Introduction

Poxvirus DNA replication occurs within specialized regions of the cytoplasm of infected cells by incompletely understood mechanisms. Self-priming, primer-dependent, and recombination models have all been proposed (Moss and De Silva, 2006). One model takes into account the hairpins at the ends of the linear, double-stranded poxvirus genome (Baroudy et al., 1982a) and resembles the rolling hairpin strand displacement mechanism proposed for paroviruses (Baroudy et al., 1982b; Moyer and Graves, 1981). The evidence for nicking near the ends of the genome (Esteban and Holowczak, 1977b; Pogo et al., 1984, 1981; Pogo, 1980) and the resolution of concatemeric forms of DNA by a virus-encoded Holiday junction endonuclease (Garcia et al., 2000; Garcia and Moss, 2001) are compatible with a rolling hairpin mechanism. However, both linear minichromosomes with specific telomere ends (Du and Traktman, 1996) and closed circular DNA molecules without poxvirus-specific sequences replicate efficiently in infected cells (De Silva and Moss, 2005; DeLange and McFadden, 1986; Merchlinsky and Moss, 1988). In addition, there are reports of VACV DNA covalently linked to RNA (Olgiati et al., 1976) and of short nascent DNA segments resembling Okazaki fragments that could be chased into larger molecules in vivo (Esteban and Holowczak, 1977a). The recent finding of a DNA primase encoded by VACV (De Silva et al., 2007) provides a mechanism for the formation of RNA primers that could be used for initiation of DNA replication or lagging strand synthesis (Frick and Richardson, 2001).

The VACV DNA primase is encoded by the D5R gene (VACVWR110), which is conserved in all sequenced poxviruses (De Silva et al., 2007). D5 (the protein encoded by D5R) was shown to be required for DNA replication more than 20 years ago (Evans and Traktman, 1992; Roseman and Hruby, 1987; Seto et al., 1987). Analysis of the C-terminal region of D5 and homologs in other poxviruses as well as some distantly related large DNA viruses, revealed a domain characteristic of helicase superfamily III members of the AAA+ class of nucleoside triphosphatases (NTPases) (Gorbalenya and Koonin, 1989; Iyer et al., 2001). Furthermore, in vitro studies demonstrated DNA-independent NTPase activity (Evans et al., 1995) and mutations in the active site eliminated the ability of D5R to complement a temperature sensitive mutant (Boyle et al., 2007). The possibility of a second enzymatic activity in the N-terminal segment of D5 was suggested by a motif present in the archaeo-eukaryotic primase superfamily (Iyer et al., 2005). Recently, recombinant D5 was shown to have primase activity in vitro and mutation of the primase active site, which had no effect on NTPase activity, eliminated the ability of D5R to complement a conditional lethal mutant in vivo (De Silva et al., 2007). In the present study, we characterized the products of the D5 primase and provided information on the substrate and template specificities.

Results

In vitro products of the VACV DNA primase

We previously demonstrated the DNA primase activity of purified recombinant D5 in a reaction containing single stranded dX174 or M13 phage DNA template, Mg2+ and the four rNTPs including either [α-32P]CTP or [α-32P]UTP (De Silva et al., 2007). Prior to analysis on
denaturing 20% polyacrylamide gels, the products were treated with phosphatase, which reduces the background of unincorporated radioactive nucleotides and slows the migration of RNA of less than six nucleotides (Holstege et al., 1997). The RNase-sensitive products labeled with $\alpha^{-32}$P]CTP consisted of a discrete band that migrated at the position of a phosphorylated 14-nucleotide marker and more slowly migrating products that extended to the top of the gel (Fig. 1A). Of the total radioactivity, approximately 20% migrated with the 14-nucleotide marker. Synthesis of these products was proportional to enzyme concentration and a specific activity of 3.6 nmol CMP incorporated in 30 min per nmol of enzyme with a $\phi$X174 DNA template was determined (Fig. 1B).

Before proceeding to further analyses of the primase products, we determined the effects of altering the divalent and monovalent cation concentrations, which can affect activity and processivity of polymerases. Our standard primase assay contains 6 mM Mg$^{2+}$. In the experiment shown in Fig. 1C, Mg$^{2+}$ and Mn$^{2+}$ were varied from 0 to 10 mM, while total rNTPs were maintained at 1.5 mM. No primase activity was detected in the absence of added divalent cation. The effects of Mg$^{2+}$ and Mn$^{2+}$ were slightly different. Activity was greater with Mn$^{2+}$ and peaked at 6 mM, whereas activity with Mg$^{2+}$ increased up to 10 mM. The products appeared heterogeneous at all divalent cation concentrations, although the proportion of the material migrating with the 14-nucleotide marker was about 5-fold higher with Mn$^{2+}$ compared to Mg$^{2+}$. Our standard primase assay contains 60 mM K$. However, we found that K$ decreased activity at concentrations above 15 mM (Fig. 1D). The greater activity with Mn$^{2+}$ than Mg$^{2+}$ and the inhibitory effect of monovalent cations are similar to the properties of eukaryotic DNA primases (Kirk and Kuchta, 1999).

VACV D5 forms dinucleotide and longer RNA products

Phosphatase-treatment reduces the mobility of products less than 6 nucleotides long on high concentration polyacrylamide gels (Holstege et al., 1997). For these products, the mobility initially increases with size and is affected by base composition. Thus, the phosphatase-treated dinucleotide products of an archaeon primase migrated with a 14-nucleotide marker (Lao-Sirieix and Bell, 2004). Similarly, we found that corresponding mobility VACV DNA primase product(s) labeled with $\alpha^{-32}$P]CTP and treated with phosphatase migrated as a band that overlapped with GpC and ApC on a denaturing 20% polyacrylamide gel (Fig. 2A). Although the product migrating as a dinucleotide was sensitive to RNase, the more rapidly migrating materials were insensitive (De Silva et al., 2007) and were not analyzed further.

The labeled material near the top of the gel was previously shown to be sensitive to RNase and nuclease P1 but insensitive to DNase (De Silva et al., 2007). To investigate the possibility that the retarded electrophoresis was partly due to protein association that survived the formamide treatment, we treated the samples with proteinase K or proteinase K followed by phenol chloroform extraction prior to electrophoresis. However, these treatments had no effect suggesting that association with protein was not affecting the electrophoretic mobility of the RNA (data not shown). To determine the size range of the large RNA products, samples were resolved on a formaldehyde/1%
Reactions contained either $[^{32}\text{P}]$dCTP or $[^{32}\text{P}]$CTP in the presence of rNTPs or dNTPs or both. Incorporation of $[^{32}\text{P}]$CTP into dinucleotide or longer products was insignificant when rNTPs were replaced with dNTPs, suggesting that neither dATP or dCTP can initiate oligonucleotide synthesis (Fig. 4). In addition dNTPs reduced the incorporation of $[^{32}\text{P}]$dCTP in the presence of rNTPs. Nevertheless, $[^{32}\text{P}]$dCTP was incorporated into a discrete band that co-migrated with a 24-nucleotide marker and longer products in the presence of rNTPs but not dNTPs (Fig. 4). Based on the pattern, we presume that the “24-nucleotide” product is GpCd and ApCd, which have slower migrations than GpC and ApC on 20% polyacrylamide gels (Lao-Sirieix and Bell, 2004). Both the presumed dinucleotide and longer RNA products were sensitive to nuclease P1, whereas the more rapidly migrating material was not (data not shown). Also, dNTPs reduced the incorporation of $[^{32}\text{P}]$dCTP in the presence of rNTPs (Fig. 4). The dNTPs appeared to have a greater effect on reducing the amount of long polynucleotides than of dinucleotides.

**Homooligomer template specificity of VACV primase**

Defined length homooligomers were tested for their ability to serve as templates. Primase assays were performed using synthetic 18-mers of dA, dC, dG, and dT with complementary rNTPs. Each reaction was divided into two aliquots and one was treated with nuclease P1 before gel electrophoresis. Oligo(dT) was by far the best template resulting in a range of nuclease P1-sensitive products that migrated between the phosphorylated markers of 16 and 87 nucleotides (Fig. 5). Products longer than the template could arise from primase slippage, primer reannealing, or terminal nucleotidyl transferase activity. Oligo(dT) was a much poorer template than oligo (dC) and no nuclease P1 sensitive material was obtained with either oligo(dG) or oligo(dA) (Fig. 5). There was, however, some undefined nuclease-resistant material at the origin in all cases.

**Discussion**

DNA primases catalyze the synthesis of RNA in at least four discrete steps: template binding, NTP binding, phosphodiester bond formation,
and chain extension (Frick and Richardson, 2001). Most primases do not exhibit template specificity, although they may have sequence preferences, as they must initiate RNA synthesis frequently during lagging strand synthesis. In this context, the VACV DNA primase was able to use single stranded phage DNAs as well as oligo(dC) and to a lesser extent oligo(dT) as templates. Extensive studies with bacteriophage T7 and other primases indicate the presence of separate NTP binding sites for initiation and elongation. Primer synthesis is initiated by the formation of a dinucleotide with release of pyrophosphate. The product is then transferred to the initiation site allowing the synthesis and couple primer synthesis with DNA replication.

Fig. 4. Incorporation of ribo- and deoxyribonucleotides. Primase assays were performed using [α-32P]CTP (lanes 1–4) or [α-32P]dCTP (lanes 5–8) supplemented with combinations of 1.5 mM rNTPs and/or 1.5 mM dNTPs. Positions of 32P-end labeled oligonucleotide markers are indicated on the left. Origin indicates the top of the well; filled arrow represents dinucleotide product formed with [α-32P]CTP; open arrow represents the presumed dinucleotide product formed with [α-32P]dCTP.

In an infected cell, D5 primase and NTPase activities function in conjunction with other viral proteins. It is known that the A20 protein interacts with both D5 and the D4 uracil DNA glycosylase (McCraith et al., 2000) and that A20 and D4 form a processivity factor for the DNA polymerase (Stanitsa et al., 2006). These proteins presumably form a complex that allows DNA synthesis at the replication fork. It will be interesting to attempt to reconstitute this complex in vitro and couple primer synthesis with DNA replication.

Materials and methods

Chemicals

Adenylyl (3'→5') cytidine [ApC], guanylyl (3'→5') cytidine [GpC], nucleoside P1, isobutyric acid, and concentrated ammonia were purchased from Sigma-Aldrich (St. Louis, MO). Isopropanol for making denaturing polyacrylamide gels were obtained from National Diagnostics Inc. (Atlanta, GA). Ribonucleotide and deoxyribonucleotide triphosphates were from Roche Diagnostics Corp. (Indianapolis, IN). The 18mer oligo(dA), oligo(dC), oligo(dG), and oligo(dT) were purchased from US Biologicals (Swampscott, MA). Fluor coated plates were from Ambion Inc. (Austin, TX). Isopropanol and hydrochloric acid were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Proteinase K and phenol/chloroform were from Invitrogen (Carlsbad, CA).

We previously determined that ATP or GTP was required for VACV primer synthesis and that omission of GTP reduced activity more than omission of ATP (De Silva et al., 2007). An explanation for this was found upon nearest neighbor sequencing of the dinucleotide products labeled with [α-32P]CTP, which revealed four times more G than A and neither C nor U in the first position. Although we do not know whether a similar ratio of G to A would be found by nearest neighbor analysis with other labeled ribonucleoside triphosphates, preferential initiation with G can also explain the finding that the template activity of oligo(dC) was much higher than oligo(dT) and that oligo(dG) and oligo(dA) were inactive. The VACV primase resembles other primases in regard to initiation with a purine (Frick and Richardson, 2001). The VACV primase was unable to initiate oligonucleotide synthesis in the presence of dNTPs, though dNTPs could be incorporated in the presence of rNTPs. Thus, it seems likely that the VACV primase discriminates between rNTPs and dNTPs in the initiation site but to a lesser degree in the elongation site, similar to that of bacterial and eukaryotic DNA primases (Conaway and Lehman, 1982; Rowen and Kornberg, 1978) but different from archael DNA primases, which appear to have additional functions including gap filling and 3'-terminal nucleotidyl transferase activities (Bocquier et al., 2001; Lao-Sirieix and Bell, 2004; Le Breton et al., 2007). Although DNA primases typically form short RNAs (Frick and Richardson, 2001), the VACV DNA primase synthesized RNAs that were several kb long, like archael (Bocquier et al., 2001; Lao-Sirieix and Bell, 2004) and some viral (Mikhaylov and Rohrmann, 2002) primases.

Fig. 5. Template recognition of homooligomer templates. Primase reactions were carried out using 100 ng of dT, dC, dG, and dA 18mer homooligomers in the presence of the complementary [α-32P]NTP and 1 mM Mn2+. Products were treated with calf intestinal alkaline phosphatase and then with (+) or without (-) nuclease P1. An autoradiograph of a 20% polyacrylamide gel is shown with the positions of 32P-end labeled oligonucleotide markers on the left.
Primase assays

Primase reactions were carried out as previously described (De Silva et al., 2007). Unless stated otherwise, reaction mixtures (5 μl) contained 25 mM Tris acetate (pH 7.5), 60 mM K acetate, 6 mM Mg acetate, 10 mM DTT, 100 μg/ml BSA, 1 mM ATP, 250 μM UTP, 250 μM GTP, 25 μM CTP, 0.66 μM [α-32P]CTP (3000 Ci/mmol) (Perkin–Elmer Life Sciences, Shelton, CT), 1 μg (0.57 pmol) φX174 phage DNA (New England Biolabs, Beverly, MA), 100 ng (1.1 pmol) recombinant D5 with a 10-histidine tag (De Silva et al., 2007). Reactions were incubated at 37 °C for 30 min, then stopped by heating at 80 °C for 10 min. Products were treated with 4 units of calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA) at 37 °C for 1 h and denatured by adding an equal volume of formamide stop solution (95% deionized formamide, 20 mM EDTA, plus bromophenol blue and xylene cyanol). Reaction products were heated at 90 °C for 2 min and analyzed on a denaturing 20% polyacrylamide, 7.5 M urea gel followed by autoradiography or analysis on a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Oligonucleotide size markers (GE Healthcare, Piscataway, NJ) or chemically synthesized oligonucleotides were 32P-end-labeled using polynucleotide kinase (New England Biolabs, Beverly, MA) and γ-32P[ATP [3000 Ci/mmol] (Perkin–Elmer Life Sciences, Shelton, CT).

Formaldehyde agarose gel electrophoresis of primase products

The primase reaction was performed as described above except the reaction was stopped by the addition of RNA loading buffer (5 μl 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-μl gel buffer (0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA [pH 7.0]). The samples were resolved 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 3 h, rinsed in diethylylpyrocatechol-treated water, incubated in ten gel volumes of 50 mM NaOH, 1.5 M NaCl for 30 min and neutralized in 0.5 M Tris–HCl (pH 7.4), 1.5 M NaCl for 20 min before transfer to 10× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate pH 7.0). After 45 min incubation, the RNAs were transferred to a nylon membrane by capillary action. The analysis was carried out as previously described (Grosjean et al., 2007). The analysis was carried out as previously described (Grosjean et al., 2007). The analysis was carried out as previously described (Grosjean et al., 2007). The analysis was carried out as previously described (Grosjean et al., 2007).

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