Identification of a DNA-binding domain and an active-site residue of pseudorabies virus DNase

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The pseudorabies virus (PRV) DNase gene has an open reading frame of 1476 nt, capable of coding a 492-residue protein. A previous study showed that PRV DNase is an alkaline exonuclease and endonuclease, exhibiting an *Escherichia coli* RecBCD-like catalytic function. To analyse its catalytic mechanism further, we constructed a set of clones truncated at the N-terminus or C-terminus of PRV DNase. The deleted mutants were expressed in *E. coli* with the use of pET expression vectors, then purified to homogeneity. Our results indicate that (1) the region spanning residues 274–492 exhibits a DNA-binding ability 7-fold that of the intact DNase; (2) the N-terminal 62 residues and the C-terminal 39 residues have important roles in 3′-exonuclease activity, and (3) residues 63–453 are responsible for 5′- and 3′-exonuclease activities. Further chemical modification of PRV DNase revealed that the inactivation of DNase by diethyl pyrocarbonate, which was reversible on treatment with hydroxylamine, seemed to be attributable solely to the modification of histidyl residues. Because the herpesviral DNases contained only one well-conserved histidine residue, site-directed mutagenesis was performed to replace His^{371} with Ala. The mutant lost most of its nuclease activity; however, it still exhibited a wild-type level of DNA-binding ability. In summary, these results indicate that PRV DNase contains an independent DNA-binding domain and that His^{371} is the active-site residue that has an essential role in PRV DNase activity.

Key words: catalysis, mutant, RecBCD, site-directed mutagenesis.

INTRODUCTION

Pseudorabies virus (PRV), the causative agent of Aujeszky's disease in pigs [1], is a member of the Alphaherpesvirinae [2]. PRV DNA is synthesized by a rolling-circle mechanism, yielding 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 the genes for thymidine kinase, ribonucleotide reductase, uracil glycosylase, deoxyuridine triphosphate nucleotidohydrolase and DNase [4–8].

A herpesviral mutant lacking DNase can synthesize wild-type levels of viral DNA, indicating that DNase has little or no role in herpesviral DNA replication [9]. Analysis of this mutant suggested that DNase is required for the efficient processing of viral DNA replication intermediates and for the egress of capsids from the nucleus but that it is not essential for viral DNA synthesis [10–12]. A previous study indicated that PRV DNase could cleave the double-stranded and single-stranded DNA species under alkaline conditions in the presence of Mg^{2+} ions. In addition it exhibits an *Escherichia coli* RecBCD-like catalytic function with 5′-exonuclease, 3′-exonuclease and endonuclease activities, suggesting that PRV DNase might have a role in the process of recombination that occurs during infection with PRV [13]. Several studies also indicated that similar modes of action are present in herpesviral DNases purified from different sources [14,15]. A structure–function analysis of the herpesviral DNase showed that mutations at the N-terminus of gammaherpesvirus (Epstein–Barr virus; EBV) eliminate its exonuclease activity [16]; however, other studies indicated that the N-terminus of herpes simplex virus type 1 (HSV-1) DNase might not have a role in its enzymic activity [17]. Goldstein and Weller [18] showed also that the region spanning residues 325–340 of HSV-1 DNase exhibits an important role in exonuclease activity. Despite these and other biochemical analyses, the functional domain and the active-site residue of herpesviral DNase remain to be defined.

To address this question, we constructed sequential deleted clones to identify the functional domain of PRV DNase. Chemical modification and site-directed mutagenesis were performed to analyse the active-site residue of PRV DNase. The catalytic mechanism of PRV DNase is also discussed.

MATERIALS AND METHODS

Construction of PRV DNase truncated clones

The cloning plasmid pGEM-B2S2A1 and the expression plasmid pET-DNase were constructed as described previously [8,13]. To establish a set of clones truncated at the N-termini of PRV DNase, pGEM-B2S2A1 was digested with *Not*I and *Sph*I, then deleted with the Erase-a-Base® system (Promega). The unidirectional deleted plasmids were then digested by *Hin*dIII and *Xho*I, eluted and ligated to the prokaryotic expression vector pET-28(+) to create the DNase N-terminally truncated clones N1(Δ1–62), N3(Δ1–119), N4(Δ1–181), N5(Δ1–223), N6(Δ1–273) and N7(Δ1–387) (Figure 1). To construct a set of C-terminally truncated mutants, the *Stu*I–*Xho*I, *Alw*NI–*Bam*HI, *Apa*I–*Bam*HI and *Hph*I–*Bam*HI fragments of pET-DNase were inserted into the vector pET-28(+) to create C1(Δ454–492), C2(Δ378–492), C3(Δ296–492) and C4(Δ184–492) respectively. An internal deleted mutant (I II-del), lacking residues 97–387, was built by deleting an 870 bp *Bal*I fragment of pET-DNase and ligating itself. All the plasmids created in this study were confirmed as in-frame constructions by sequencing.

Abbreviations used: DEPC, diethyl pyrocarbonate; EBV, Epstein–Barr virus; HSV-1, herpes simplex virus type 1; PRV, pseudorabies virus; UL12, alkaline nuclease.

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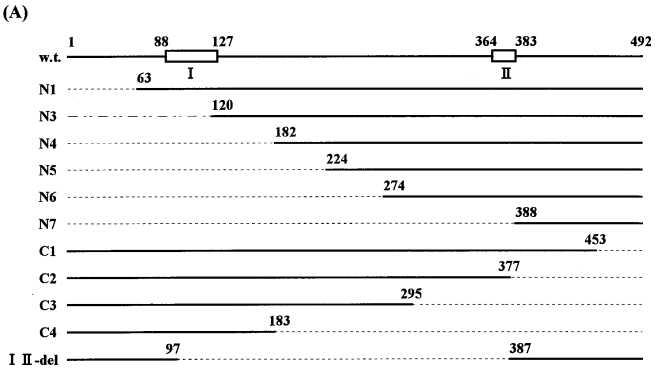

(B)

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Conserved region I
 Residues          88                                    126
 PRV               EAATRAQSESDLWTLLRRGLATASTVRWGADGPRFPPTW
 HSV-1             EAATQNQADCQLWALLRRGLTTASTLRWGPQGPCFSPQW
 VZV               ESETRGQGDNAIWTLLRRNLITASTLKWSVSGPVIPPQW
 HCMV              EKESRGQSRNSVWHLLRMDTVSATKFYEAFVSGCLPGAA
 EBV               ESMTRGQSENLMWDILRNGGIISSSKLLSTIKNGPTKVF

 Alphaherpesvirus E--T--Q-----W-LLRR-L-TAST--W---GP---P-W
 Herpesvirus       E-----Q-----W--LR----------------------

Conserved region II
 Residues          364                384
 PRV               PVFANPRHANFRQILVQSYVV
 HSV-1             PVFANPRHPNFKQILVQGYVL
 VZV               PVFANPRHANFKQIAVQTYVL
 HCMV              PAFVNPRHQYYFQMLIQQYVL
 EBV               NIFINPRHNYFYQVLLQ-YKI
 Alphaherpesvirus PVFANPRH-NF-QI-VQ-YV-
 Herpesvirus       --F-NPRH----Q---Q-Y--
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Figure 1 Construction of truncated mutants of PRV DNase

(**A**) Structures of truncated mutants of PRV DNase. The structure of wild-type DNase is shown at the top, with open boxes designating conserved regions I (residues 88–127) and II (residues 364–383). The diagrams illustrate the relative positions and codon numbers of truncated sites. Broken lines indicate the deleted regions. (**B**) Sequences of conserved region I and II of herpesviral DNase counterparts. Sequences from HSV-1 [33], varicella-zoster virus (VZV) [34], human cytomegalovirus (HCMV) [35] and EBV [35] were aligned with PRV sequence [8] by using the program PileUp. The highly conserved residues in alphaherpesviruses and herpesviruses are shown at the bottom.

Expression and purification of recombinant wild-type and truncated PRV DNases

Recombinant protein was expressed in *Escherichia coli* BL21 (DE3) pLysS strain by transforming pET-DNase or truncated mutant to produce an N-terminal fusion with six histidine residues. The proteins were purified as described previously [13] and stored at −70 °C until further analysis.

Nuclease activity assay

For analysis of the DNase activity, 2 pmol of purified enzyme was mixed with 1 μg of nick-translated ^{3}H-labelled pUC18 DNA in DNase buffer [50 mM Tris/HCl (pH 9.0)/2 mM $MgCl_2$/10 mM 2-mercaptoethanol] and incubated at 37 °C for 10 min. The undigested DNA was precipitated with trichloroacetic acid and the radioactivity of acid-soluble nucleotides was counted with a scintillation counter.

For analysing the 5′- and 3′-exonuclease activities, the reaction mixture, containing 0.2 pmol of purified protein, 0.5 μg of unlabelled, cognate DNA, 50 mM Tris/HCl, pH 9.0, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol in a final volume of 20 μl, was mixed with 0.1 μg of 5′- or 3′-labelled DNA respectively. After incubation for 1 min at 25 °C, 2 μl of 10× stop buffer [50 % (v/v) glycerol/1 % (w/v) SDS/0.1 % Bromophenol Blue/100 mM EDTA] was added to terminate the reaction. Samples were analysed on a 10 % (w/v) polyacrylamide gel and the degradative products were determined by densitometric scanning. The radiolabelling of the ends of double-stranded DNA was performed as described previously [13]. For analysis of endonuclease activity, 0.5 μg of pUC19 DNA was mixed with 2 pmol of purified protein, incubated at 37 °C and analysed by agarose-gel electrophoresis. The amount of DNA banding on an agarose gel was quantified by densitometric scanning.

Nitrocellulose filter binding assay

The formation of protein–DNA complexes was measured by using an alkali-treated nitrocellulose filter as described previously [13]. In brief, a 100 μl mixture containing 100 ng of ^{3}H-labelled DNA and 0.2 nmol of protein in DNA-binding buffer [50 mM Tris/HCl (pH 9.0)/10 mM 2-mercaptoethanol] 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 a scintillation counter.

Chemical modification and assay of restoration by hydroxylamine

PRV DNase (1 nmol), in 50 mM potassium phosphate buffer, pH 6.0, was incubated at 25 °C with various concentrations of diethyl pyrocarbonate (DEPC). Aliquots were withdrawn at 5, 10, 15 and 30 min; the reaction was stopped by the addition of 1 μl of 0.1 M imidazole, pH 6.0. The residual activities were subsequently determined by nuclease activity assay as described above. For the assay of restoration by hydroxylamine, the PRV DNase, modified as described above, was mixed with hydroxylamine to a final concentration of 20 mM and incubated at 4 °C for 5 h. The DNase activity was determined under standard assay conditions. The DEPC used in this study was freshly diluted with 100 % ethanol; the ethanol concentration in the reaction mixture did not exceed 2.5 % (v/v).

Spectral analysis

The DEPC-modified DNase was monitored spectrophotometrically by Beckman DU®-64 spectrophotometer. The absorbance was measured in the range 230–300 nm at 20 °C.

Preparation of uracil-containing single-stranded DNA

Uracil-containing single-stranded DNA was produced by transforming pET-DNase into *E. coli* CJ236 strain, which lost its deoxyuridine triphosphate nucleotidohydrolase and uracil glycosylase activities. In brief, the cells were inoculated into 5 ml of Luria–Bertani broth overnight and then refreshed in 50 ml Luria–Bertani broth at 37 °C with shaking. When D_{600} reached 0.3, 20 multiplicities of infection of helper phage M13KO7 were added and incubated for a further 8 h. The culture medium was then centrifuged for 5 min at 17000 ***g*** and 4 °C; the supernatant containing the transducing particles was collected. The supernatant was subsequently treated with 10 μg/ml RNase A/10 units/ml DNase I/2 mM $MgCl_2$ at 25 °C for 10 min, then precipitated with 3.5 M ammonium acetate/20 % (w/v) poly(ethylene glycol) (molecular mass 6 kDa). The pellet was resuspended in 200 μl of high salt buffer [0.3 M NaCl/0.1 M

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Tris/HCl (pH 8.0)/1 mM EDTA], incubated on ice for 30 min and centrifuged at 12000 *g* for 2 min to remove the low-molecular-mass DNA. The supernatant was extracted by phenol/chloroform (1:1, v/v); the uracil-containing single-stranded DNA was precipitated by ethanol.

Site-directed mutagenesis

Site-directed mutagenesis was performed as described previously [19]. Uracil-containing single-stranded DNA (0.3 pmol) was annealed with 6 pmol of 5′-kinase primer in 20 mM Tris/HCl (pH 8.0)/2 mM $MgCl_2$/50 mM NaCl. The second-strand DNA was then synthesized by the addition of 4 μl of 10 × synthesis buffer [4 mM deoxyribonucleotides/175 mM Tris/HCl (pH 8.0)/37.5 mM $MgCl_2$/5 mM dithiothreitol/7.5 mM ATP], 3 units of T4 DNA ligase and 1 unit of T4 DNA polymerase, followed by sequential incubations on ice for 5 min, at 25 °C for 5 min and at 37 °C for 90 min. The double-stranded DNA was then transformed into *E. coli* strain NM522, to destroy the uracil-containing strand by uracil glycosylase activity and to allow the mutated strand to be amplified. The primer (CGAAAGTTGG-CCGCTCGCGGGTTCG) used in this study was designed to displace the histidine residue with alanine and create a *Bsr*BI site at the same time.

RESULTS

Expression and purification of wild-type and truncated PRV DNases

The recombinant PRV DNase was purified from the *E. coli* BL21 (DE3) pLysS strain transformed with a pET plasmid carrying the wild-type or truncated DNase gene. After induction with isopropyl β-D-thiogalactoside, all 11 truncated mutants and a wild-type DNase were expressed in insoluble forms and revealed by SDS/PAGE (Figure 2). The proteins were then solubilized with urea and purified to homogeneity by nickel-affinity chromatography for further assays.

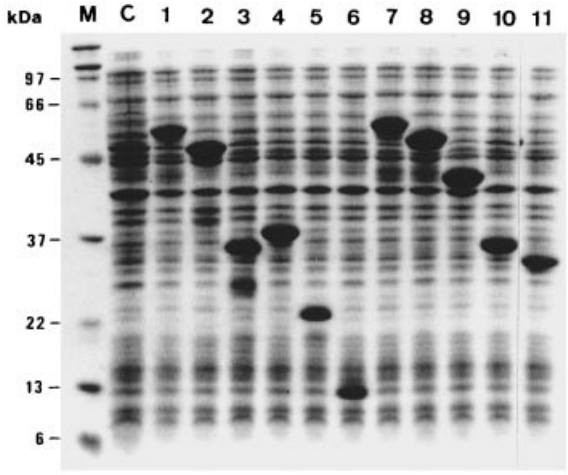

Figure 2 SDS/PAGE analysis of polypeptide components of truncated DNase preparations

The preparations of isopropyl β-D-thiogalactoside-induced *E. coli* were analysed by SDS/PAGE and stained with Coomassie Brilliant Blue. Lanes 1–11 contained N1, N3, N5, N4, N6, N7, C1, C2, C3, C4 and I II-del respectively. Lane C was loaded with the preparation of uninduced *E. coli* containing pET-DNase. The molecular masses of protein standards (lane M) are indicated at the left.

Table 1 Enzymic activities and DNA-binding abilities of wild-type and truncated PRV DNases

Equimolar amounts of each protein were assayed for their nuclease and DNA-binding activities. The relative activity of each protein was compared with that of wild-type DNase, set arbitrarily at 100.

Protein	DNase activity (%)	5′-Exonuclease activity (%)	3′-Exonuclease activity (%)	DNA-binding ability (%)
DNase	100	100	100	100
N1	87	89	23	21
N3	11	27	5	13
N4	9	11	8	14
N5	5	8	6	660
N6	6	1	6	680
N7	6	2	7	16
C1	80	73	35	20
C2	11	10	8	11
C3	9	11	8	11
C4	6	8	5	10
I II-del	10	5	6	23
H371A	11	7	8	87

Enzymic activity of truncated PRV DNases

The purified proteins were assayed for their DNase activities by using random-labelled, 5′-labelled or 3′-labelled double-stranded DNA as a substrate. Table 1 shows that the truncated mutants retained 23 % or 35 % 3′-exonuclease activity when residues 1–62 or 454–492 were removed. Mutant N3, which lacked residues 1–119, retained 27 % 5′-exonuclease activity. All mutants lacking residues 1–181 or 378–492 lost their 5′- and 3′-exonuclease activities. Moreover, the remaining nuclease activity of each mutant was consistent as the incubation time was prolonged, suggesting that the enzyme was rate-limiting for nuclease activity. These findings indicated that the N-terminal 62 residues and the C-terminal 39 residues had important roles in 3′-exonuclease activity, whereas residues 63–453 were responsible for 5′- and 3′-exonuclease activities.

DNA-binding ability of truncated PRV DNases

A nitrocellulose filter binding assay was performed to analyse the DNA-binding ability of truncated PRV DNases. Table 1 shows that mutants N5 and N6 exhibited a 7-fold increase in the DNA-binding ability over the wild-type DNase. A truncated mutant containing residues 274–387 displayed a loss of DNA-binding ability (results not shown). Taken together, these results suggested that PRV DNase contained an independent DNA-binding domain that was located in residues 274–492.

Effect of DEPC and hydroxylamine on PRV DNase activity

DEPC can modify different nucleophiles (such as amine, alcohol, thiols, imidazole and guanido groups), producing the carbethoxyl derivatives [20,21]. At pH 6.0, DEPC is mostly specific for histidine; however, it also reacts to a smaller extent with lysine and tyrosine. The modified residues could be further differentiated by spectral analysis and an assay for restoration with hydroxylamine. The O-carbethoxylation of tyrosine would result in a significant decrease in the absorbance of the modified protein at 278 nm, whereas the N-carbethoxylation of histidine shows an increase in absorbance at 240 nm [22]. Additionally, lysine-modified enzymes cannot recover their activity in the presence of hydroxylamine, whereas histidine-modified enzymes

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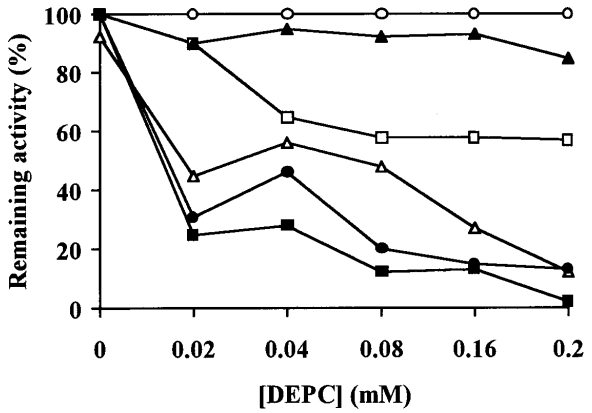

Figure 3 Effect of DEPC on PRV DNase activity

PRV DNase was mixed with various concentrations of DEPC and incubated at room temperature for 0 (○), 5 (□), 10 (△), 15 (●) or 30 (■) min; the remaining activity was analysed. An assay for restoration with hydroxylamine (▲) was performed by incubating 20 mM hydroxylamine and DEPC-inactivated DNase together at 4 °C for 5 h and subsequently assaying its enzymic activity.

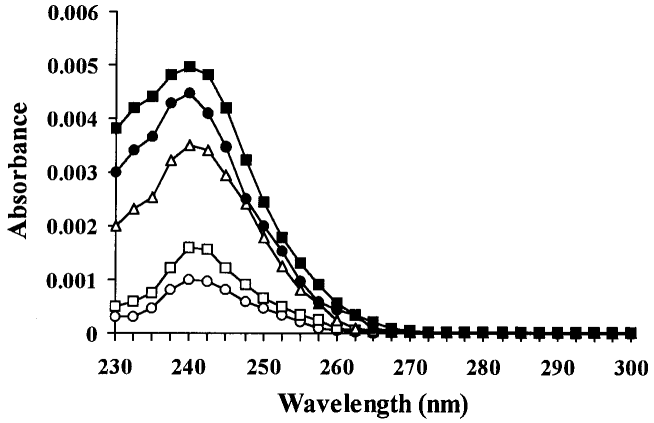

Figure 4 Spectral analysis of DEPC-inactivated PRV DNase

PRV DNase was mixed with 0.2 mM DEPC, incubated at 25 °C for 1 (○), 5 (□), 10 (△), 30 (●) or 60 (■) min and subsequently measured at 230–300 nm. The spectrum was recorded after subtraction of the blank consitsing of the reaction mixture without DEPC.

retrieve their function after treatment with hydroxylamine [23]. Carbethoxylation of PRV DNase by DEPC resulted in a loss of enzymic activity; the inactivation was concentration- and time-dependent (Figure 3). No loss of activity was observed in the control samples. The DNase activity was lost completely when the concentration of DEPC reached 0.2 mM; this lost activity could be recovered after treatment with hydroxylamine (Figure 3), suggesting that the residue modified by DEPC and yielding a response in PRV DNase activity might be histidine.

Further spectral analysis showed that the carbethoxylation of PRV DNase, as a result of treatment with DEPC, was accompanied by an increase in the absorbance of the modified protein at 240 nm but not at 280 nm (Figure 4). In addition the absorbance reached a limit after incubation for 60 min. It indicated that the loss of PRV DNase activity resulted from the modification of a histidine rather than a tyrosine residue.

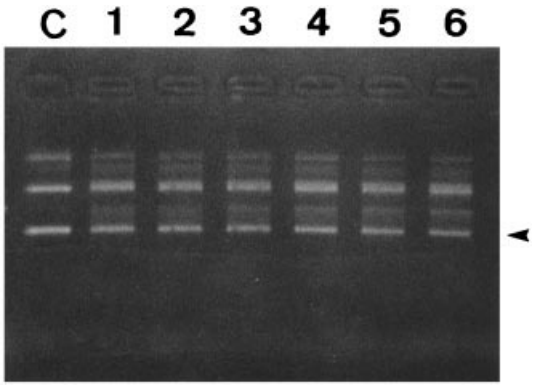

Figure 5 Endonuclease activity assay of site-directed mutant H371A

The reaction mixture, containing 0.5 μg of pUC19 DNA and H371A, was incubated at 37 °C for 30, 60, 120, 180, 240 and 300 min (lanes 1–6 respectively) and analysed by agarose-gel electrophoresis. Lane C contained BSA in place of DNase in the assay. The closed circular supercoiled pUC19 DNA is indicated with an arrowhead.

Characterization of site-directed mutant H371A

A comparison of DNase counterparts from several different herpesviruses shows that His^{371} of PRV DNase is highly conserved in herpesviral DNases (Figure 1), suggesting His^{371} as a candidate for site-directed mutagenesis. The primer for site-directed mutagenesis was designed to change His^{371} to a non-polar alanine residue and to create a new restriction enzyme site (*Bsr*BI) for screening. A total of 45 transformants were obtained. Five clones in this population were mutants, as judged by restriction enzyme digestion and sequencing analysis, and designated H371A. Mutant H371A displayed a loss of exonuclease and endonuclease activities (Table 1 and Figure 5). However, H371A exhibited a similar DNA-binding ability to that of a wild-type DNase (Table 1). The mobility-shift effect of H371A was also observed (Figure 5). These findings indicate that His^{371} is the active-site residue involved in PRV DNase activity but not in DNA-binding ability.

DISCUSSION

This paper describes the DNA-binding domain and the active-site residue of PRV DNase (Figure 6). An analysis of truncated mutants revealed that the N-terminal 62 residues, corresponding

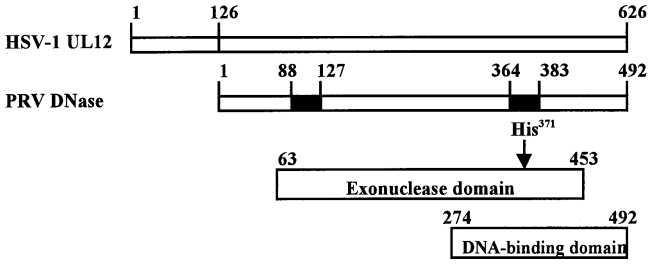

Figure 6 Summary diagram illustrating known functional features of PRV DNase

The structure of PRV DNase and the relative positions and codon numbers of HSV-1 UL12 are shown at the top, with filled boxes designating conserved region I (residues 88–127) and II (residues 364–383). The regions responsible for exonuclease and DNA-binding activities are shown below. Boundary amino acid positions for functional properties are numbered.

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to residues 126–192 of HSV-1 UL12 (alkaline nuclease), and the C-terminal 39 residues have important roles in 3′-exonuclease activity, whereas residues 63–453 are responsible for 5′- and 3′-exonuclease activities. Similar results have been reported previously: Bronstein et al. [24] showed that an N-terminally truncated version of HSV-1 UL12, designated UL12.5, retains endonuclease and exonuclease activities, whereas Goldstein et al. [18] showed that the N-terminus of the HSV-1 UL12 might not have a role in its enzymic activity. In the only other reported structure–function analysis of an alkaline nuclease counterpart, mutations in the N-terminus of EBV alkaline nuclease eliminate exonuclease activity [16]; however, these mutations are in a region that is not well conserved in the herpesviral counterparts. These inactivating residues might be EBV-specific.

The truncated mutants were analysed further for their DNA-binding abilities. Mutant N6 in this study exhibited a 7-fold increase in DNA-binding ability, suggesting that PRV DNase contains a DNA-binding domain located at residues 274–492. Moreover, all mutants except N5 and N6 lost their DNA-binding abilities. We speculate that the N-terminus and C-terminus of PRV DNase are flexible regions that are responsible for leading DNA to DNase or fixing the DNA on the DNA-binding region of DNase. A similar structure is observed on the Klenow fragment [25,26]. Although the truncation was at the N-terminus and the C-terminus, it results in a dysfunction of flexibility and subsequently blocks the interaction between DNA and DNase. The exact mechanism of the DNA-binding ability of PRV DNase remains to be elucidated.

In RNases such as RNase T1 [27], RNase T2 [28] and RNase A [29], and also in pancreatic DNase I [30] and S1 nuclease [31], histidine has been implicated in the active site of the enzyme. The chemical modification and site-directed mutagenesis used in this study indicated that PRV DNase is a member of the histidine nuclease family and that His^{371} is the active-site residue of PRV DNase. A previous study suggested that pancreatic DNase I interacts with the minor groove of DNA by van der Waals interactions or hydrogen-bonding and subsequently attacks the phosphodiester bond by a catalytic triad (Glu^{78}–His^{134}–water) [32]. The carboxylate anion of Glu^{78} accepts a proton from His^{134}, which in turn accepts a proton from the water molecules; as a nucleophile this then attacks the phosphorus atom, cleaving the phosphodiester bond. Although a comparison of PRV DNase and DNase I revealed a low similarity between their amino acid sequences, the involvement of His^{371} of PRV DNase and Asp^{340} of HSV-1 UL12 in the herpesviral DNase activities [18] suggests that a similar mechanism might occur in PRV DNase, in which the histidine and aspartic residues take the roles of the histidine and glutamic residues of DNase I respectively. Further studies designed to examine the catalytic mechanism of PRV DNase are in progress.

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