Journal of Biochemistry and Molecular Biology, Vol. 31, No. 5, September 1998, pp. 498–502

JOURNAL OF Biochemistry and Molecular Biology

© BSRK & Springer-Verlag 1998

HBV Polymerase Residues Asp[429] and Asp[551], Invariant at Motifs A and C are Essential to DNA Binding

Younhee Kim†, Young Bin Hong and Guhung Jung*

Department of Biology Education, Seoul National University, Seoul 151–742, Korea

†Present Address: Department of Oriental Medicine, Semyung University, Checheon, Chungbuk 390–711, Korea

Received 10 June 1998, Accepted 30 June 1998

HBV polymerase shares several regions of amino acid homology with other DNA-directed and RNA-directed polymerases. The amino acid residues Asp[429], Gly[518], Asp[551], Lys[585], and Gly[641] in the conserved motifs A, B′, C, D, and E in the polymerase domain of HBV polymerase were mutated to alanine or histidine by *in vitro* site-directed mutagenesis. Those mutants were overexpressed, purified, and analyzed against DNA-dependent DNA polymerase activity and affinity for DNA binding. All those mutants did not show DNA-dependent DNA polymerase activities indicating that those five amino acid residues are all critical in DNA polymerase activity. South-Western analysis shows that amino acid residues Asp[429] and Asp[551] are essential to DNA binding, and Gly[518] and Lys[585] also affect DNA binding to a certain extent.

Keywords: Conserved motifs, DNA binding, HBV DNA polymerase, Polymerase active site, Site-directed mutagenesis.

Introduction

Hepatitis B virus (HBV) is a member of the family *hepadnaviridae*. The virus causes chronic hepatic insufficiency, cirrhosis, and hepatocellular carcinoma (HCC). The number of virus carriers is estimated to exceed 300 million worldwide (Ganem and Varmus, 1987; Beasley, 1988). Hepadnavirus replication involves the synthesis of viral DNA from an RNA pregenome and the process is mediated by viral polymerase encoded by a Polymerase Open Reading Frame (P ORF) (Summers and Mason, 1982; Ganem and Varmus, 1987). Mutational and sequence analyses between the coding region of the P ORF and those of several retroviral reverse transcriptases (RTs) revealed that they share sequence homology and that there are four domains within the P ORF (Toh *et al.*, 1983; Khudyakov and Makhov, 1989): a terminal protein in the N-terminus followed by a spacer region and then a reverse transcriptase (RT) and a C-terminal RNase H domain (Radziwill *et al.*, 1988; Li *et al.*, 1989; Chang *et al.*, 1990; Faruqi *et al.*, 1991). Hepatitis B viral polymerase is a multifunctional enzyme containing protein priming activity (Wang and Seeger, 1992; Zoolim and Seeger, 1994; Lanford *et al.*, 1995), DNA-dependent DNA polymerase, RNA-dependent DNA polymerase, and RNase H activities (Ganem and Varmus, 1987; Khudyakov and Makhov, 1989).

Comparison of the five known polymerase structures of Klenow fragment of pol I (Beese *et al.*, 1993), HIV-1 reverse transcriptase (Jacobo-Molina *et al.*, 1993), bacteriophage T7 RNA polymerase (Sousa *et al.*, 1993), DNA polymerase β (Pelletier *et al.*, 1994), and *Taq* DNA polymerase (Eom *et al.*, 1996) demonstrated that even far-related polymerases may have analogous polymerization active sites with a small number of crucial side chains in common (Joyce and Steitz, 1994). On the basis of amino acid sequence similarities, DNA-directed and RNA-directed polymerases have several conservation motifs identified by Delarue *et al.*, Poch *et al.*, and Mendez *et al.* as shown in Fig. 1 (Sousa, 1996). Motifs A and C are conserved irrespective of polymerase-template or substrate specificity, reflecting a direct role for these structures in the activity common to all polymerases: phosphodiester bond formation. Two Asp residues in motifs A and C bind and present two metal ions in the appropriate geometrical arrangement to catalyze a phosphoryl transfer reaction at the active site (Steitz *et al.*, 1994). Conservation of motifs T/DxxGR, B in the DNA-directed polymerases, and

* To whom correspondence should be addressed.
Tel: 82-2-880-7773; Fax: 82-2-880-7773
E-mail: drjung@plaza.snu.ac.kr

†Present Address: Department of Oriental Medicine, Semyung University, Checheon, Chungbuk 390–711, Korea

Motif designation	T/DxxGR	A	B	C		
DNA-directed polymerase						
DNA polymerase (pol I-like, pol α-like)	htxxGR	DhxxhEh	KhhxxxxhYG	hxD		
RNA polymerase (phage, mitochondrial)	hDhRGRhY	PhxxDxxCxGhQHh	RxhxK+xVMThxYG	hHDSFGT		
Large subunit of the multimeric RNAPs	VDhSGRSV					
HBV polymerase		LDVSAAFYHI	LGFRKIPMGGGLSP	YMDDVVL	GIHLNPNK	VGLLGF
RNA-directed polymerase						
DNA polymerase		hDhxxxhxxh	hxhx−xhQGxxxSP	YhDDhhh	Ghxh.xxxK	hxhLGh
RNA polymerase		DhxxxhD	SGxxxx.h	hhxGDDxhh	GxxhxxxK	
Motif designation		A	B′	C	D	E

Fig. 1. Patterns of conserved motifs in nucleic acid polymerase. Residues underlined are invariant. Other residues are well conserved: h, hydrophobic residue; +, positively charged residue; ×, any residue; ., a sequence gap (Sousa, 1996).

conservation of motifs B′ in the RNA-directed polymerases, reflects a role for these elements in template-strand binding. Motif E, conserved only in the DNA-synthesizing polymerases within the RNA-directed class of enzymes, reflects the role of this structural element in primer contacts (Sousa, 1996).

In this paper, we report a mutational analysis of amino acid residues Asp^{429}, Gly^{518}, Asp^{551}, Lys^{585}, and Gly^{641} corresponding to the most representative homology regions of motifs A, B′, C, D, and E in the polymerase domain of HBV polymerase, with the construction and comparison of wild and mutant proteins in the corresponding residue of HBV polymerase.

Materials and Methods

Nucleotides Unlabeled nucleotides were purchased from Pharmacia P-L Biochemicals (Uppsala, Sweden). Oligonucleotides used in mutagenesis were synthesized in Integrated DNA Technology Inc. (Coralvillo, USA). Homopolymer template poly(dA)·oligo$(dT)_{12-18}$ was obtained from Boehringer Mannheim Biochemicals (Indianapolis, USA), and [α-^{32}P]dTTP (3000 Ci/mmol) was purchased from Amersham International Plc (Buckinghamshire, UK).

Molecular clones and expression of mutant MBP-fused HBV polymerase The polymerase gene of HBV sequences (subtype *adr*; Rho *et al.*, 1989) was cloned in frame (pMPLX) as previously described (Lee *et al.*, 1993). To improve the expression of MBP (maltose binding protein) fused-HBV polymerase in *E. coli*, a six histidine tag was introduced to the C-terminus of the HBV polymerase, designated pMPH, as described previously (Kwak *et al.*, 1996). Plasmid pMPH was used for site-directed mutagenesis, carried out according to Kunkel (1987). The presence of desired mutations was confirmed by the dideoxy nucleotide chain-termination method (Sanger *et al.*, 1977). MBP-fused mutant proteins were expressed in *E. coli* strain NM522. Purification of MBP-fused mutant proteins was performed according to the manufacturer's recommendation (New England Biolabs, Inc., Beverly, USA) with some modifications as described previously (Jeong *et al.*, 1996).

DNA polymerase assay The standard enzyme reaction (50 μl) contained 50 ng of homopolymer template poly(dA)·oligo $(dT)_{12-18}$ (Boehringer Mannheim Biochemicals), 2 μCi of [α-^{32}P]dTTP (3000 Ci/mmol, Amersham), 50 mM Tris·HCl (pH 7.4), 0.1% NP-40, 4 mM $MnCl_2$, 1 mM DTT, and 75 mM NaCl. Reaction was started by adding 0.5 μg of the purified mutant fusion proteins and incubated at 37°C for 1 h, and the reaction was stopped by adding 50 mM EDTA. The reaction mixture was blotted onto Whatman DE81 filters and dried. The filters were then washed three times with 0.5 M Na_2HPO_4, once with water, and once with 95% ethanol, and dried. Radioactivity was measured by liquid scintillation counting. The DNA polymerase activity of MBP-fused P or mutant protein was calculated by subtracting the activity when the purified MBP was used in the polymerase reaction.

DNA binding First, double-stranded DNA was prepared and labeled. Purified plasmid pMAL-c_2 DNA was digested with restriction endonucleases *Eco*RI and *Bgl*I, and a 180 nucleotide *Eco*RI–*Bgl*I DNA fragment was purified. The DNA fragment was then labeled by incubating DNA with Klenow fragment and [α-^{32}P]dTTP and dATP, dGTP, dCTP, and dTTP. Second, 50 μg of the crude extract containing MBP-fused P or mutant proteins was separated in 8% SDS-PAGE and then electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell). After transfer, the separated proteins were renatured by treating with and gradually removing 6 M guanidium hydrochloride. The membrane was then incubated with Z buffer (25 mM HEPES-KOH, pH 7.6, 100 mM KCl, 12.5 mM $MgCl_2$, 10 mM $ZnSO_4$, 10% glycerol, 0.1% NP-40, 1 mM DTT, and 0.25% bovine serum albumin) containing ^{32}P-labeled template DNA for 30 min. Nonbinding template DNA was removed by washing the membrane with Z buffer. The nitrocellulose was then dried, exposed to X-ray film, and autoradiographed.

Results and Discussion

Site-directed mutagenesis in conserved motifs A, B′, C, D, and E of HBV polymerase and purification of point mutants Amino acid residues Asp^{429}, Gly^{518}, Asp^{551}, Lys^{585}, and Gly^{641} corresponding to the most representative homology regions of motifs A, B′, C, D, and E in the polymerase domain of HBV polymerase, are invariant and known to be involved in polymerase activity in other reverse transcriptases. The relative positions of these five amino acid residues in the P ORF is shown in Fig. 2. All those residues were mutated to alanine or histidine and designated D429A, G518A, D551A, D551H, K585A, and G641A, respectively. Wild-type and mutant fusion proteins were purified by amylose column chromatography and separated by 8% SDS-PAGE, and the Coomassie blue-stained gel is shown in Fig. 3. With some degradation products, purified wild-type and mutant fusion proteins are shown as a major band marked by an arrow with their molecular weights. The presence of the MBP-fusion proteins was verified by Western blot analysis using an anti-MBP monoclonal antibody and the pattern of bands was exactly the same between Coomassie-stained gel and

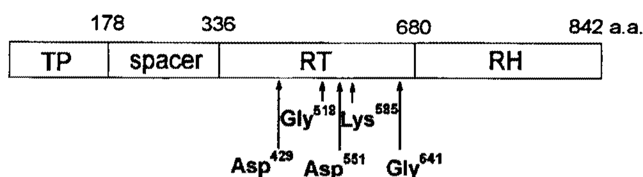

Fig. 2. Relative positions of mutation in five point mutants of HBV polymerase used in this experiment. The open box indicates the HBV *pol* open reading frame (ORF). From the N-terminus, polymerase ORF begins with the terminal protein (TP) followed by spacer region, reverse transcriptase (RT), and RNase H (RH) domains. The numbers shown are those of amino acids starting from the start codon of the HBV *pol* open reading frame.

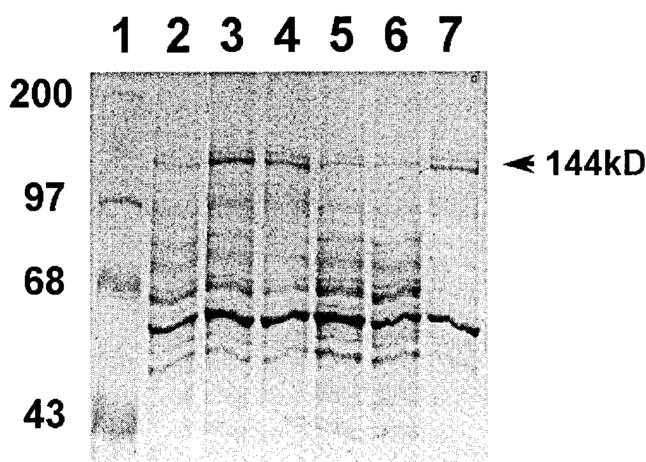

Fig. 3. Wild-type and mutant forms of HBV polymerase fused with MBP. Wild-type and mutant forms of HBV polymerase fused with MBP were overexpressed and purified as described in Materials and Methods. Each purified protein was separated in 8% SDS-PAGE and a Coomassie blue-stained SDS-PAGE is shown. Lane 1 is molecular weight standards. The size of protein is shown in kDa at the left. Wild-type (lane 2), and point mutants (lane 3, D429A; lane 4, G518A; lane 5, D551A; lane 6, K585A; lane 7, G641A) are shown. The arrow indicates full-length proteins of wild-type and mutant forms of HBV polymerase fused with MBP.

immunoblot analyses, indicating that these fusion proteins are very unstable (data not shown).

Polymerization activity of wild-type and mutant HBV DNA polymerases Under the standard reaction conditions described in Materials and Methods, DNA-dependent DNA polymerase activities of the purified mutant fusion proteins were compared with that of wild-type HBV polymerase fused with MBP as shown in Table 1. All the five mutant fusion proteins showed little polymerase activities indicating that these five residues in motifs A, B′, C, D, and E are critical in polymerase activity.

Affinity for DNA binding Motifs A and C were conserved irrespective of polymerase-template or substrate specificity, reflecting a direct role for these structures in the activity common to all polymerases: phosphodiester bond formation. In the structure of HIV-1 RT complexed with primer-template, the fingers subdomain and elements from motif B′ contact the template strand (Jacobo-Molina *et al.*, 1993). To study the affinity for DNA binding of each HBV mutant fusion proteins, the purified proteins were analyzed by South-Western analysis (Fig. 4). Lane 1 is

Table 1. Relative DNA polymerase activity of each point mutant D429A, G518A, D551A, K585A, or G614A compared with that of wild-type HBV polymerase.

Constructs	Relative activity (%)
wild-type HBV polymerase	100
D429A	4
G518A	0.1
D551A	0.1
D551H	0.1
K585A	0.1
G641A	9

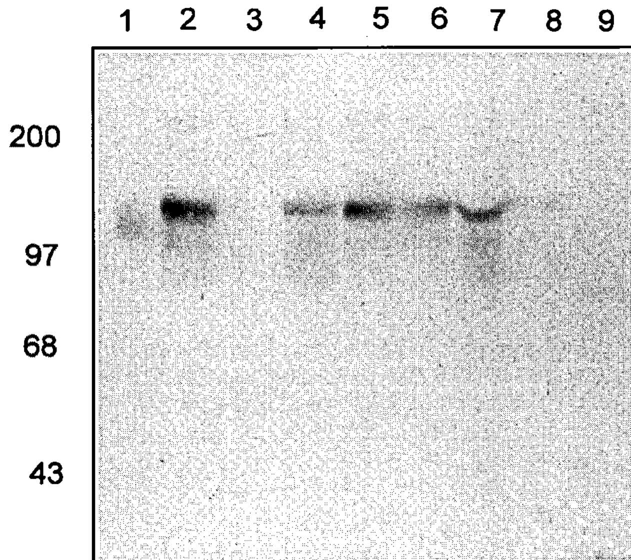

Fig. 4. South-Western analysis of wild-type and mutant forms of HBV polymerase fused with MBP. Purified wild-type and mutant proteins fused with MBP were separated in 8% SDS-PAGE and renatured proteins were reacted with a ^{32}P-labeled *Eco*RI–*Bgl*II DNA fragment spanning 180 nucleotides as described in Materials and Methods. The autoradiographed gel is shown. The size of protein is drawn in kDa according to the prestained markers at the left. Lane 1 is *E. coli* DNA polymerase I as a positive control; lane 2, HBV polymerse fused with MBP; lane 3, D429A; lane 4, G518A; lane 5, D551A; lane 6, K585A; lane 7, G641A; lane 8, D551H; lane 9, RNase H domain of HBV polymerase fused with MBP, as a negative control.

E. coli DNA polymerase I as a positive control and lane 9 contains a negative control showing that MBP does not have DNA binding activity. Since both *E. coli* DNA polymerase I and HBV DNA polymerase bound to the 180 nucleotide *Eco*RI–*Bgl*I DNA fragment, it appears that HBV DNA polymerase does not have sequence specificity for DNA binding. When 250 nucleotide DNA of poly(dA)·poly(dT) was used as a probe, the same result was obtained (data not shown). Change of amino acid Asp^{429} to alanine drastically changed the DNA binding ability of the mutant protein, indicating that amino acid Asp^{429} in motif A is involved in DNA binding in HBV polymerase. When amino acid Asp^{551} in motif C was changed into alanine, the mutant showed DNA binding activity in the same way as the wild-type polymerase did, but when it was changed to histidine, which has a positive charge, the mutant protein did not bind to the template strand. That is, introduction of two positive charges into motif C drastically changed the affinity for DNA binding. The band intensities of G518A (lane 4) and K585A (lane 6) were rather decreased compared to the control (lane 2), meaning that motifs B′ and D participate in DNA-binding directly or indirectly. The results presented in this paper indicate that residues Asp^{429} and Asp^{551} of the HBV polymerase in the conserved motifs A and C are involved in DNA binding, corresponding to an enzyme–DNA complex competent for polymerization. Similarly, Gly^{518} and Lys^{585} in the conserved motifs B′ and D also affect DNA binding to a certain extent. Further analyses are needed to reveal the clear role of those residues in conserved motifs.

Acknowledgements We are thankful for the financial support of the Korea Research Foundation made in the program year of 1995. Y. Kim was the recipient of a postdoctoral fellowship from the Korea Research Foundation.

References

Beasley, R. P. (1988) Hepatitis B virus — the major etiology of hepatocellular carcinoma. *Cancer* **61**, 1942–1956.

Beese, L. S., Derbyshire, V. and Steitz, T. A. (1993) Structure of DNA polymerase I Klenow fragment bound to duplex DNA. *Science* **260**, 352–355.

Chang, L. J., Hirsch, R. C., Ganem, D. and Varmus, H. E. (1990) Effects of insertional and point mutations on the functions of the duck hepatitis B virus polymerase. *J. Virol.* **64**, 5553–5558.

Eom, S. H., Wang, J. and Steitz, T. A. (1996) Structure of *Taq* polymerase with DNA at the polymerase active site. *Nature* **382**, 278–281.

Faruqi, A. F., Roychoudhury, S., Greenberg, R., Israel, J. and Shih, C. (1991) Replication-defective missense mutations within the terminal protein and spacer/intron regions of the polymerase gene of human hepatitis B virus. *Virology* **64**, 764–768.

Ganem, D. and Varmus, H. E. (1987) The molecular biology of the hepatitis B viruses. *Annu. Rev. Biochem.* **56**, 651–693.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H. and Arnold, E. (1993) Crystal structure of human inmmunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**, 6320–6324.

Jeong, J.-H., Kwak, D.-S., Rho, H.-M. and Jung, G. (1996) The catalytic properties of human hepatitis B virus polymerase. *Biochem. Biophys. Res. Commun.* **223**, 264–271.

Joyce, C. M. and Steitz, T. A. (1994) Polymerase structures and function: variations on a theme? *Annu. Rev. Biochem.* **63**, 777–822.

Khudyakov, Y. E. and Makhov, A. M. (1989) Prediction of terminal protein and ribonuclease H domains in the gene P product of hepadnaviruses. *FEBS Lett.* **243**, 115–118.

Kunkel, T. A., Roberts, J. D. and Zabour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* **154**, 367–382.

Kwak, D.-S., Kim, J.-Y., Lee, Y.-I. and Jung, G. (1996) Expression and purification of human hepatitis B virus polymerase with a dual affinity tag. *Korean J. Genetics* **18**, 131–138.

Lanford, R. E., Notvall, L. and Beames, B. (1995) Transcomplementation of nucleotide priming and reverse transcription between independently expressed TP and RT domains of the hepatitis B virus reverse transcriptase. *J. Virol.* **69**, 4431–4439.

Lee, H.-J., Kwon, Y.-T., Rho, H.-M. and Jung, G. (1993) Expression of hepatitis B virus polymerase gene in *E. coli*. *Biotechnol. Lett.* **15**, 821–826.

Lee, Y.-I., Hong, Y.-B., Kim, Y., Rho, H.-M. and Jung, G. (1997) RNase H activity of human hepatitis B virus polymerase expressed in *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **233**, 401–407.

Li, J.-S., Cova, L., Buckland, R., Lambert, V., Deleage, G. and Trepo, C. (1989) Duck hepatitis B virus can tolerate insertion, deletion, and partial frameshift mutation in the distal pre-S region. *J. Virol.* **63**, 4965–4968.

Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H. and Kraut, J. (1994) Structures of ternary complexes of rat DNA polymerase β, a DNA template-primer, and ddCTP. *Science* **264**, 1891–1903.

Radziwill, G., Zentgraf, H., Schaller, H. and Bosch, V. (1988) The duck hepatitis B virus polymerase is tightly associated with the viral core structure and unable to switch to an exogenous template. *Virology* **163**, 123–132.

Rho, H.-M., Kim, K., Hyun, S.-W. and Kim, Y.-S. (1989) The nucleotide sequence and reading frames of a mutant hepatitis B virus subtype *adr*. *Nucleic Acids Res.* **17**, 2124.

Sanger, F., Nickelen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.

Sousa, R., Chung, Y. J., Rose, J. P. and Wang, B.-C. (1993) Crystal structure of bacteriophage T7 RNA polymerase at 3.3 Å resolution. *Nature* **364**, 593–599.

Sousa, R. (1996) Structural and mechanistic relationships between nucleic acid polymerases. *TIBS* **21**, 186–190.

Steitz, T. A., Smerdon, S. J., Jager, J. and Joyce, C. M. (1994) A unified polymerase mechanism for nonhomologous DNA and RNA polymerases. *Science* **266**, 2022–2025.

Summers, J. and Mason, W. S. (1982) Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. *Cell* **29**, 403–415.

Toh, H., Hayashida, H. and Miyata, T. (1983) Sequence homology between retroviral reverse transcriptase and putative polymerase of hepatitis B virus and cauliflower mosaic virus. *Nature* **305**, 827–829.

Wang, G. H. and Seeger, C. (1992) The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. *Cell* **71**, 663–670.

Zoulim, F. and Seeger, C. (1994) Reverse transcription in hepatitis B viruses is primed by a tyrosine residue of the polymerase. *J. Virol.* **68**, 6–13.