Recombinant pseudorabies virus DNase exhibits a RecBCD-like catalytic function

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The pseudorabies virus (PRV) DNase gene has previously been mapped within the PRV genome. To characterize further the enzymic properties of PRV DNase, this enzyme was expressed in *Escherichia coli* with the use of a pET expression vector. The protein was purified to homogeneity and assayed for nuclease activity *in vitro*. Recombinant PRV DNase exhibited an alkaline pH preference and an absolute requirement for Mg^{2+} ions that could not be replaced by Ca^{2+} and Na^{+} ions. Further studies showed that PRV DNase exhibited endonuclease, 5′-exonuclease and 3′-exonuclease activities in both single-stranded and double-stranded DNA. This activity occurred randomly and no significant base preference was demonstrated. The multiple biochemical activities of PRV DNase are similar to the activities of *Neurospora crassa* endo-exonuclease and *E. coli* RecBCD, two additional enzymes that are involved in recombination. Taken together, the similarity of action between *N. crassa* endo-exonuclease, *E. coli* RecBCD, and PRV DNase suggests that PRV DNase might have a role in the process of recombination that occurs during PRV infection.

INTRODUCTION

Pseudorabies virus (PRV), the causative agent of Aujeszky's disease in pigs [1], is a member of Alphaherpesvirinae [2]. PRV DNA is synthesized by a rolling circle mechanism, which yields concatemers that are cleaved into monomers and packaged into capsids [3]. A variety of genes involved in nucleotide metabolism are expressed during PRV DNA replication. These include thymidine kinase, ribonucleotide reductase, uracil glycosylase, deoxyuridine triphosphate nucleotidohydrolase and DNase genes [4–8].

The PRV DNase gene has an open reading frame of 1476 nucleotides, capable of coding a 492-residue protein with 44.1 % identity with the counterpart of herpes simplex virus type 1 (HSV-1) [8]. The HSV-1 nuclease is thought to provide nucleotides for HSV-1 DNA synthesis by degrading host cellular DNA [9]. HSV-1 mutant, carrying a deletion in the nuclease gene, is able to synthesize near wild-type levels of viral DNA [10]. Furthermore, studies with the mutant also show the inability to process viral DNA genomes correctly and the failure of DNA-containing capsids to migrate into the cytoplasm [11,12]. Taken together, these studies demonstrate that the nuclease is required for efficient processing of viral DNA replication intermediates and the egress of capsids from the nucleus, but is not essential for viral DNA synthesis. Nevertheless, the catalytic mechanism of DNase, which is involved in nucleotide metabolism, genome process, capsid egress and other biological functions, remains to be elucidated.

The PRV DNase exhibits a RecBCD-like catalytic function described in this study. The recombinant PRV DNase was overexpressed in *Escherichia coli* by using a pET expression vector and was purified to homogeneity. The biochemical properties and the mode of action of PRV DNase were analysed *in vitro*. The possible role of PRV DNase during PRV infection is discussed.

MATERIALS AND METHODS

Construction of recombinant plasmid

The PRV DNase gene, 2.4 kb of an *Apa*I–*Sal*I fragment in PRV *Bam*HI fragment 2, was obtained as described previously [8]. The resulting plasmid was sequentially digested with *Sau*I, filled in by T4 DNA polymerase and digested with *Xho*I to yield a fragment 1.5 kb in size. This fragment was then inserted into a histidine-tagged expression plasmid pET-28a(+) (Novagen), which was treated with *Eco*RI (filled in) and *Xho*I to create the pET-DNase. The recombinant plasmid has lost the first 10 residues of the DNase open reading frame; these have been replaced by 34 residues derived from pET-28a(+).

Expression and purification of recombinant PRV DNase

Recombinant protein was expressed in *E. coli* BL21(DE3)pLysS strain by transforming the pET-DNase to produce an N-terminal fusion with six histidine residues. Cells were grown in 50 ml of Luria–Bertani broth agitated at 37 °C until D_{600} reached 0.6. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and the cells were pelleted 2 h after induction. The cell pellet was washed with PBS (137 mM NaCl/2.7 mM KCl/4.3 mM Na_2HPO_4/1.4 mM KH_2PO_4) and stored at −70 °C until fractionation. Once thawed, the pellet was resuspended in 5 ml of buffer A [50 mM Tris/HCl (pH 8.0)/1 mM EDTA/100 mM NaCl/1 mM dithiothreitol/0.1 mM PMSF] containing 20 % (w/v) sucrose and 1 mg/ml lysozyme. The resuspended pellet was incubated on ice for 20 min, refrozen at −70 °C and then thawed at 37 °C. After sonication, the suspension was centrifuged at 20000 ***g*** for 10 min at 4 °C. The pellet was collected, resuspended in 5 ml of buffer A containing 1 % (v/v) Triton X-100 and incubated on ice for 15 min. After centrifugation, the pellet was resuspended in 5 ml of binding

Abbreviations used: ds, double-stranded; HSV-1, herpes simplex virus type 1; IPTG, isopropyl β-D-thiogalactoside; PRV, pseudorabies virus; ss, single-stranded.

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buffer [5 mM imidazole/0.5 M NaCl/20 mM Tris/HCl (pH 7.9)/8 M urea]. The suspension was incubated at room temperature for 2 h with continuous mixing, then centrifuged at 20000 ***g*** for 10 min. The PRV DNase was purified from the supernatant by nickel-affinity chromatography, with 8 M urea present throughout the procedure. The protein in the final column eluate was pooled and renatured by sequential dialysis. The protein was dialysed overnight against renaturing buffer A [50 mM Tris/HCl (pH 8.0)/2 mM EDTA/500 mM NaCl/0.1 mM PMSF/4 M urea] and sequentially dialysed with renaturing buffer B [50 mM Tris/HCl (pH 8.0)/0.1 mM PMSF] containing 0 %, 15 % and 25 % (v/v) glycerol. Finally, the protein was dialysed overnight against the storage buffer [50 mM Tris/HCl (pH 8.0)/20 % (v/v) glycerol/0.5 mM dithiothreitol/0.2 % (v/v) Nonidet P40/0.1 mM PMSF]. The purified DNase was stored at −70 °C until further analysis. Protein was analysed by SDS/PAGE and was quantified with a Bradford assay (Bio-Rad).

DNase activity assay

The reaction mixture, containing 0.1 μg of purified enzyme, 1 μg of ^{3}H-labelled pUC18 DNA, 50 mM Tris/HCl, pH 9.0, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol in a final volume of 100 μl, was incubated at 37 °C for 10 min. The undigested DNA was precipitated by adding 5 μl of 5 mg/ml BSA and 100 μl of 5 % (w/v) trichloroacetic acid. The supernatant, which represented the acid-soluble nucleotides, was collected and the radioactivity of the nucleotides was counted by scintillation spectroscopy. One unit of DNase activity is defined as the amount of enzyme required to convert 1 μg of double-stranded DNA into acid-soluble material in 10 min at 37 °C.

Agarose gel electrophoresis assay

Various amounts of DNase were mixed with 0.1 μg of DNA in DNA binding buffer [50 mM Tris/HCl (pH 9.0)/10 mM 2-mercaptoethanol]. After a 5 min incubation at 37 °C, 2 μl of tracking buffer [20 % (w/v) Ficoll/40 μg/ml Bromophenol Blue] was added and a 15 μl sample was applied to a horizontal 1.2 % (w/v) agarose gel. Electrophoresis was carried out with TAE buffer (40 mM Tris/acetate/2 mM EDTA) and stained with ethidium bromide solution [1 M NaCl/10 mM Tris/HCl (pH 7.5)/1 mM EDTA/20 μg/ml ethidium bromide].

Nitrocellulose filter binding assay

The formation of protein–DNA complexes was measured by using alkali-treated nitrocellulose filter as described previously [13]. Briefly, nitrocellulose filters (Hybond®-C super; Amersham) were soaked in 0.5 M KOH for 20 min at room temperature, washed with distilled deionized water and stored in 100 mM Tris/HCl, pH 7.6, at 4 °C. Neither single-stranded DNA (ssDNA) nor double-stranded DNA (dsDNA) was retained by the alkali-treated filter, but protein–DNA complex was efficiently retained. The reaction mixture contained 100 ng of ^{3}H-labelled DNA and various amounts of DNase in the DNA binding buffer. A 100 μl sample of this mixture was incubated at 37 °C for 5 min and then applied to the alkali-treated nitrocellulose filters by using Bio-Dot microfiltration units (Bio-Rad). The filters were washed twice with DNA binding buffer and dried; the radioactivity was determined with the scintillation counter.

Radiolabelling of the ends of ssDNA and dsDNA

Homo-oligonucleotides (20-mer) of A, T, G or C were synthesized (Genemed Biotechnologies). The oligonucleotides were labelled at their 5′ end by T4 polynucleotide kinase, with [γ-^{32}P]ATP as the substrate. To obtain the poly(dA)·poly(dT) or poly(dG)·poly(dC) duplex, equimolar amounts of two complementary oligonucleotides were mixed, heat-denatured at 80 °C for 5 min, then reannealed by slow cooling to room temperature. For labelling the 5′ end of DNA, pUC18 DNA was linearized by *Sma*I, dephosphorylated by calf intestinal alkaline phosphatase, then rephosphorylated with ^{32}P by T4 polynucleotide kinase, using [γ-^{32}P]ATP as the substrate. For labelling the 3′ end of DNA, pUC18 DNA was digested by *Sma*I and *Eco*RI. The 3′-recessive end, created by *Eco*RI, was filled by using Klenow fragment, [α-^{32}P]dATP and dTTP.

Exonuclease activity assay

The reaction mixture, containing 0.1 μg (approx. 10000 c.p.m.) of end-^{32}P-labelled DNA, 0.5 μg of unlabelled, cognate DNA, 10 ng of purified enzyme, 50 mM Tris/HCl, pH 9.0, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol in a final volume of 20 μl, was incubated at 25 °C for 0, 20 or 60 s. The reaction was terminated by adding 2 μl of 10 × stop buffer (50 % glycerol/1 % SDS/0.1 % Bromophenol Blue/100 mM EDTA). Samples were analysed on a 10 % (w/v) polyacrylamide gel at 100 V and the degradative products were examined by autoradiography.

RESULTS

Expression and purification of the recombinant PRV DNase

The recombinant PRV DNase was purified from the *E. coli* BL21(DE3)pLysS strain transformed with a pET plasmid carrying the PRV DNase gene. After induction with IPTG, a 53 kDa protein was observed by SDS/PAGE analysis (Figure 1). The amount of purified protein recovered was approx. 5 mg per 100 ml of bacterial culture.

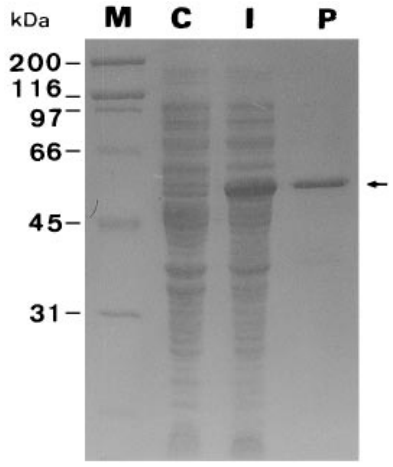

Figure 1 SDS/PAGE analysis of the polypeptide components of DNase preparations

The preparations of uninduced *E. coli* containing pET-DNase (lane C), IPTG-induced *E. coli* containing pET-DNase (lane I) and purified recombinant PRV DNase (lane P) were analysed by SDS/PAGE and stained by Coomassie Brilliant Blue. The molecular masses of protein standards (lane M) are shown at the left. The 53 kDa recombinant PRV DNase is indicated by the arrow.

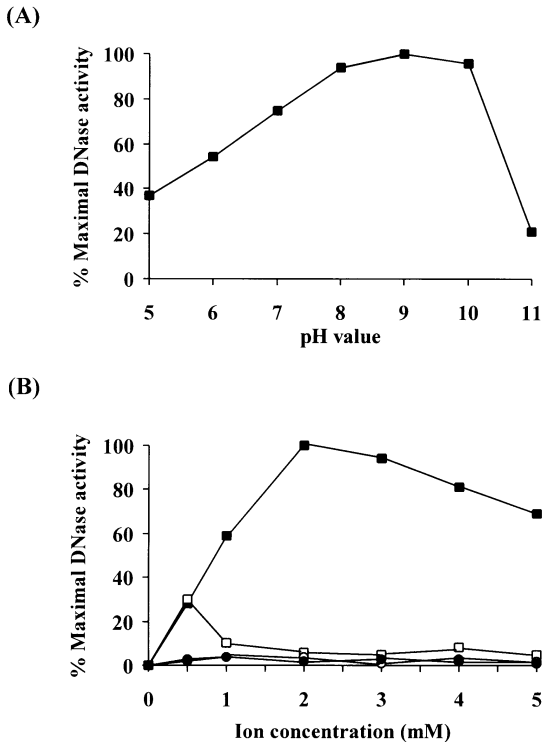

Figure 2 Biochemical properties of PRV DNase

(**A**) Effect of pH on enzyme activity. The following buffers were used to construct the pH curve: acetic acid (pH 5), sodium phosphate (pH 6), Tris/HCl (pH 7–9) and 3-(cyclohexylamino)-1-propanesulphonic acid (pH 10 and 11). (**B**) Effect of varied divalent and monovalent cations, and their concentration on enzyme activity: Mg^{2+} (■), Mn^{2+} (□), Ca^{2+} (●), Na^{+} (○).

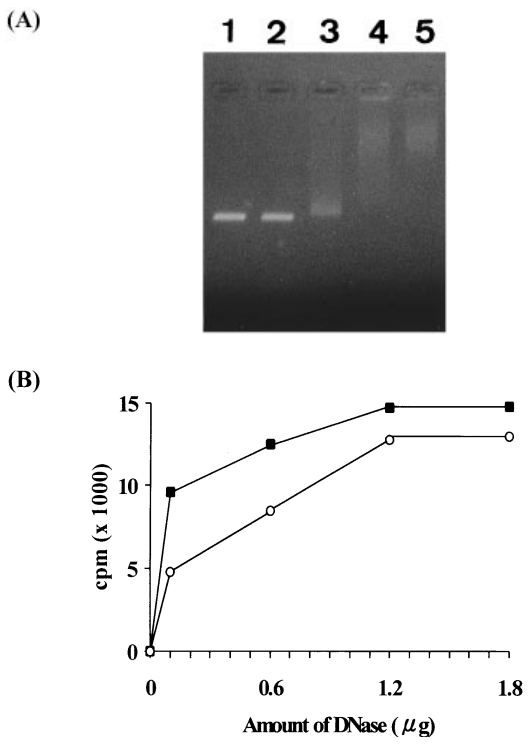

Figure 3 Binding of PRV DNase to DNA

(**A**) Agarose gel electrophoresis analysis of PRV DNase–dsDNA complexes. pUC18 dsDNA (100 ng) was mixed with 0 μg (lane 2), 0.2 μg (lane 3), 1 μg (lane 4) or 5 μg (lane 5) of recombinant PRV DNase, or 1 μg (lane 1) of BSA. (**B**) Nitrocellulose filter binding assay of PRV DNase–DNA complexes. ^{3}H-labelled pUC18 dsDNA (100 ng) (■) or pUC18 ssDNA (100 ng) (□) was mixed with various amounts of recombinant PRV DNase. The ssDNA was prepared by denaturing the pUC18 dsDNA at 100 °C for 5 min and cooling immediately on ice. The assays were performed as described in the Materials and methods section.

Biochemical properties of recombinant PRV DNase

Figure 2(A) shows an optimum DNase activity at pH 9.0. An inhibiting pH value seemed to be reached at pH 11.0. The effects of various divalent or monovalent ions on the activity of the purified enzyme are shown in Figure 2(B). There was an absolute requirement for Mg^{2+} ions for optimal enzyme activity, with an optimum concentration of 2 mM. Mn^{2+} ions gave an optimum at a concentration of 0.5 mM; however, the DNase activity remained limited to approx. 30% of that measured at the Mg^{2+} optimum. Ca^{2+} and Na^{+} ions could not replace Mg^{2+}. Taken together, the PRV DNase is an alkaline DNase with an absolute requirement for Mg^{2+}.

DNA-binding ability of recombinant PRV DNase

Figure 3(A) shows the results obtained by the electrophoresis of PRV DNase and DNA complexes at increasing protein-to-DNA ratios. The broad smears and shift band indicated the binding of PRV DNase to DNA. The nitrocellulose filter binding assay showed that PRV DNase possesses a similar affinity for ssDNA and duplex DNA (Figure 3B). Additionally, DNase bound to DNA in a dose-dependent manner, which suggests that the binding of DNase to DNA is co-operative. The saturation of dsDNA by PRV DNase occurs with a protein-to-DNA ratio of 12:1 (w/w). On the basis of this result, it can be estimated that the stoichiometry of binding of PRV DNase to dsDNA is one PRV DNase molecule per seven nucleotides.

Substrate preference of recombinant PRV DNase

We first examined the DNase activity on single-stranded homopolynucleotides. These results showed that recombinant PRV DNase hydrolysed various polymeric substrates in the order poly(dT) > poly(dA) = poly(dC) > poly(dG) (Figure 4A). In addition, the degradation of double-stranded polynucleotides was greater for poly(dA)·poly(dT) than for poly(dG)·poly(dC) (Figure 4B). Taken together, these results confirm that DNase exhibits both single-stranded and double-stranded DNase activity that is independent of significant base preference.

Endonuclease and exonuclease activities of recombinant PRV DNase

The endonuclease and exonuclease activities of PRV DNase were investigated by incubating the DNase with either supercoiled or linear pUC18 DNA. Endonucleolytic cleavage of supercoiled DNA occurred gradually as incubation time increased (Figure 5A). We further examined the release of 5′ and 3′ end-labelled nucleotides from dsDNA and ssDNA (Figure 5B and 5C). In these experiments, both 5′-labelled and 3′-labelled DNAs were hydrolysed effectively by DNase. Additionally, the 3′ end-labelled nucleotides are released by the enzyme, providing evidence that PRV DNase is not a phosphatase. Therefore these results show that the PRV DNase possesses endonuclease, 5′-exonuclease and 3′-exonuclease activities on dsDNA and ssDNA.

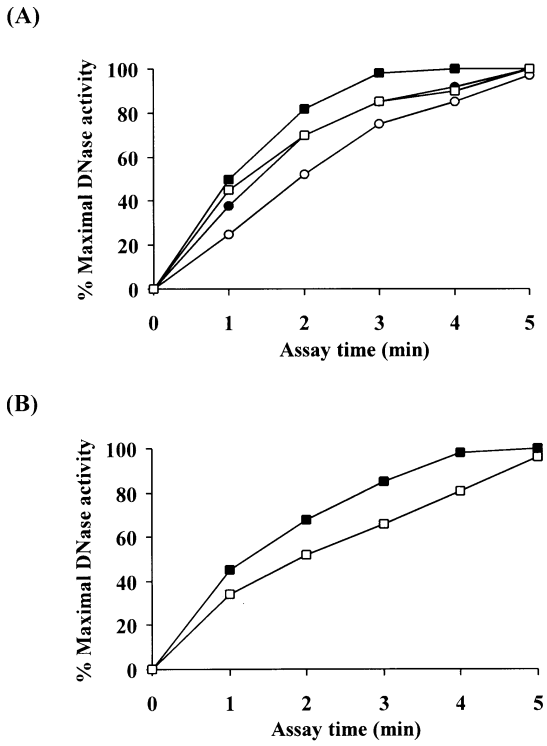

Figure 4 Substrate preference of PRV DNase

(**A**) ssDNA specificity of PRV DNase. 5′-Labelled poly(dT) (■), poly(dC) (□), poly(dA) (●) or poly(dG) (○) was hydrolysed by PRV DNase at the time indicated. (**B**) dsDNA specificity of PRV DNase. 5′-Labelled poly(dA)·poly(dT) (■) or poly(dG)·poly(dC) (□) duplex was used as a substrate. The single-stranded oligonucleotides were 20-mer synthetic homonucleotides. The poly(dA)·poly(dT) or poly(dG)·poly(dC) duplex was formed by mixing equimolar amounts of the two complementary oligonucleotides, heating to 80 °C for 5 min, and then cooling slowly to room temperature. Further descriptions of these assays are provided in the Materials and methods section.

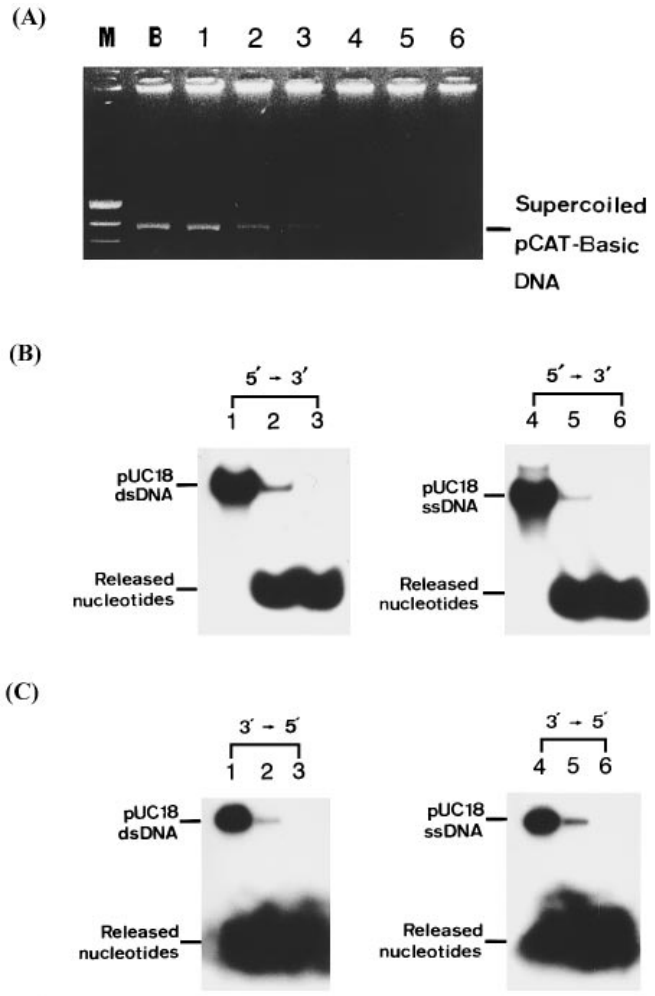

Figure 5 Endonuclease and exonuclease activities of PRV DNase

(**A**) Endonuclease activity of PRV DNase. Supercoiled pCAT-basic DNA (1 μg) was used as a substrate. The reaction mixtures were incubated at 37 °C for 15 s (lane 1), 45 s (lane 2), 2 min (lane 3), 3 min (lane 4), 4 min (lane 5) or 5 min (lane 6) before analysis by agarose gel electrophoresis. Lane B represents a reaction mixture that contains all the components minus PRV DNase. Lane M contained the lambda DNA/*Hin*dIII DNA size marker. (**B**) 5′-Exonuclease activity assay. 5′-Labelled pUC18 dsDNA (lanes 1–3) and pUC18 ssDNA (lanes 4–6) were hydrolysed by PRV DNase for 0 s (lanes 1 and 4), 20 s (lanes 2 and 5) and 60 s (lanes 3 and 6) at 25 °C. (**C**) 3′-Exonuclease activity assay. 3′-Labelled pUC18 dsDNA (lanes 1–3) and pUC18 ssDNA (lanes 4–6) were hydrolysed by PRV DNase for 0 s (lanes 1 and 4), 20 s (lanes 2 and 5) and 60 s (lanes 3 and 6) at 25 °C. The top band represents the labelled DNA substrate; the bottom band represents the radiolabelled nucleotides released by the enzyme. 5′-Labelled or 3′-labelled ssDNA was prepared by denaturing the respective labelled dsDNA at 100 °C for 5 min and subsequent cooling immediately on ice. Samples were separated on a 10% (w/v) polyacrylamide gel and proteins were detected by autoradiography.

DISCUSSION

This report describes the enzymic properties of recombinant PRV DNase produced by an overexpression system in *E. coli*. Previously, herpes viral DNase has been purified from virus-infected cells and from the soluble fraction obtained from the *E. coli* overexpression procedure [9,14]. These sources of DNase have proved to be difficult to work with because of the resulting low yields of DNase expression in virus-infected cells, and tedious and lengthy chromatographic separation methods. To solve these problems we expressed an N-terminal fusion with six histidine residues in *E. coli*, solubilized the inclusion bodies by urea, purified the protein by affinity chromatography and re-natured the protein by sequential dialysis. The histidine tag did not significantly alter the PRV DNase activity, on the basis of the findings that: (1) no detectable recombinant PRV DNase was purified by affinity chromatography after eliminating the denaturant, suggesting that the histidine tag was folded into the interior of protein structure upon renaturation (results not shown); (2) purified recombinant PRV DNase exhibits similar activity to that of herpes viral DNases purified from virus-infected cells.

The PRV DNase has an alkaline pH preference and an absolute requirement for Mg^{2+}. Additionally, we have demonstrated that Ca^{2+} and Na^{+} ions cannot replace the Mg^{2+} in PRV DNase activity. It has been demonstrated that most nucleases require divalent ions as cofactors for full promotion of catalytic action, and monovalent metal ions cannot replace divalent metal ions to stimulate the enzymic activity [15,16]. The Ca^{2+} ion usually participates in the mechanism of phosphodiester bond cleavage involving the nucleophilic activation of an active-site water molecule by general base catalysis, which is independent of pH [17]. Physiologically Mg^{2+} ion is used, but it can be replaced *in vitro* with Mn^{2+} ion, which leads to decreased specificity and catalytic activity in restriction enzyme [18]. Although the Mn^{2+} ion could substitute for Mg^{2+} as an enzymic cofactor, the PRV

DNase activity remains limited to approx. 30 % of that measured at the Mg^{2+} optimum. The effect of divalent cations on protein function requires further study.

Recombinant PRV DNase exhibits endonuclease, 5′-exonuclease and 3′-exonuclease activities that hydrolyse both ssDNA and dsDNA. By comparing the substrate (ssDNA or dsDNA) and mode of action (exonuclease or endonuclease) of PRV DNase with known nucleases, we found that the enzymic activities of PRV DNase are comparable to those of *Neurospora crassa* endo–exonuclease and *E. coli* RecBCD, two additional enzymes that are involved in recombination. The role of RecBCD in the recombination pathway in *E. coli* has been well studied [19]. The RecBCD enzyme complex functions by cutting and unwinding one strand of DNA that is located near the *chi* sequence, to generate an ssDNA tail. The RecA protein then assembles on this strand and forms a D-loop to initiate strand exchange with the homologous DNA. As demonstrated by the present study, PRV DNase has a catalytic function that is comparable to that of RecBCD. In addition, it has been demonstrated that HSV-1 ICP8, an ssDNA-binding protein, can also promote homologous pairing and strand transfer by interacting with HSV-1 nuclease [20,21]. Thus we speculate that the mechanism of herpes viral recombination is similar to that of the RecBCD pathway in *E. coli*, where the herpes viral nuclease and ICP8 correspond to the RecBCD and RecA proteins of *E. coli*. Because the recognition and unwinding processes vary from Rec proteins, we hypothesize that other proteins are involved in herpes viral recombination. Further experiments designed to examine the exact roles of PRV DNase, DNA-binding protein and other proteins in recombination will be required to understand more fully the recombination mechanism that occurs during PRV infection.

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