Site-directed mutagenesis in a conserved motif of Epstein–Barr virus DNase that is homologous to the catalytic centre of type II restriction endonucleases

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Sequence alignment of human herpesvirus DNases revealed that they share several conserved regions. One of these, the conserved motif D203…E225XK227 (D…EXK) in the sequence of Epstein–Barr virus (EBV) DNase, has a striking similarity to the catalytic sites of some other nucleases, including type II restriction endonucleases, λ exonuclease and MutH. The predicted secondary structures of these three residues were shown to resemble the three catalytic residues of type II restriction endonucleases. Site-directed mutagenesis was carried out to replace each of the acidic residues near the motif by residues with different properties. All substitutions of D203, E225 and K227 were shown to cause significant reductions in nuclease activity. Six other acidic residues, within the conserved regions, were also replaced by Asn or Gln. Five of these six variants retained nuclease activity and mutant D195N alone lost nuclease activity. The four charged residues, D195, D203, E225 and K227, of EBV DNase were found to be important for nuclease activity. Biochemical analysis indicated that the preference for divalent cations was altered from Mg2+ to Mn2+ for mutant E225D. The DNA-binding abilities of D203E, E225D and E225Q were shown to be similar to that of wild-type. However, K227 mutants were found to have variable DNA-binding abilities: K227G and K227N mutants retained, K227E and K227D had reduced and K227R lost DNA-binding ability. Comparison of the biochemical properties of the corresponding substitutions among EBV DNase and type II restriction enzymes indicated that the D…EXK motif is most likely the putative catalytic centre of EBV DNase.

INTRODUCTION

Many herpesviruses encode an alkaline deoxyribonuclease (DNase), which was originally characterized by its high pH optimum in vitro (Morrison & Keir, 1968). The DNases of various herpesviruses have similar biochemical properties, regardless of whether the enzymes are expressed in mammalian cells, Escherichia coli or using recombinant baculovirus. Biochemical studies of these herpesvirus DNases has shown that they accept both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) as substrate and exhibit both endonuclease and exonuclease activities, a high pH optimum, an absolute requirement for divalent cations and sensitivity to high salt concentration and polyamines (Clough, 1979, 1980; Hoffmann & Cheng, 1979; Hoffmann, 1981; Baylis et al., 1989, 1991; Chen et al., 1993; Bronstein & Weber, 1996; Sheaffer et al., 1997; Kehm et al., 1998). In the case of Epstein–Barr virus (EBV) DNase, dsDNA is digested processively but ssDNA distributively (Lin et al., 1995). To digest the substrate, the enzyme first converts supercoiled DNA to nicked open circular DNA, then nicks open circular DNA to linear DNA and subsequently degrades the linear DNA (Hoffmann, 1981; Stolzenberg & Ooka, 1990; Lin et al., 1995). The endonuclease activity of this enzyme seems to have a structural preference but no base sequence specificity (Lin et al., 1995; Kehm et al., 1998). Exonuclease digestion of DNA is in the 5′→3′ direction and the products are 5′-monophosphate nucleosides (Lin et al., 1995; Sheaffer et al., 1997). Despite these biochemical analyses, the role(s) of the enzyme remains unclear. In the case of EBV DNase, the enzyme was induced in parallel to EBV DNA polymerase (Cheng et al., 1980) and interacted with viral DNA replication components, DNA polymerase, EA-D and mDBP (Daibata & Sairenji, 1993; Lin et al., 1995; Zeng et al., 1997). Studies of infection with a DNase null mutant of herpes simplex virus (HSV) indicated that this enzyme was required for efficient egress of capsids from the nucleus (Shao et al., 1993) and for processing non-linear or
branched viral DNA intermediates (Martinez et al., 1996). From these results, it seems possible that herpesvirus DNases play roles in the conformational or maturational changes required for egress of DNA-containing capsids and in resolution of recombination intermediates.

Herpesvirus DNases are conserved, and alignments of amino acid sequences reveal that the DNases share several conserved regions (Martinez et al., 1996; Goldstein & Weller, 1998; Liu et al., 1998; see Fig. 1). Furthermore, human cytomegalovirus (HCMV) DNase can substitute for HSV-1 DNase and complement the growth of an HSV-1 DNase deletion mutant (Gao et al., 1998). In the absence of structural information from crystallographic studies, little is known about the cleavage mechanism and active site(s) for catalysis by herpesvirus DNases. In previous studies, we tried to locate the regions important for DNase activity by deleting amino acid residues serially from both ends of EBV DNase; a drastic loss of DNase activity was observed in mutants with small deletions and substitutions (Lin et al., 1994; Liu et al., 1998). We extended our mutational analysis to internal regions, including four conserved regions and

![Figure 1](image-url)

Fig. 1. Multiple sequence alignment and secondary structure prediction of the conserved D...EXK region of human herpesvirus DNases. (a) Sequence alignment was performed using the DIALING 2.1 program. The numbers in the sequences indicate the position of residues relative to each herpesvirus DNase. Amino acids were coloured according to the degree of conservation and amino acid properties. The acidic amino acids (D or E) conserved in all sequences are shaded red and those partially conserved in five or more are shaded in pink. The other amino acids conserved in eight or more sequences are shaded in yellow and those in less than eight are shaded green. The mutated residues and substitution amino acids are shown on the top of sequences. The aligned human herpesvirus DNases include P3HR1 and B95-8 (derived from different strains of EBV), HSV-1, HSV-2, VZV, HCMV, HHV-6, HHV-7 and HHV-8 DNases. Accession numbers for each sequence are given in Methods. The secondary structure elements are shown below the sequences: β-strands as arrows and α-helices as cylinders. The secondary structure elements of EBV DNase were predicted using the PHDsec program. (b) The secondary structure elements of EcoRI, EcoRV, PvuII, BamHI, λ-exonuclease and MutH are shown according to their X-ray crystal structures. The catalytic residues are shaded.
two non-conserved regions of herpesvirus DNases. DNA-binding and nuclease activities were abolished in all six internal deletion mutants, except that one mutant, with a deletion of residues 138–152, retained an intermediate activity to bind DNA (Liu et al., 1998). It is difficult to propose a mechanism for DNA cleavage by EBV DNase and to assign amino acid residues for an active centre(s) in catalysis simply from analyses of deletion and substitution mutations.

In this study, we have tried to define functional residues by comparing conserved regions of herpesvirus DNases with other nucleases for which crystal structures have been elucidated. We identified a conserved motif, D203…E225K227 (D…EXK), which has been shown to be a catalytic centre in some nucleases, including type II restriction endonucleases, λ exonuclease and MutH (Kovall & Matthews, 1999). Analysis of the crystallographic data revealed structural homologies in the active centres, despite the lack of sequence similarities (Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). Conserved acidic residues in the D…EXK motif have been implicated in metal-ion binding, while the lysine residue is probably involved in stabilization of the charged pentacoordinate transition state or participates directly in catalysis (Pingoud & Jeltsch, 1997). In addition, the D…EXK motif was found in many nucleases by Aravind et al. (2000), using the SEALS program package. Three conserved motifs were aligned and predicted as catalytic residues in these nucleases as well as herpesvirus DNases. In order to investigate the significance of the D…EXK motif in herpesvirus DNases, site-directed mutagenesis was used to manipulate this region in EBV DNase. The results demonstrated that D203, E225 and K227 of EBV DNase play a critical role in catalysis. Based on these data, we hypothesize that herpesvirus DNases have a similar catalytic centre to the type II restriction endonucleases.

**METHODS**

**Plasmids and site-directed mutagenesis.** pDNase5, a cDNA clone from P3HR1 cells, encodes the full-length (470 amino acids) wild-type EBV DNase subcloned into pET3a (Lin et al., 1994). The mutants in this study were derived from pDNase5 and generated by site-directed mutagenesis and subcloning strategies. Site-directed mutagenesis was performed using two-step PCR mutagenesis, as described previously (Liu et al., 1998). Each mutant was generated by two consecutive PCR reactions and the corresponding regions of pDNase5 replaced with the recombinant PCR product. For example, in the construction of D195N, the two primary PCR fragments of 516 bp and 346 bp were amplified using primer pairs IDP11 and APD195N, and SPD195N and IDP11, respectively (Table 1). The two primary PCR fragments were mixed and annealed through the sequences of APD195N and SPD195N (Table 1). The secondary PCR reaction was subsequently carried out by mixing 10 ng of each primary PCR fragment as template DNA, along with IDP11 and IDP1 as outer primers. A recombinant PCR product of 839 bp was amplified.

After treatment with Stul and Apal, the PCR-amplified fragment was used to replace the Std–Apal region of pDNase5. The resulting plasmid, D195N, was verified by DNA sequencing. For protein purification of wild-type and mutant DNases, the cDNA inserts were subcloned from pET3b to pET15b by digestion with Ndel and HindIII, as described previously (Tsai et al., 1997). The advantage of pET15b is that it carries a stretch of six histidine residues at the N terminus of the recombinant protein.

**Expression of recombinant EBV DNase and purification of His-tagged DNase.** Purification of wild-type and mutant proteins was carried out as described previously (Lin et al., 1994). Briefly, a 2 ml culture of *E. coli* BL21(DE3) pLysS (Novagen) harbouring a particular plasmid was grown to exponential phase (OD600 = 0.4–0.6) and induced for 2 h with 1 mM IPTG. Cells were harvested and lysed with 250 μl 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 1 mM DTT and 10% glycerol. After sonication, 5 μl of prepared lysate was assayed at various dilutions for nuclease activity (Chen et al., 1982; Lin et al., 1994). The reaction mixture contained 50 mM Tris/HCl, pH 8.0, 4 mM β-mercaptoethanol, 4 mM MgCl2, and 3 μg *E. coli* [14C]DNA (2 × 104 c.p.m.). The reaction mixture was incubated at 37 °C for 1 h and then stopped by the addition of 25 μl sheared calf thymus DNA (2 mg ml−1) followed by 25 μl 50% trichloroacetic acid. DNase activity was defined as the amount of enzyme that converted [14C]DNA to acid-soluble material. The relative nuclease activity was normalized with the DNase protein concentration, as described previously (Liu et al., 1998). The nuclease activity of each clone minus that of cells harbouring pET3a gave the net nuclease activity. The specific nuclease activity was calculated by dividing the net activity of each clone by the intensity of each expressed protein in enhanced chemiluminescence (ECL) Western blot analysis (Tsai et al., 1997), which was quantified by scanning with a densitometer. The relative nuclease activity is the specific nuclease activity of each clone divided by that of pDNase5.
The bound proteins were then eluted with 5 bed vols of TGI-100 (20 mM Tris/HCl, pH 8.0, 10% glycerol, 100 mM imidazole). The collected TGI-100 eluate was sequentially collected into four fractions (TGI-100-1, -2, -3, -4). The protein in each fraction was analysed by 10% SDS-PAGE.

**Plasmid DNA cleavage assay.** For the plasmid DNA cleavage assay, reaction mixtures contained 125 ng of purified DNase, 0.5 µg of pEGFP-C1 plasmid DNA (Invitrogen), 50 mM Tris/HCl, pH 8.0, 4 mM β-mercaptoethanol and 4 mM MgCl₂ or 4 mM MnCl₂. The reactions were incubated at 37°C, aliquots were taken at the indicated times and analysed by agarose gel electrophoresis. Similar assays were carried out using linear plasmid DNA, which was cut by HindIII.

**Expression of the DNase protein by in vitro transcription/translation and assay of DNA-binding activity.** For measuring the DNA-binding ability of EBV DNase, [35S]methionine-labelled DNase and dsDNA cellulose chromatography were used as described previously (Liu et al., 1998). Using a TNT T7 coupled reticulocyte lysate system (Promega), DNA templates of pDNase5 and its mutants were transcribed with T7 RNA polymerase and translated in a rabbit reticulocyte lysate. Each 0.25 µg of purified plasmid DNA was used as an *in vitro* transcription/translation (IVT) template in a final volume of 12.5 µl. The IVT-expressed polypeptides were radio-labelled in the reaction mixture containing [35S]methionine. After 90 min of *in vitro* translation at 30°C, the [35S]methionine-labelled polypeptides were diluted 1:10 in buffer A (50 mM Tris/HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, 10% glycerol) containing 100 µg RNase A. An aliquot (12.5 µl) was retained as starting material and the remainder was applied to a 200 µl bed volume of dsDNA–cellulose (Sigma) in a Bio-Spin column (Bio-Rad) equilibrated in buffer A. The column was washed with 300 µl buffer A and then eluted with 400 µl step gradients of 100, 200, 300, 400 and 500 mM NaCl in buffer A. Five µl of starting material and 20 µl of eluates were mixed with SDS-PAGE sample buffer (100 mM Tris/HCl, pH 6.8, 4% SDS, 5% β-mercaptoethanol, 20% glycerol, 0.05% bromophenol blue) and analysed by 10% SDS-PAGE and fluorography.

**RESULTS**

Multiple sequence alignment and secondary structure prediction of the conserved D...EXK region of human herpesvirus DNases

The critical amino acid residues of EBV DNase were predicted by sequence alignment with other human herpesvirus DNases. The alignment revealed that these enzymes share several conserved regions. Comparison of these conserved regions with those of other nucleases, with defined crystal structures, revealed that a conserved motif,
D203...E225X K227 (in the EBV DNase sequence), has a striking similarity to the catalytic sites of various nucleases, including type II restriction endonucleases, exonuclease and MutH (Fig. 1a, b). The secondary structures of this motif were predicted and indicated that D203 was located in a loop and E225 and K227 were located in a β-sheet. The predicted secondary structures involving these three residues were shown to resemble three catalytic residues of the type II restriction endonucleases, exonuclease and MutH (Fig. 1b). Based on these similarities, it seems possible that D203, E225 and K227 of EBV DNase are involved in catalysis.

Analysis of the nuclease activity of EBV DNase mutants

To investigate the possible functional roles of the three conserved residues, each was replaced by residues with different properties using site-directed mutagenesis. Residue D203 was changed to Asn, Lys, Gln, Glu or His; residue E225 was changed to either Asp or Gln and residue K227 was changed to Glu, Asp, Gly, Asn or Arg (Fig. 1a). In addition, the other acidic residues (D195, E209, D213, D219, E238 and D240) within conserved regions also were replaced by Asn or Gln (Fig. 1a). Wild-type and mutant DNases were cloned into the pET3a vector and expressed in E. coli BL21(DE3) pLysS. The recombinant EBV DNase was intact and contained no additional domains derived from the vector. The nuclease activities of wild-type mutants and the vector control were measured. All mutants with substitutions of D203, E225 and K227 showed drastically reduced nuclease activity (Fig. 2). Five of the six other acidic residue mutants, E209Q, D213N, D219N, E238Q and D240N, retained their nuclease activities (> 75 %) and only the mutant D195N lost nuclease activity (3%) (Fig. 2). Residue D195 was not conserved in the alignment of herpesvirus DNases but a conserved Asp residue was found nearby in other herpesvirus DNases (Fig. 1a). Based on the analysis of nuclease activity, we found that four charged residues of EBV DNase, D195, D203, E225 and K227, may play important roles in catalysis.

Biochemical characterization of EBV DNase mutants D203E, E225D, E225Q and K227E

EBV DNase mutants were cloned into the pET15b vector and tagged with six histidines to characterize further their biochemical properties. His-tagged wild-type DNase and its mutants were expressed and purified by chromatography on a His-Bind column. The purified enzymes are shown in Fig. 3. The purified enzymes were incubated with supercoiled or linear DNA to determine the endonuclease and exonuclease activities, respectively. In addition, the effect of divalent cations was examined. In the case of His-tagged wild-type EBV DNase, a DNA cleavage pattern was observed with the appearance initially of nicked open circular DNA, then linear DNA and subsequently degraded DNA (Fig. 4a). DNA was degraded more rapidly in the presence of Mg2+ than Mn2+ (Fig. 4a). In the presence of Mg2+, Ca2+ was inhibitory for DNase activity (data not shown). These biochemical properties of His-tagged DNase were essentially the same as those described previously (Hoffman & Cheng, 1979; Lin et al., 1995). Therefore, the His-tagged DNase retains the same enzymatic characteristics as the intact DNase. His-tagged DNase mutants, as well as His-tagged wild-type DNase, were characterized with respect to a variety of reaction conditions. In the case of the D203E and K227E mutants, cleavage of supercoiled DNA or linear DNA could not be detected in the presence of Mg2+ or Mn2+ (Fig. 4b, e; Fig. 5a, b). For the E225Q mutant, cleavage of supercoiled DNA was not detected in the presence of...
Mg\(^{2+}\) (Fig. 4d), whereas supercoiled DNA was converted to linear DNA, but not degraded further, in the presence of Mn\(^{2+}\) (Fig. 4d). Degradation of linear DNA by E225Q was not detected in the presence of Mg\(^{2+}\) or Mn\(^{2+}\) (Fig. 5a, b), indicating that E225Q retained slight endonuclease activity in the presence of Mn\(^{2+}\) but had lost exonuclease activity. In the case of E225D, both supercoiled and linear DNA were degraded in the presence of Mn\(^{2+}\) but not Mg\(^{2+}\) (Fig. 4c; Fig. 5a, b), indicating an altered requirement for divalent cations from Mg\(^{2+}\) to Mn\(^{2+}\). Interestingly, the linear DNA was degraded more rapidly in the presence of Mn\(^{2+}\) by E225D than by wild-type DNase (Fig. 5b). Substitution of E225 by aspartic acid did not change the acidic residue, but decreased the length of the side chain, suggesting that residue E225 is involved in the structure of the metal binding site.

**DNA-binding ability of DNase mutants**

To characterize the DNA-binding ability of DNase mutants, DNase mutant polypeptides were expressed and labelled with \(^{35}\)Smethionine using an IVT system. The IVT-expressed polypeptides were applied to a dsDNA–cellulose column and the strength of DNA binding was determined by elution with NaCl step gradients (Liu et al., 1998). As shown in Fig. 6, the IVT-expressed wild-type DNase bound efficiently to dsDNA–cellulose and the protein could be detected predominantly in the fractions eluted by 400 and 500 mM NaCl. The elution profiles of D203E, E225D and E225Q were similar to that of wild-type (Fig. 6). Although these mutants were defective in cleaving DNA, their DNA-binding abilities were not affected, suggesting that other changes might contribute to the loss of nuclease activity for

**Fig. 4.** Digestion of circular DNA by EBV DNase and mutants. Purified protein of wild-type (a), D203E (b), E225D (c), E225Q (d) and K227E (e) mutants was incubated with pEGFP-C1 DNA in the presence of MgCl\(_2\) or MnCl\(_2\). Samples taken at the times indicated were electrophoresed through 0-7 % agarose. OC and SC indicate the respective positions of the open circular and supercoiled forms of pEGFP-C1 DNA.
these mutants. The IVT-expressed polypeptides from K227 mutants were shown to have different abilities to bind DNA. K227G and K227N mutants were detected predominantly at the 400 mM NaCl fraction and K227E and K227D were at the 200 and 300 mM NaCl fractions (Fig. 6). However, mutant K227R, which maintained the positive charge, was detected predominantly in the 100 mM NaCl fraction (Fig. 6), indicating that it had almost completely lost DNA-binding ability. One reason that K227R loses its nuclease activity may be through the impairment in DNA binding. Since K227G retained and K227R lost DNA-binding ability, the positive charge of K227 may not be essential for DNA binding and K227 may play a role in catalysis distinct from DNA binding.

**DISCUSSION**

In common with other herpesviruses, EBV encodes a DNase that is expressed during the lytic cycle. It has been demonstrated that herpesvirus DNases have significant sequence homology and similar biochemical properties and there are, therefore, likely to be conserved catalytic residues. To identify them, we compared the conserved residues of herpesvirus DNases with the catalytic residues of other nuclease for which crystal structures have been determined. We found a conserved motif, D203...E225XK227, in EBV DNase, which is homologous to the catalytic centres of some nucleases, including type II restriction endonucleases, λ exonuclease and MutH (Kovall & Matthews, 1998, 1999). The D...EXK motif was also found in Holliday junction resolvases and related nucleases (Aravind et al., 2000). In addition to the D...EXK motif, a Gly/Leu-rich region was found near the first acidic residue of EBV DNase, similar to EcoRI, PvuII and BamHI (Fig. 1b). Furthermore, a cluster of hydrophobic residues between D203 and E225 of EBV DNase also paralleled EcoRI and BamHI (Fig. 1b). Prediction of the secondary structure of the domain including D203, E225 and K227 suggested that D203 was located in a loop and E225 and K227 in a β-sheet, similar to the location of catalytic residues of type II restriction enzymes (Fig. 1a, b). These similarities are strong enough to propose that D203, E225 and K227 of EBV DNase correspond to three functional residues of type II restriction enzymes.

To test this hypothesis, each of the three conserved residues and flanking acidic residues were replaced by site-directed mutagenesis. Residue D203 was changed to Asn, Lys, Gln, Glu or His, residue E225 was changed to Asp or Gln, and
residue K227 was changed to Glu, Asp, Gly, Asn or Arg. All of these mutants exhibited significantly reduced nuclease activities (Fig. 2), underlining the importance of these three residues. Similar mutagenesis studies have been performed on EcoRI, EcoRV, BamHI and PvuII (Selent et al., 1992; Dorner & Schildkraut, 1994; Grabowski et al., 1995; Nastri et al., 1997). Mutation of any of the three corresponding residues of these restriction enzymes abolished nuclease activity. Mutant D430E of HSV-1 alkaline nuclease, corresponding to the D203E mutant of EBV DNase, lost exonuclease activity but retained endonuclease activity (Goldstein & Weller, 1998). According to crystallographic studies, the two acidic residues form a binding site for the catalytically essential metal ion and the Lys residue orients the water and assists in the nucleophile attack on the phosphorus atom (Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). Corresponding amino acid residues D203 and E225 of EBV DNase were proposed to bind the metal phosphorus atom (Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). Mutation of any of the three corresponding residues of these restriction enzymes abolished nuclease activity. Mutant D430E of HSV-1 alkaline nuclease, corresponding to the D203E mutant of EBV DNase, lost exonuclease activity but retained endonuclease activity (Goldstein & Weller, 1998). According to crystallographic studies, the two acidic residues form a binding site for the catalytically essential metal ion and the Lys residue orients the water and assists in the nucleophile attack on the phosphorus atom (Pingoud & Jeltsch, 1997; Kovall & Matthews, 1999). Corresponding amino acid residues D203 and E225 of EBV DNase were proposed to bind the metal ion. In a plasmid DNA cleavage assay, Mn$^{2+}$ could activate the E225D mutant (Fig. 4c), indicating a change of preference for divalent cations from Mg$^{2+}$ to Mn$^{2+}$ and implying that the E225 residue is involved in the formation of the metal binding site. Because the radial size of Mn$^{2+}$ is larger than Mg$^{2+}$ and the side chain of Asp is shorter than that of Glu, the change in cation preference of the E225D mutant is most likely attributable to the short side chain of Asp forming a larger metal binding site, fitting Mn$^{2+}$ better than Mg$^{2+}$. Unlike E225D, D203E and K227E were not activated by Mn$^{2+}$. Interestingly, a K92E mutant of EcoRV also became active in the presence of Mn$^{2+}$ (Selent et al., 1992) and an E98Q mutant of MnlI endonuclease switched its cofactor requirement from Mg$^{2+}$ to Mn$^{2+}$ (Lagunavicius & Siksnys, 1997). Unlike the K92E mutant of EcoRV, K227E of EBV DNase lost nuclease activity, even in the presence of Mn$^{2+}$, which is similar to a K113E mutant of EcoRI (Grabowski et al., 1995). In addition to D203, E225 and K227, six other acidic residues near the D...EXK motif were also replaced by Asn or Gln. Mutational analysis showed that five of the six acidic residue mutants retained, and only mutant D195N lost, nuclease activity (Fig. 2). Although the D195 residue of EBV DNase is not conserved, a conserved Asp residue was aligned nearby in other herpesvirus DNases (Fig. 1a). The E238 residue of EBV DNase is partially conserved (Asp or Glu in the other herpesvirus DNases, shown in Fig. 1a), but the E238Q mutant retained nuclease activity, indicating that the carboxylate group of E238 was not required for catalysis. Many metal-dependent nucleases contain more than two catalytic acidic residues (Asp or Glu) to form metal binding sites at their active sites, such as type II restriction endonucleases (Pingoud & Jeltsch, 1997), the 3'→5' exonuclease activity of the Klenow fragment (Bernad et al., 1989), T4 RNase H and RAD2 (Mueser et al., 1996) and hFEN-1 (Shen et al., 1997). In the alignment of human herpesvirus DNases, wholly conserved acidic residues include E107, E166, D203 and E225 in the EBV sequence. The role of E166 in EBV DNase was predicted to involve catalysis (Aravind et al., 2000). The residue E107 could not be predicted in the absence of homology. However, the presence of a D(...DEXK motif in the active sites of type II restriction endonucleases and other nucleases provided the information to predict the roles of D203 and E225. Although amino acid sequences of type II endonucleases and other nucleases did not exhibit any significant sequence identity, local structures, particularly in catalytic sites, showed striking resemblance (Kovall & Matthews, 1999). It was suggested that these nucleases with structural homology were diverged from a common ancestor (Kovall & Matthews, 1999). Furthermore, Glu113 of BamHI substitutes for the conserved Lys and the active site was characterized as a D...EXE motif (Newman et al., 1994). The (D...EXK(D/E) motif may exhibit a common function for nucleases and many nucleases contain such a motif in their active sites, such as the 3'→5' exonucleases of prokaryotic and eukaryotic DNA polymerases, T4 RNase H and RAD2, bacterial RecJ exonucleases, hFEN-1 and Holliday junction resolvases and related nucleases (Bernad et al., 1989; Morrison et al., 1991; Mueser et al., 1996; Moser et al., 1997; Shen et al., 1997, 1998; Aravind & Koonin, 1998; Aravind et al., 2000). These similarities indicate that the catalytic (D/E)X(K/D/E) motif is conserved in the evolution of nucleases. Corresponding to this concept, Nael was found to be a likely evolutionary bridge between DNA endonuclease and topoisomerase (Huai et al., 2000). Double-stranded DNA–cellulose chromatography was used to determine the DNA-binding characteristics of the mutants. Wild-type EBV DNase, expressed by IVT, bound efficiently to dsDNA and was eluted with 500 mM NaCl (Fig. 6). The DNA-binding characteristics of the D203E, E225D and E225Q mutants were similar to that of wild-type (Fig. 6). The D203 and E225 mutants of EBV DNase bound but failed to cleave DNA, similar to D91A and E111A of EcoRI, D74A and D90A of EcoRV, and D94A and E111A of BamHI (Selent et al., 1992; Dorner & Schildkraut, 1994; Grabowski et al., 1995). K227 mutants of EBV DNase showed variable ability to bind DNA: K227G and K227N mutants lost the ability to bind DNA, similar to K70A of PvuII and K70A of PvuII lost nuclease activity but bound to DNA (Selent et al., 1992; Grabowski et al., 1995; Nastri et al., 1997), similar to the K227G mutant of EBV DNase. However, the K227R mutant of EBV DNase lost the ability to bind DNA, similar to K70R of PvuII (Nastri et al., 1997). Mutational analysis of K227 mutants of EBV DNase demonstrated that biochemical alteration of K227 mutants was similar to their counterparts in EcoRI and PvuII, underlining the similarity of these residues. Based on the studies of site-directed mutagenesis and biochemical properties of corresponding substitutions, it is suggested that the conserved motif D...EXK of herpesvirus DNases is
most likely the putative catalytic centre, and is homologous to those of type II restriction endonucleases.

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