Pakistan J. Zool., vol. 37(3), pp. 165-168, 2005.

The Cloning of PreS Gene from Pakistanian isolates of Hepatitis B Virus into a High Expression Vector pKK223-3

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Abstract.- Hepatitis B virus is regarded as one of the main etiologic factors involved in the development of human hepatocellular carcinoma. HBV has eight different genotypes that are geographically distributed. Hepatitis B surface antigen is composed of three related envelope proteins that are synthesized by alternate use of three translational start codons and a common stop codon. PreS region at the 5′ end of envelope gene is highly immunogenic with five known epitopes and hepatocellular binding sites. For cloning and expression studies of PreS gene, primers were designed that contained restriction sites and a termination codon at 3′ end. Pakistanian HBV PreS gene was PCR amplified and cloned in a T-A cloning vector. Clones were screened and positive clones were double digested with HindIII and EcoRI. Gel purified insert was cloned in high expression vector pKK223-3. Cloning was confirmed with restriction analysis and PCR amplification. Such clones would be useful for expression study of PreS product in bacterial cell, for large-scale production of viral antigen for diagnostic purposes and possibly vaccine production.

Key Words: Hepatitis B virus, T-A cloning, PreS region.

INTRODUCTION

Hepatitis B virus (HBV) is the smallest known DNA virus that can infect man. It causes serious health problem with more than 500 million carriers. It is the major cause of liver cancer and 62% of all the reported cancers are caused by HBV. IARC (1994) reports south Asia as area of intermediate endemicity (2-7%), while reports from Pakistan show 10-30% endemicity (Malik, 1995). HBV has a complex genomic organization. It has four overlapping reading frames S, C, P and X, which encode seven viral proteins. The Surface gene (S) has three initiation codons and hence three envelope proteins, namely Major, Middle and Large which are found in the serum. These proteins can induce protective antibodies (Neurath *et al*., 1989; Nunez *et al*., 2001). Pre-S region of surface gene is highly immunogenic and is the most hypervariable part of the HBV genome. Immunogenic response of Pre-S region is able to provide protection from HBV infection (Milich, 1989). Pre-S region has five known epitopes (Kuroki *et al.,* 1990; Mimms *et al.,* 1990) and two hepatocellular binding sites (Pontisso *et al*., 1989). Brind *et al.* (1997) have provided *in vivo* evidence for re-infection of the liver by HBV particles lacking PreS envelope protein expression.

Hepatitis B virus on the basis of sequence heterogeneity has eight genotypes (A-H) that are geographically distributed. This sequence heterogeneity may reduce efficacy of vaccines, commercial detection kits and currently available therapeutic antibodies. The PreS gene is the most divergent amongst all regions of hepadnavirus genomes. Therefore, the product of this gene may play a role in species specificity of HBV and may be involved in attachment of HBV to susceptible cells (Fan *et al.,* 2001). In that case, synthetic peptide analogs corresponding to selected segments of the translational product of PreS gene are promising for eliciting protection against HBV infection (Milich, 1989).

During this study Pre-S region from Pakistani HBV isolate was cloned in an expression vector that may be a step towards HBV vaccine production.

MATERIALS AND METHODS

Primer designing

Primers were designed by aligning flanking

0030-9923/2005/0003-0165 $ 8.00/0

sequence of PreS region of HBV sequences from different genotypes taken from NCBI website. Primer sequences are as follows:

NHBF1 5' ACTgaattcGGTCACCATATTCTTGG 3'
NHBR1 5' CAACAaggcttAAAGTCCTAGGAATCCTGATG 3'

Isolation of DNA

HBV DNA was extracted according to the protocol described by Persing *et al*. (1993). 10 µl of extracted DNA was used for PCR.

PCR amplification

HBV DNA was amplified using 10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM Mg Cl_2, 200µM dNTPs, 1µM each primer and 2.5U Taq DNA polymerase in 25 µl reaction. Programme used for PCR was 5 min initial denaturation at 95 °C, then 35 cycles with 50 sec denaturation at 95 °C, 50 sec annealing at 55 °C, 1 min extension at 72 °C and a final extension at 72 °C for 10 min.

Gel purification

PCR product was purified from agarose gel in 1X TAE buffer using Gene Clean® II kit BIO101.

Cloning

Purified PCR product was ligated in T/A cloning vector (Fermentas) in 3:1 insert vector ratio according to the protocol given. Ligation mix was transformed in DH5α. Transformants were screened using X-Gal/IPTG and ampicilin selection. Plasmids were prepared from white colonies according to Sambrook *et al*. (1989).

Restriction analysis

Restriction enzymes EcoRI and HindIII were used for double digestion. Restricted fragments were analyzed on 1% agarose gel and HBV Pre-S insert was purified as above.

Cloning in pK223-3

pK223-3 was also prepared with double digestion with EcoRI and HindIII. 0.18 pmol of vector were ligated with 0.54 pmol of insert using 5U of T4 DNA Ligase (Fermentas) in 20 µL reaction supplemented with BSA. *E. coli* DH5α were transformed with the ligation mixture. Later plasmids were isolated and cloning was confirmed with restriction analysis and PCR amplification.

RESULTS AND DISCUSSION

HBV genome has 10% sequence heterogeneity both at nucleotide and amino acid level (Lauder *et al*., 1993). It has only three reported highly conserved regions within the whole genome. HBV sequences from GenBank were aligned to find the conserved region for primer designing. Flanking the Pre-S region only two patches of 27 bp were found conserved among all GenBank sequences from seven different genotypes. These were used to design a primer pair using PRIMER software. HBV DNA extracted from local isolates were PCR amplified using the primer gave 0.6 Kb PCR product (Fig. 1). PCR product was cloned in T/A cloning vector. Confirmation of cloning with restriction and PCR is shown in Figures 2 and 3, respectively.

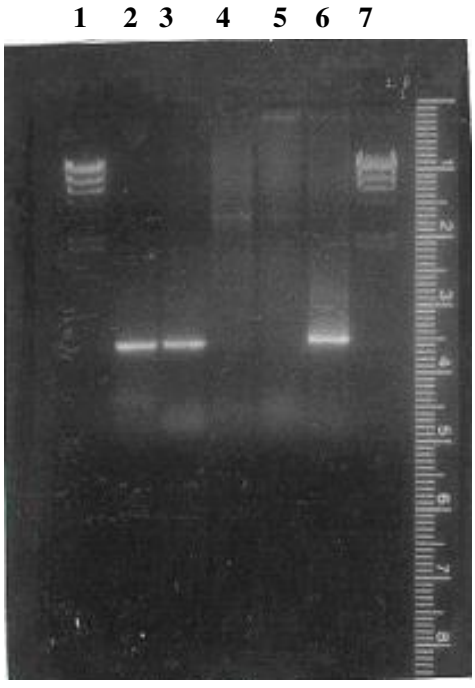

Fig. 1. PCR amplification of PreS region of Hepatitis B virus; Lane 1& 7, Marker λ HindIII; Lane 2,3 & 6, PCR products of 0.6kb PreS region of HBV.

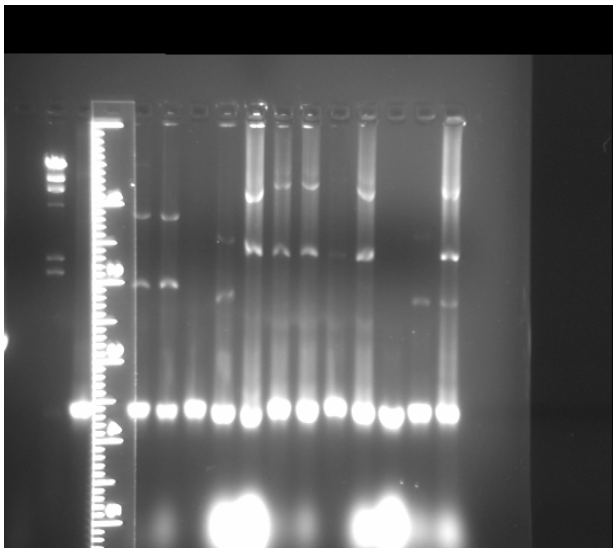

Fig. 2. Confirmation of PreS HBV clones through PCR; Lane 1 Marker λ HindIII; Lane 2, positive control; Lane 3, negative control, Lanes 4-15, PCR product from PreS region from Pakistanian HBV clones.

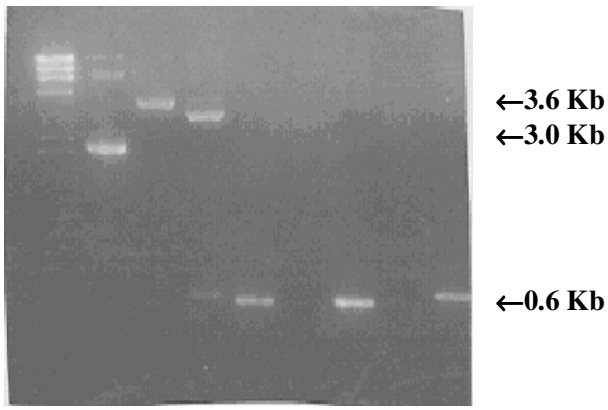

Fig. 3. Confirmation of cloning in T/A cloning vector through restriction analysis and PCR; Lane 1 Marker λ Hindi III; Lane 2, uncut plasmid; Lane 3, single cut with EcoRI; Lane 4, double digestion with EcoRI and HindIII; Lane 5, PCR from clone, Lane 6 Negative control for PCR; Lane 7 positive control for PCR.

The restriction fragment 0.6 Kb was purified and ligated with an expression vector pK223-3. After transformation only few white colonies were produced on LB ampicillin plate. Cloning was confirmed with restriction analysis and PCR using the same primers as shown in Figure 4. Antibodies against PreS region are stable and long lasting (Kazaks *et al*., 2004; Madalinski *et al.,,* 2004). Similarly, vaccination based on recombinant HBsAg derived from a non-Asian HBV isolate may not be ideal for mass vaccination of an Asian population due to geographical distribution of HBV subtypes and 10% or greater sequence variation between geographically different isolates of the same subtype.

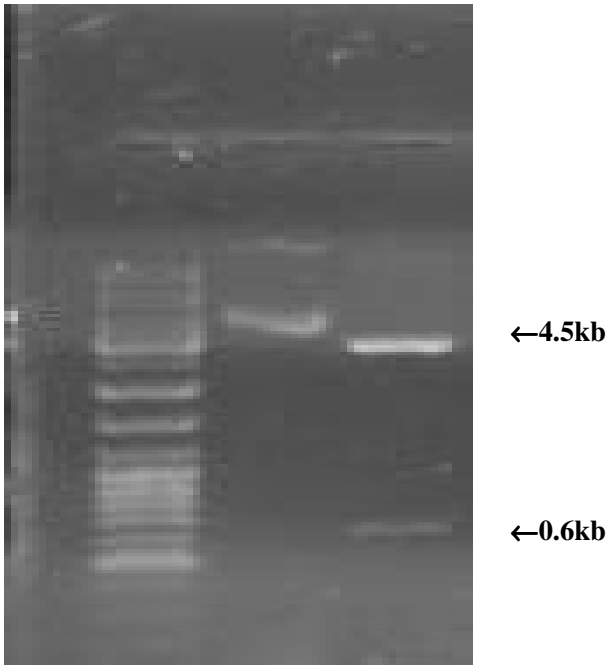

Fig. 4. Confirmation of cloning in pK233-3. Lane 1, marker; Lane 2; Uncut pK233-3; Lane 3, restriction digestion of PreS clone with EcoRI and HindIII.

Therefore, such clones would be useful for expression study of PreS product in bacterial cell, for large-scale production of viral antigen for diagnostic purposes and possibly vaccine production.

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(Received 17 November 2004, revised 3 May 2005)