figure 5. The primer consists of ribo- and deoxynucleotides. For these primase reactions we either used as label 6 nM [α-^{32}P]dATP or 6 nM [α-^{32}P]ATP. The reactions contained as template substrate C and were supplemented with combinations of 10 μM dNTPs and 10 μM rNTPs. In the presence of ribonucleotides and deoxynucleotides, efficient incorporation (numbers below the gel) is seen for α-dATP whereas α-ATP is only poorly incorporated (see text for details).

measurements using substrates D and E (Table 1). Substrate D was 17-nt long and contained the GTG motif and supported primer synthesis, substrate E contained the modified motif GAG with a single base exchange and therefore did not serve as template for primer synthesis.

Since wild-type ORF904 has a C-terminal helicase domain which presumably binds DNA sequence unspecifically and could therefore mask the DNA binding of the primase domain, a deletion mutant comprising amino acids 40–370 of the protein was used in the binding assays. This protein retains full primase activity and does not contain any part of the helicase domain, making it suitable to investigate the binding properties of the primase domain alone.

The oligonucleotide with the recognition sequence (substrate D) was bound with a K_d of 225 ± 5 nM, whereas the affinity for DNA without intact motif was found to be 5-fold lower, with a K_d of 1200 ± 80 nM (Figure S2). The differences in affinity therefore correspond to the observed sequence specificity of the primase reaction. Since the oligodeoxynucleotide with the mutated motif is still bound by the protein, however, other factors are presumably important in the sequence-specific initiation, as well.

Why is the primase reaction ATP dependent?

We have previously shown that the primer synthesized by ORF904 is alkali stable and DNase I sensitive, suggesting that it is made up in part or entirely of deoxynucleotides. We reconfirmed the former results by assembling primase reactions in the presence of dNTPs and rNTPs using either [α-^{32}P]dATP or [α^{32}P]ATP as label. As substrate we used substrate C in order to maximize incorporation of the label (Figure 5).

With [α-^{32}P]-dATP as label, an efficient primer synthesis was observed only in the presence of ribonucleotides and deoxynucleotides. Without ribonucleotides a primer was not formed, which is in agreement with our observation that ATP (or another ribonucleotide, see subsequently) is required for primer synthesis. In the presence of ribonucleotides but without cold deoxynucleotides, a small amount of primer was synthesized. In this reaction only labelled dATP was present without competing unlabelled dNTPs. Therefore the incorporation seen represented only a marginal amount of primer synthesized. The full-length primer synthesis was low because the low concentration of dATP effectively limited primer synthesis. Most of the radioactivity in this lane was found just above the unincorporated dATP. This species could

be a dinucleotide formed between a ribonucleotide and dATP (see subsequently).

When [α-^{32}P]ATP was used as label we observed incorporation, but ribonucleotides alone did not support complete primer synthesis, since a full-length primer was seen only when deoxynucleotides were present. Even in the presence of only labelled ATP, no primer synthesis was seen, thus reinforcing the conclusion that a primer or a truncation product consisting solely of ribonucleotides cannot be formed by the enzyme.

These results strongly suggest that both ribo- and deoxynucleotides are required for synthesis and that both are incorporated into the primer. The amount of label incorporation (50% with [α-^{32}P]dATP versus 5% with [α-^{32}P]ATP, lanes dNTPs/rNTPs) also clearly shows that deoxynucleotides make up the majority of the primer. In principle, it is possible that the primase has only low discriminatory power and randomly incorporates ribonucleotides and deoxyribonucleotides with a clear preference for the latter. However such a stochastic behaviour does not explain the strict requirement for both bases for efficient primer synthesis.

Primer synthesis can be divided into two phases: the formation of the initial dinucleotide and the extension of the dinucleotide to the full-length primer. We reasoned that the formation of the dinucleotide might require a ribonucleotide whereas the extension reaction is exclusively restricted to deoxynucleotides. This model is quantitatively and qualitatively consistent with the above incorporation results.

The dinucleotide could be formed of two ribonucleotides. However we do not observe a dinucleotide in the absence of deoxynucleotides. On the contrary, in the presence of [α-^{32}P]dATP and rNTPs a dinucleotide is formed (Figure 5, lane 3). In addition the amount of α-ATP incorporation is only a tenth of the incorporation with α-dATP (5% versus 50%). With two ribonucleotides in the primer a ratio of 1:4 would be expected. We therefore consider the possibility unlikely that the dinucleotide is made up of only ribonucleotides.

In a mixed dinucleotide the ribonucleotide could be the first or second base. With the use of [γ-^{32}P]ATP as label it is easy to differentiate between these two possibilities since the radioactivity will only be incorporated in the primer when ATP is the first base. This is indeed the case (Figure 6 and see below). The experiment thus suggested that a dinucleotide consisting of a ribonucleotide at the first position and a deoxynucleotide at the second position is formed and then extended by the addition of further deoxynucleotides to the full-length primer. This interpretation also agrees quite well with the amount of incorporation (50% with [α-^{32}P]dATP versus 5% with [α-^{32}P]ATP as label), since the ratio would be expected to be 7:1 (with 7 dATP and 1 rATP in the full-length primer).

Different ribonucleotides can serve as first base of the primer

We observed label incorporation when the reaction mixture contained 5 nM [γ-^{32}P]ATP, dNTPs, the template and the enzyme (Figure 6, upper panel, lane 1). In a control experiment with excess unlabelled ATP (1 mM) no incorporation was seen, as would be expected. In contrast, ADP and AMP as well as AMPcPP (α, β-methyleneadenosine 5′-triphosphate) and γ-S-ATP (adenosine 5′-[γ-thio]triphosphate) did not compete for the labelled ATP and label incorporation was observed. Only the analogues AMPPcP (β,γ-methyleneadenosine 5′-triphosphate) and AMPPnP (adenosine 5′-(β,γ-imido)triphosphate) competed with the labelled ATP and prevented label incorporation.

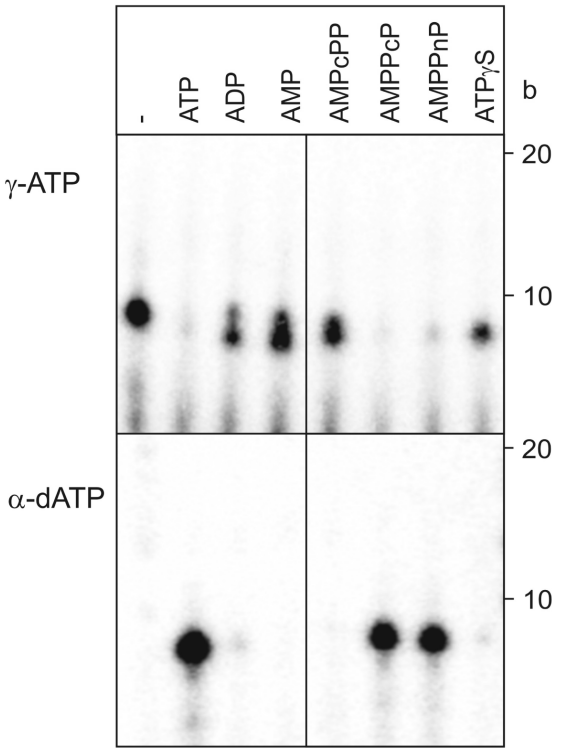

Figure 6. ATP is incorporated as first base in the primer. Primase reactions in the presence of 10 μM dNTPs and oligodeoxynucleotide C were assembled in the presence of [γ-^{32}P]ATP (upper panel) or [α-^{32}P]dATP (lower panel). The reactions also contained 1 mM of the nucleotide indicated above the panel. The label of [γ-^{32}P]ATP is incorporated in the primer showing that ATP can serve as the first base of the primer (upper panel). ATP, AMPPcP and AMPPnP are efficient competitors for the labelled ATP and prevent incorporation. In contrast when [α-^{32}P]dATP provides the label, ATP, AMPPcP or AMPPnP can be shown to support primer synthesis (see text for details).

The behaviour of the non-hydrolysable ATP analogues is somewhat unexpected: The ATP is not hydrolysed during the primase reaction and the inability of the analogues to compete is therefore not related to their inability to be hydrolysed. Nevertheless the analogues exhibit different qualities regarding their effectiveness in primer initiation. AMPcPP and γ-S-ATP behave like ADP or AMP and are not accommodated by the ribonucleotide-binding site of the enzyme. It appears therefore that there are strict structural requirements for the first base. We reconfirmed that only ATP, AMPPcP and AMPPnP can serve as first base and support the synthesis of a primer by performing the primase assay in the presence of [α-^{32}P]dATP (Figure 6, lower panel).

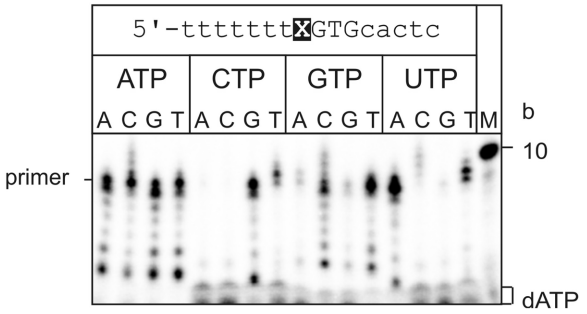

Figure 7. All ribonucleotides can serve as first nucleotide of the primer. Primase reactions in the presence of 10 μM dNTPs and [α-^{32}P]dATP were assembled. Oligodeoxynucleotides which differed at the position 5′ to the GTG were used as templates. The reactions were carried out in the presence of 1 mM of the indicated ribonucleotides. Full-length primers were synthesized with each ribonucleotide as first base. Preference for the cognate base is observed for CTP and UTP, whereas GTP is inserted opposite a T and to a lesser extent opposite the cognate base C.

Next we asked whether other ribonucleotides could also be accepted as first base of the primer. Since the first base is likely to base pair with the template, we performed these experiments with all four possible templates which differ in the position of the likely initiation site of primer synthesis. Results of the previous experiments imply an initiation at the first base 5′ to the GTG. We therefore varied the base in this position and also varied the ribonucleotide present in the reaction mixture. As can be seen in Figure 7, ATP can serve as first base with all four templates. In the reactions with the ribonucleotides CTP or UTP, a large amount of primer is synthesized when the template contains the cognate base 5′ of the GTG. The other substrates yield less or no primer. In contrast to this situation, however, with GTP primer synthesis appears to be most efficient when a thymine was present at the position in question, rather than the cognate base cytosine. On the whole, these results show a clear dependency between the initiating ribonucleotide and the first template base and provide independent evidence that the primer synthesis initiates complementary to the base 5′ of the GTG recognition site.

Fidelity of the primase reaction

The fidelity of the primase reaction was assayed using a template that contained all four bases successively upstream of the GTG recognition sequence (substrate F, Table 1). We then determined whether misincorporation occurs when the cognate base is absent from the reaction mix (Figure 8A). For this experiment [α-^{32}P]dATP was used as a label, which will be incorporated at the second position of the expected primer. When ATP and dATP were present only a dinucleotide is formed, indicating that dATP was not misincorporated at the third position opposite the cytosine. Addition of dGTP to the reaction allowed the efficient synthesis of a trinucleotide along with a minor, barely visible, amount of a longer product of 7 nt. When the reaction mix contained additionally dCTP (and was therefore only lacking dTTP) the majority of product synthesized was a tetranucleotide and again, a minor amount of a longer product was visible. When all four dNTPs were present, a full-length primer (8 nt) and a run-off product of 10 nt were synthesized. This suggests that the sequence of the primer synthesized by ORF904 is predominantly a reverse complement of the template. We attribute the minor by-product that is shorter than the full-length primer to slippage (see subsequently).

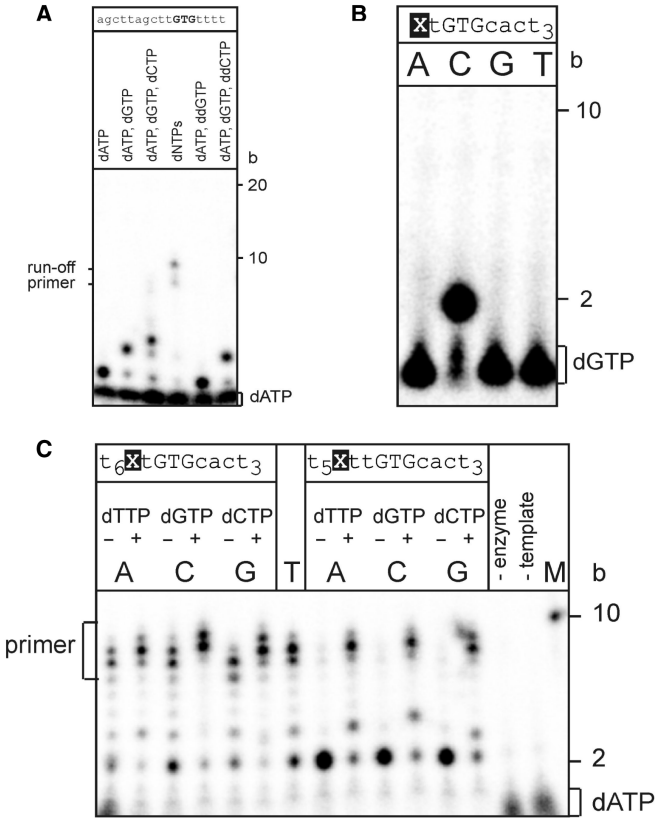

Figure 8. Fidelity of the primase. (**A**) Primase reactions were assembled in the presence of [α-^{32}P]dATP, 8 μM template and 1 mM ATP. Ten micromolar of each of the indicated dNTP or ddNTP were also added. Truncated primers (2–4 bases) show that non-cognate deoxynucleotides are not accepted by the primase for primer synthesis. The dideoxynucleotides tested are not incorporated to any extent. (**B**) Four templates differing in the first base of the template (position 2 of the formed primer) were assembled in the presence of [α-^{32}P]dGTP and 1 mM ATP. A dinucleotide (dGprAppp) is only formed with the cognate template (lane C). (**C**) Seven templates with two position varied (corresponding to positions 2 and 3 of the formed primer) were investigated in the presence and absence of the cognate deoxynucleotide. All reactions contained [α-^{32}P]dATP and 1 mM ATP. Shorter primers were synthesized in the absence of the cognate dNTP when position 2 was varied indicating slippage. Slippage was much less pronounced when the cognate deoxynucleotide of position 3 was missing. In these reactions aborted primers (dinucleotides) were the major product.

Additionally tests investigated whether dideoxynucleotides (ddNTPs) are incorporated by the primase. The same template was used to determine whether the addition of cognate ddNTPs leads to an elongation of the primer. When dATP and ddGTP were added, only a dinucleotide was observed, showing that ddGTP cannot be incorporated by the primase. The same results were obtained when the mix contained ddCTP in addition to dATP

Table 2. Kinetic properties of the primase activity of the replication protein ORF904

	Dinucleotide				Full-length primer			
	K_m (μM)	V_{max} (pmol dATP min^{-1})	K_{cat} (mol dATP min^{-1} mol $enzyme^{-1}$)	(dinucleotide min^{-1} $enzyme^{-1}$)	K_m (μM)	V_{max} (pmol dATP min^{-1})	K_{cat} (mol dATP min^{-1} mol $enzyme^{-1}$)	(primer min^{-1} $enzyme^{-1}$)
dATP	35 ± 15	0.38 ± 0.07	9.5 ± 1.75	9.5 ± 1.75	32 ± 9.5	0.38 ± 0.192	9.5 ± 4.8	1.35 ± 0.64
ATP	nd	nd			150 ± 40	0.27 ± 0.002	6.75 ± 0.05	0.96 ± 0.007
Template	nd	nd			200 ± 60	0.23 ± 0.024	5.75 ± 0.6	0.82 ± 0.09

and dGTP; the product had the same length as if only dATP and dGTP were present (Figure 8A), indicating that ddCTP is not incorporated either. Furthermore, an excess of dideoxynucleotides did not appear to compete with the incorporation of dNTPs (data not shown). In conclusion, neither ddNTPs nor non-cognate dNTPs are readily incorporated into the primer.

The infidelity of primases can be caused by genuine misincorporation opposite of a template base or by slippage. As short primer/template duplexes are unstable, slippage could be the major contributor to primase infidelity. To assess the relative contribution of genuine misincorporation versus slippage, we took advantage of the strict sequence dependence of primer synthesis and analysed the fidelity at the second position of the primer with two different types of templates. To measure genuine misincorporation, we used a short primase template that allows the synthesis of a dinucleotide. Only when the cognate base was present in the template, a radioactive dinucleotide was formed (Figure 8B). The lack of a radioactive dinucleotide for the other templates indicates that the misincorporation does not occur to an appreciable rate. We estimate that we would be able to detect misincorporation at least at a rate of 1/100 of the correct nucleotide. The second type of template allows the synthesis of a full-length primer. With these templates primer synthesis took place with relatively high efficiency, even when the cognate deoxynucleotide opposite the base at position 2 was not present in the reaction mixture (Figure 8C, left). In all three cases the primers synthesized under these conditions were shorter than the primers synthesized when the deoxynucleotide is present. We therefore suggest that primer synthesis is possible in these cases because the ternary complex of primase, template and initiating ribonucleotide can move forward so that the second base of the primer is incorporated opposite the third position of the template. The slippage should be disfavoured when a longer primer has already been synthesized. In fact, when templates are used where the missing nucleotide must be incorporated at position 3, the synthesis of long primers is very inefficient and only dinucleotides are observed (Figure 8C, right).

On the whole, these experiments suggest that slippage at the second position of the template could be the main cause for incorrectly synthesized primers whereas genuine misincorporation does not occur at a high rate.

Kinetics of dinucleotide formation and primer synthesis

Our data showed that the primase could initiate primer synthesis with an ATP regardless of the base 5′ of the GTG motif and then proceed to synthesize a primer made up of dNTPs. We investigated the kinetics of this reaction using substrates C and G (Table 1). With substrate C, the addition of ATP and dATP to the reaction will yield a full-length primer of 8 nt. We have measured the apparent K_m and V_{max} for the incorporation of dATP, ATP and for the template with this reaction. In the case of template G in the presence of dATP, only a dinucleotide is synthesized thus enabling us to study the kinetics of the formation of the initiating dinucleotide. With this template the apparent K_m for dATP was determined (Table 2 and Figure S3). The apparent K_m for dATP was ~35 μM for the dinucleotide as well as for the full-length primer synthesis. This value is similar to the reported K_m of a number of other eukaryotic primases, and of the *Sulfolobus* primase, for rNTPs (9,10).

At ~150 μM, the K_m for ATP is with somewhat higher than the K_m for dATP whereas the K_m for template is 200 nM. The maximal velocity values in the range of 0.2–0.4 pmol dATP/min correspond to a primer formation rate of about 1 full-length primer per minute per enzyme. A similar rate has been observed for *E. coli* DnaG when interacting with DnaB helicase (11) and for calf thymus primase (12). In contrast the observed rate compares favourably to the synthesis rate of the *Sulfolobus* primase (0.1 pmol dATP/min/μg which equals about 0.001 primers per minute per enzyme).

The rate of dinucleotide formation (~10 min^{-1}) is higher than the rate of full-length primer synthesis (~1.4 min^{-1}), suggesting that the dinucleotide formation is not the rate-limiting step for full-length primer synthesis. Rather dinucleotide formation and primer extension by a single nucleotide appear to proceed with the same velocity (5–10 min^{-1}).

Although the extension of the primer seems to be a rather slow process the primase does not appear to dissociate from the DNA. The products of the primase reaction are mainly full-length primer. This was examined by measuring primase activity at two different dNTP concentrations (1 and 10 μM) over time (Figure 9).

Truncated products were observed mainly at the low dNTP concentration of 1 μM. Even at short incubation times and at 10 μM dNTP, which is a third of the K_m, the majority of the products were full-length primers.

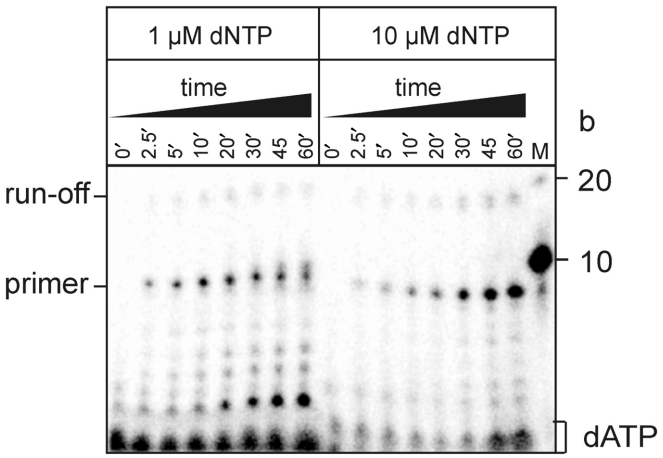

Figure 9. Time course of primase synthesis with ORF904 and substrate A. Four micromolar of template and 0.04 μM of protein were incubated with either 1 or 10 μM of dNTPs/[α^{32}P]dATP and 1 mM ATP. Samples were taken at the indicated times and electrophoresed. At the higher concentration of dNTPs, only a minority of products is shorter than the 8-nt full-length primers, whereas at the lower dNTP concentration the amount of truncated primers is increased.

Therefore even under non-saturating dNTP concentrations, the processivity of the primase to complete primer synthesis is high once a dinucleotide is formed.

DISCUSSION

We have investigated the primer synthesis by the replication protein ORF904 from the cryptic plasmid pRN1 from *Sulfolobus islandicus* using oligodeoxynucleotides as short defined templates. We found that there is a strict template dependence of the primase activity. One oligodeoxynucleotide yielded a short primer that was strictly dependent on ATP and displayed similarities to the primer that had been observed using single-stranded plasmid DNA as a template. This oligodeoxynucleotide was used to further investigate primer formation by ORF904. By mutating systematically the nucleotides of the oligonucleotide, the motif 5′-GTG-3′ was found to be of great importance. It is known that a number of bacterial and viral primases have preferred initiation sites and the recognition sites consist usually of a trinucleotide (9). For example the *E. coli* DnaG protein requires 5′-CTG-3′ to initiate a primer (13), the T7 primase 5′-GTC-3′ (14) and SP6 primase 5′-GCA-3′ (15). Eukaryotic primases usually do not exhibit such sequence specificity but exceptions have been observed. The primase from mouse requires the trinucleotide 5′-CC(C/A)-3′ for efficient primer synthesis (16) and the viral herpes simplex helicase-primase has a somewhat relaxed dependence on a trinucleotide made up of 5′-pyr-pyr-G-3′ for the synthesis of longer primers (17). The sequence specificities of archaeal primases have not been investigated extensively, but for the *Sulfolobus* primase the highest primase activity has been reported with a thymine-rich bubble substrate (18).

The specificity of ORF904 for the GTG trinucleotide is very strict, which is rather unusual for primases. For example, the alteration of the respective recognition sequence usually leads to a decrease in the velocity of the primase reaction [e.g. mouse primase (16)] or as is the case for herpes simplex primase to a decrease of the length of the synthesized primers and the primer synthesis rate (17). Some primases, e.g. the T7 primase, do however display similarly stringent sequence requirements and do not initiate a primer in the absence of a recognition site (19).

Shortening of the primase template revealed that ORF904 also accepts very short templates: a dinucleotide primer can be formed with a minimal template length of six nucleotides, i.e. two nucleotides upstream and one downstream of the recognition motif GTG. From the lengths of the primer formed and run-off products obtained with shortened oligodeoxynucleotides, we inferred that the primer is likely to be initiated at the first base upstream of the recognition trinucleotide. Thus, no part of the recognition site is copied into the primer and the site is therefore entirely cryptic.

In contrast, prokaryotic and viral primases initiate the primer at the central nucleotide of the recognition sequence (9) whereas eukaryotic primases have been found to start primer synthesis some distance upstream of their recognition motif as in the case of mouse primase (16).

The utilization of nucleotides during primer formation by ORF904 has been assessed using oligodeoxynucleotides with the recognition sequence. With γ-labelled ATP it could be shown that a ribonucleotide is incorporated as the first moiety of the primer. We could also show by exchanging the bases at the position of the template opposite the first nucleotide of the primer that the other ribonucleotides can also serve as first base of the primer. Contrary to ATP they are generally only efficiently incorporated when base pairing with the template at this position is possible. This preference for ATP is in accord with the observation that primases generally prefer to initiate a primer with a purine (9,20).

Primases are known to accept NTPs with modified triphosphate groups as first base of a primer (9,21,22) and likewise ORF904 does seem to be able to initiate a primer using certain modified ATP analogues. But the fact that AMPPcP and AMPPnP are incorporated while AMPcPP and γ-S-ATP are not indicates that there are certain other structural requirements that have to be met by the first nucleotide of the primer.

The rest of the primer is exclusively made up of dNTPs. For other archaeal primases such as the primase from *Sulfolobus solfataricus* (10), the primase from *Pyrococcus horikoshi* (23) and the small primase subunit from *Pyrococcus furiosus* (24) it has been shown that *in vitro* dNTPs are also utilized to generate primers or are even the preferred substrates for primer synthesis. In these cases however, in contrast to the substrate requirements of ORF904, a combination of dNTPs and rNTPS is not required for efficient primer synthesis. It rather appears that the active sites of these archaeal primases are able to accept both rNTPs and dNTPs. For the *Sulfolobus* primases the kinetic data suggests that *in vivo* an RNA

primer is formed. On the other hand, the pyrococcal primases appear to prefer dNTP and might synthesize a DNA primer *in vivo*. Interestingly the activity of the small catalytic primase subunit of *Pyrococcus furiosus* is modulated by the addition of the large primase subunit. If both subunits act together the efficiency to synthesize RNA primers is increased (25). The ability to incorporate dNTPs has also been observed for bacterial primases such as DnaG from *E. coli* (22). The physiological relevance of the dNTP incorporation is not known.

The length of the primer that ORF904 synthesizes is 8 nt using single-stranded plasmid DNA or oligodeoxynucleotides as template. Longer products that are also observed seem to be the result of an elongation of the primer rather than an additional specific primase product. These primer multimers are usually synthesized as multiples of the unit-length primer (9) but we never observed any specific primase products at multiples of 8.

It is not clear which part of the protein is responsible for the recognition of the GTG motif. By using different deletion mutants, we could show that the first 370 amino acids of ORF904 are sufficient for primer synthesis and exhibit a sequence specificity comparable to the full-length protein. The deletion mutant C255 (amino acids 1–255) that only comprises the prim/pol domain is not able to synthesize a primer either on single-stranded circular DNA or on oligodeoxynucleotides. Although DNA polymerase activity is seen for the deletion mutants C255 and C370, the primer synthesized by C370 is not efficiently elongated in the primase assays presented here. The deletion mutant C526 on the other hand elongates the primer as the wild type protein does, though it does not contain any part of the helicase domain, which is localized between amino acids 546 and 797. Therefore the central part of the protein from amino acids 370–526 seems to play a part in the observed elongation of a primer. For the T7 gene 4 product, which possesses a similar, but not evolutionary related, organization with an N-terminal primase and a C-terminal helicase domain, it could also be shown that the primase domain when expressed alone can synthesize primers with rates and sequence specificity as the full-length protein (26).

The substrate requirements of the prim/pol domain are very strict. Initiation is possible only with a ribonucleotide and some analogues and extension is only possible with deoxynucleotides. These findings are in line with models that postulate that priming requires two distinct sites: an initiation site and an elongation site (21). For the formation of the initial dinucleotide, both sites of the primase are occupied and catalysis takes place: the nucleotide at the elongation site loses its pyrophosphate group, and its α-phosphate group reacts with the 3′ hydroxyl group of the nucleotide in the initiation site. Next the primase moves along the template, and the nucleotide in the extension site now occupies the initiation site and a new nucleotide can bind to the empty extension site. According to this model, the replication protein ORF904 accepts only ribonucleotides at the initiation site and only deoxynucleotides at the extension site. This behaviour is in sharp contrast to the archaeal primases investigated. These enzymes appear to accept ribo- and deoxynucleotides for initiation and extension and are therefore able to synthesize DNA, RNA and mixed primers.

Very often primases display a rather low fidelity, which is usually explained by the fact that the synthesized primer is excised after the replication and replaced with DNA by a more accurate DNA polymerase. Low fidelity has been shown both for prokaryotic primases [*E. coli* (13)] and archaeal-eukaryotic primases [*Drosophila* (27) and Herpes simplex virus (28)] that readily misincorporate ribonucleotides with rates as high as 1 in 7. In contrast the bovine primase has misincorporation rates between 1/200 and 1/1600 (29). This distribution of tasks between DNA polymerases (high fidelity and processivity) and primases (less accurate initiation) is an elegant solution to the problem that priming is probably intrinsically more inaccurate because of the low stability of the duplex of a short primer with the template strand. For example, slippage of the initiating nucleotide or dinucleotide would lead to an incorrect primer sequence.

Here we show that the replication protein ORF904 synthesizes *in vitro* a primer consisting of an initiating ribonucleotide followed by seven deoxynucleotides. Therefore in the context of the plasmid replication, it is improbable that the DNA primer synthesized by ORF904 can be recognized later and specifically removed. Accordingly we find that that the primase will only reluctantly incorporate non-cognate deoxynucleotides. We do not observe elongation of the initiating ribonucleotide to a dinucleotide in the absence of the cognate deoxynucleotide. Likewise elongation of short primers is not possible in the absence of the cognate deoxynucleotide. However we observe considerable slippage at the second but not the third position of the primer. Our results suggest that under *in vivo* conditions (availability of all dNTPs), misincorporation occurs only at the initiating ribonucleotide and possibly also at the deoxynucleotide at position 2 due to slippage.

Our findings also have interesting evolutionary implications. According to a detailed study of Iyer and coworkers (5), the archaeo-eukaryotic primase superfamily (including the archaeo-eukaryotic primases and the prim/pol domain proteins) might have a common ancestor with the family B DNA polymerases which are the replicative polymerases of the archaea and the eukaryotes. The specialization of the cellular replication machinery with separate primase and DNA polymerase activities conceivably did not exist at the invention of DNA as genetic material. It seems plausible that an enzyme capable of *de novo* DNA polymerization was central to primordial DNA replication. We show here that the prim/pol domain is able to perform *de novo* DNA polymerization with rather high fidelity, suggesting that an enzyme with similar properties could have been able to replicate simple DNA genomes.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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