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Antigenic Properties of preS2 Region of Hepatitis B Virus Envelope Proteins

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B형 간염바이러스 표면단백질 중 preS2 부위의 항원적 특성

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ABSTRACT: The preS2 sequence of an *adr* hepatitis B virus was cloned and expressed in *Escherichia coli* as a β-galactosidase fusion polypeptide. Recombinant preS2 product interacted with the preS2-specific monoclonal antibody H8 which was induced by surface antigen particles isolated from a Korean hepatitis patient. The H8 showed only a minor cross-reactivity with recombinant preS2 product of *adw2* subtype. Determination of nucleotide sequence of the *adr* preS2 revealed that twelve amino acid residue substitutions between *adr* and *adw2* subtype sequences. The antigenic determinant to H8 must include some of these differences.

KEY WORDS□preS2, monoclonal antibody, β-galactosidase-preS2 fusion

Three different envelope proteins are encoded by the 'S' open reading frame of the hepatitis B virus (HBV) genome. These are the major (S), middle (M), and large (L) proteins (Cattaneo *et al.*, 1983). Translation of these envelope proteins starts from three different initiation codons (Stibbe and Gerlich, 1983; Heerman *et al.*, 1984). The S protein, 226 amino acids long, is encoded by the S gene. The M protein, 281 amino acids long, is encoded by the preS2 plus S sequences. The L proteins, 400 (*ad* subtypes) or 389 (*ay* subtypes) amino acids long, is encoded by the entire preS (preS1 and preS2) plus S sequences. Immunological properties of these surface antigens are the basis of classifying virus serotypes (Le Bouvier and Williams, 1975). Although preS2-containing M and L proteins are not abundantly present in hepatitis B virus particles (Heerman *et al.*, 1984), the preS2 region has been shown to be immunodominant (Neurath *et al.*, 1984; Milich *et al.*, 1985; Neurath *et al.*, 1985) and be able to elicit protective immunity in animals (Itoh *et al.*, 1986). PreS2 epitope was also indicated as one of the epitopes inducing neutralizing antibodies (Budkowska *et al.*, 1986). Knowledge of neutralizing epitopes is crucial in developing strategies for rational vaccine and anti-viral drug design. As a preliminary step towards this goal, we constructed plasmid systems of overexpressing preS2 peptide in *E. coli*. The antigenic properties of recom-

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binant preS2 were examined with an anti-preS2 monoclonal antibody.

MATERIALS AND METHODS

Materials

Restriction enzymes, *E. coli* DNA polymerase I large fragment (Klenow enzyme), T4 DNA ligase were purchased from New England Biolabs, Bethesda Research Laboratories, or Promega Corporation. ATP, [α-^{32}P]dATP, dNTPs, and DNA sequencing kit were purchased from Amersham. Ampicillin, bovine serum albumin, isopropyl-β-D-thiogalactopyanoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactoside (XGal) and 4-chloro-1-naphtol were from Sigma Chemical Company. Peroxidase-conjugated anti-mouse IgG was purchased from Kirkegaard and Perry Laboratories. All other chemicals used were reagent grade.

Strains and plasmids

E. coli JM109 [*rec*A1, *end*A1, *gyr*A96, *thi*, *hsd*R17, *sup*E44, *rel*A1, Δ(*lac-pro*AB), F'(*tra*D36, *pro*A$^+$B$^+$, *lac*I^q, *lac*Z ΔM15)] was used as a host strain for cloning. Plasmid pHBV-315 carrying the HBV genome inserted at the *Bam*HI site of pBR322 (Kim and Kang, 1984) was a kind gift from Hyen Sam Kang. Plasmid pUC9 and pUC19 were purchased from Phamacia P-L Biochemicals. Plasmids pCMHB20 and pCMHB30 were described previously (Park *et al.*, 1989) which encoded preS2 sequence of *adw*2 serotype as a fusion protein with truncated β-galactosidase.

Oligonucleotides

All the oligonucleotides were synthesized with an automated DNA synthesizer (Beckman System Plus). Modified deprotection was adopted to simplify the procedure by employing a single treatment of concentrated NH_4OH at 50 °C for 12 h. Full length DNA was purified by polyacrylamide-urea gel electrophoresis (Lo *et al.*, 1984).

Monoclonal antibodies specific to preS2 region of hepatis B surface antigen

Details of preparation of monoclonal antibody which was specific to preS2 sequence of *adr* hepatitis B surface antigen (HBsAg) was previously described (Chung and Kim, 1987). In brief, the antibody was induced by 22 nm HBsAg particles isolated from hepatitis patient serum. Antibodies were purified by HBsAg-affinty column chromatography and preS-specific antibodies were screened on the criteria that preS antigenicity is lost after digestion of HBsAg with pepsin. One out of 52 exhibited the properties described above and was named H8. Specific recognition of H8 to preS2 was then confirmed by immunoblotting to both M and L proteins of surface proteins, but not to S protein.

Cloning procedures

All the enzymatic reaction conditions and cloning procedures were performed as described by Maniatis *et al.* (1982).

Determination of nucleotide sequences

Dideoxy chain termination method (Sanger *et al.*, 1977) was carried out using supercoiled double-stranded plasmid DNA as a template (Chen and Seeburg, 1985).

Electrophoresis and Western blotting

SDS-polyacrylamide gel electrophoresis was carried out as described (Laemmli, 1970). Antigenicity of preS2 was detected by Western blotting method (Towbin *et al.*, 1979) with preS2-specific monoclonal antibody H8. The peroxidase-conjugated anti-mouse IgG was used as a second antibody.

RESULTS

Construction of expression plasmids encoding preS2 of HBsAg

Plasmid pCMHB20 and pCMHB30 were previously constructed to overproduce preS2 peptide of *adw*2 subtype as a β-galactosidase fusion polypeptide, which carried with *tac* promoter, lacZ sequence encoding N-terminal 208 and 293 amino acid residues of β-galactosidase, respectively (Park *et al.*, 1989). In order to overproduce *adr* subtype preS2 region, expression vectors were constructed as shown in Fig. 1. The 157 bp DdeI-AvaII fragment of preS2 region was isolated from pHBV-315 and ligated with two synthetic linkers to provide missing residues and stop codon. The 189 bp ligation product was then cloned into pUC9 to yield pUC9R, and from this a 176 bp DNA fragment of *adr* preS2 sequence was isolated and inserted into pUC19 to yield pUC19R. The XbaI-HindIII fragment of pUC19R was then substituted for the XbaI-HindIII of pCMHB20 to yield pCMHB(R)20. The resulting plasmid was identical to pCMHB20 except that *adr* preS2 region was substituted for *adw*2 subtype. Plasmid pCMHB(R)30 was constructed by inserting a 243 bp XbaI fragment of lacZ isolated from pCMHB30 into pCMHB(R)20 (Fig. 1).

Antigenic properties of β-galactosidase-preS2 fusion proteins synthesized in *E. coli*.

To express β-galactosidase-preS2 fusion proteins in *E. coli*, recombinant cells harboring appropriate

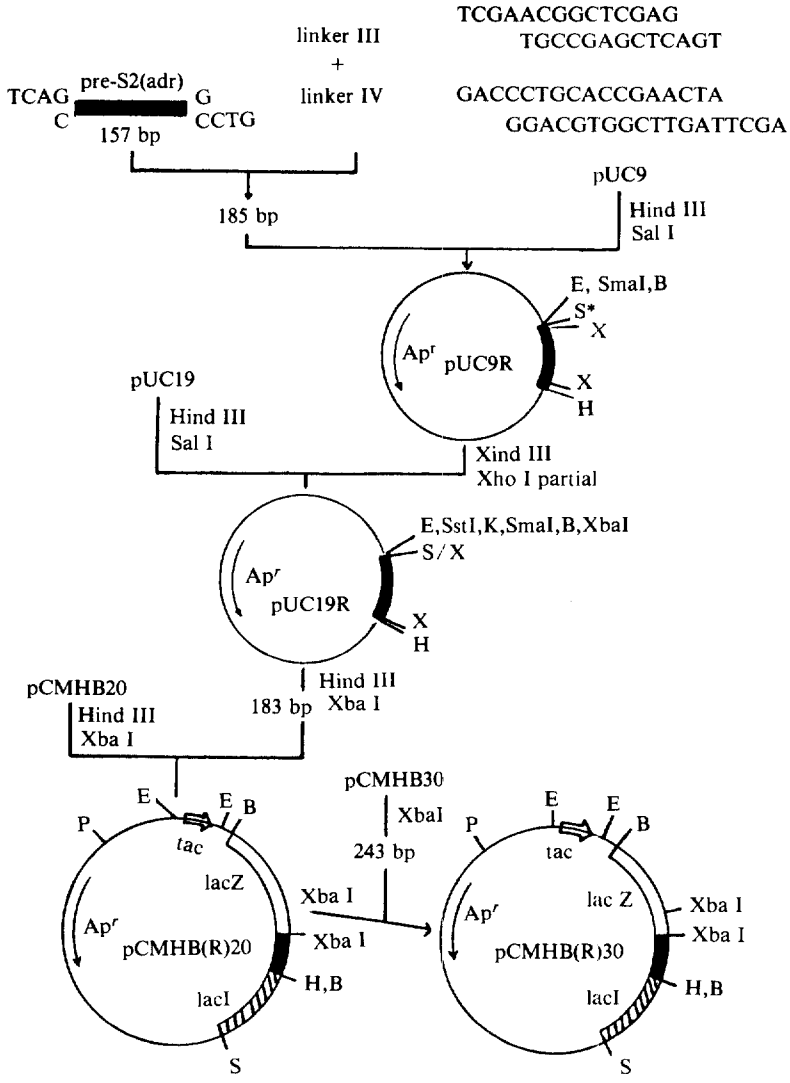

Fig. 1. *Construction of adr subtype preS2 expression vectors.*

A 157 bp DdeI-AvaII fragment encoding preS2 sequence was ligated with synthetic oligonucleotide linker III and IV. The resulting 185 bp fragment containing XhoI and HindIII adaptors at 5'- and 3'-end, respectively, was cloned at SalI and HindIII sites of pUC9 to yield pUC9R. The 176 bp fragment of *adr* preS2 sequence was obtained from pUC9R by HindIII and XhoI partial digestion and then the fragment was inserted into pUC19 between SalI and HindIII site to yield pUC19R. The XbaI-HindIII fragment of pUC19R was then substituted for the XbaI-HindIII sites of pCMHB20 to yiels pCMHB(R)20. To construct pCMHB(R)30, pCMHB30 was digested with XbaI and the isolated 243 bp fragment was inserted into XbaI site of pCMHB(R)20.

plasmids were grown and the synthesis of the fusion proteins was induced. The culture lysates were analyzed on SDS-polyacrylamide gel electrophoresis. As shown in Fig. 2a, the transformants harboring pCMHB(R)20 and pCMHB(R)30 produced fusion proteins of expected molecular size (lanes 7 and 9) with a yield of 50% of total cellular proteins, just as the cells harboring pCMHB20 and pCMHB30 (lanes 3 and 5). In order to confirm that the fusion proteins encoded by pCMHB(R)20 and pCMHB(R)30 carried the *adr* subtype preS2 sequence, Western blot analysis was performed with anti-preS2 monoclonal antibody H8, which was induced by *adr* subtype HBsAg particles. Results in Fig. 2b shows that only the fu-

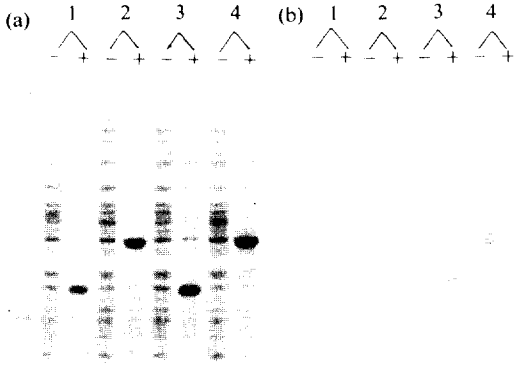

Fig. 2. *Synthesis of β-galactosidase-preS2 fusion proteins and immunoblotting of preS2 in fusion proteins.*
(a) Recombinant *E. coli* JM109 cells harboring pCMHB20, pCMHB30, pCMHB(R)20, pCMHB (R)30 plasmids were grown at 37 °C and the synthesis of recombinant proteins was induced at an early log phase (A_{600} = 0.3) by adding 2 mM IPTG. Cells were further incubated at 37 °C for 3 h and the lysates were analyzed on 10% SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1 shows molecular weight markers; from the top phosphorylase (98,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). Symbols, − and + indicates before and after induction of synthesis of recombinant proteins, respectively. Plasmids carried in each recombinant cells are also indicated: 1, pCMHB20; 2, pCMHB30; 3, pCMHB(R)20; 4, pCMHB(R)30. (b) Western blotting of (a). Immunoblotting was performed with the monoclonal antibody H8 and peroxidase-conjugated anti-mouse IgG antibody.

sion proteins encoded by pCMHB(R)20 and pCMHB (R)30 exhibited a specific interaction with H8 (lanes 7 and 9). The proteins encoded by pCMHB20 and pCMHB30 showed only a weak interaction with the same antibody.

Nucleotide Sequence Determination of an *adr* PreS2

The difference in antigenic properties seen in Fig. 2b of *adr* and *adw*2 preS2 fusion polypeptides must reside in amino acid sequence variations between two subtypes. The nucleotide sequence of *adw*2 preS2 had been reported previously (Valenzuela *et al.*, 1979). In order to identify the variation in our *adr* preS2 sequence cloned, the 157 bp DdeI-AvaII fragment (Fig. 1) containing most of preS2 was sequenced. To determine the rest of 3' portion of preS2 absent in the 157 bp fragment, a 640 bp BstEII-HincII fragment containing complete sequence of preS2 and 5' part of S was isolated from pHBV-315 and sequenced from 3' end. Fig. 3 shows the result of nucleotide sequence determination and the amino acid sequence deduced from this was shown in Fig. 4. The sequence we determined was different from those of three other *adr* serotype reported previously by Ono *et al.*, (1983), Fujiyama *et al.* (1983), and Kim and Rho (1988). Compared with the preS2 sequence of Ono *adr*, our sequence carried only two nucleotide variations (Fig. 3) without changing the amino acid sequence (Fig. 4). In comparison with preS2 sequences of Fujiyama and Kim, one and three amino acid residue substitutions were revealed, respectively. Many nucleotide variations were seen in comparison with the sequence of *adw* subtypes (Ono *et al.*, 1983; Valenzuela *et al.*, 1979), each of which yielded 12

```
                                        30                                  60
1  ATG CAG TGG AAC TCC ACC ACA TTC CAC CAA GCT CTG CTA GAT CCC AGA GTG AGG GGC CTA
2                T       A
3                        A                                  C
4                                                TC
5                T       T G C  G                      AG                C       T   G
6                T       A G                 A         AG                C       T   G


                                        90                                  120
1  TAT TTT CCT GCT GGT GGC TCC AGT TCC GGA ACA GTA AAC CCT GTT CCG ACT ACT GCC TCA
2
3    C
4              C
5      C                                 A                          C       A   T       T
6      C                                 A                          C       A   T       T

                                       150                 165
1  CCC ATA TCG TCA ATC TTC TCG AGG ACT GGG GAC CCT GCA CCG AAC
2
3                        C
4                        C
5    A     C             C                              TG A     T
6    A     C             C  G                           TG A
```

Fig. 3. *Nucleotide sequence determined for an adr subtype preS2 and its compar sion with those of other preS2 reported previously.*
1. *adr* subtype determined in this study; 2, *adr* subtype (Ono *et al.*, 1983); 3, *adr* subtype (Fujiyama *et al.*, 1983); 4, *adr* subtype (Kim *et al.*, 1988); 5, *adw* subtype (Ono *et al.*, 1983); 6, *adw*2 subtype (Valenzuela *et al.*, 1979).

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     120                                 130
1    Met Gln Trp Asn Ser Thr Thr Phe His Gln Ala Leu Leu Asp Pro Arg Val Arg Gly Leu
2
3
4                                        Val
5                        Ala Leu                     Gln
6                        Ala             Thr         Gln

     140                                 150
1    Tyr Phe Pro Ala Gly Gly Ser Ser Ser Gly Thr Val Asn Pro Val Pro Thr Thr Ala Ser
2
3
4              Pro
5       Leu                                                  Ala     Asn Ile
6       Leu                                                  Ala     Asn Ile

     160                                 170
1    Pro Ile Ser Ser Ile Phe Ser Arg Thr Gly Asp Pro Ala Pro Asn
2
3                       Ser
4                       Ser
5    His                Ser                          Val Thr Ile
6    His                Ser Ala                      Val Thr
```

Fig. 4. *Amino acid sequence of preS2 which was derivied from the nucleotide sequence in Fig. 3.*

amino acid residue substitutions among 55 preS2 residues.

DISCUSSION

The surface antigen of hepatitis B virus (HBV) has one common group determinant, *a*, and several antigenic specificities that have been designated *d, y, w,* and *r* subtypes (Le Bouvier and Williams, 1975). The subtypes *d* and *y* are mutually exclusive, and in Far-East Asia the *y* determinant is absent (Tiollais *et al.*, 1985). The *d* determinant had been previously characterized by using synthetic peptides in the region of 110-137 residue of S protein (Gerin *et al.*, 1983). Later, it was preciously located in the residue 122 (lys) of S protein by chemical modification (Peterson *et al.*, 1984). The *r* and *w* determinants which are also mutually exclusive have not been precisely located in surface antigens. The preS2 sequence we determined (Fig. 3) revealed that the virus from which the genome was derived was *adr* subtype, as has been confirmed previously in S gene sequencing (Kim and Kang, 1984). Comparison of various preS2 sequences revealed many residue substitutions between preS2 of *adr* and *adw* subtypes, while relatively small variations were seen within various *adr* subtypes (Fig. 3). The observation suggests the possibility that one of *r/w* determinants resides in preS2 region.

Construction of overexpression vectors (Fig. 2a) made it easier to characterize the antigenic properties of preS2 by simple Western blotting method. As shown in Fig. 2b, immunoblotting revealed that monoclonal antibody H8 induced by *adr* subtype HBsAg particles bound specifically to *adr* subtype preS2 fusion protein only. The result also supports the notion described above. Twelve out of 55 residues of *adr* preS2 were substituted in *adw*2 sequence as shown in Fig. 4. These sequence differences were distinguished by the monoclonal antibody H8 (Fig. 2b) and some of these different residues must be involved in antigenic structural determinant.

It has been considered that the preS2 epitope is independent of conformation and made up of a continuous sequence of amino acid residues (Neurath *et al.*, 1985). The minimum size of continuous epitopes comprize five to eight residues (Westhof *et al.*, 1984). As shown in Fig. 4, most of drastic changes are located between residue number 154 and 165, which could well be a candidate for encompassing an antigenic epitope. However Neurath and colleagues (1986) located antigenic sites of *adw*2 at amino acid resiues 120-145. Furthermore hydrophilicity plot (Hopp and Woods, 1981) of the sequence we determined also showed the maximum value between 132-137 in which leucine 132 in *adr* sequence was changed to glutamine in *adw*2 subtype. Precise location of the antigenic epitope requires further experiments. In the accompanying paper we located antigenic epitope region of preS2 by constructing serial deletions of preS2 and examining their effects on antigenicity.

적 요

adr 아형 B형 간염바이러스의 preS2 염기서열을 베타갈락토시다제 유전자에 연결시켜 클론닝한 후, 융합단백질의 형태로 대장균에서 발현시켰다. 한국인 간염환자로부터 분리된 표면항원으로 유도한 단일클론항체 H8는 preS2에 특이성을 지니고 있으며, 상기의 융합단백질과 특이적으로 결합하였다. 이 단일클론항체는 *adw*2아형 preS2 융합단백질과는 단지 낮은 수준의 결합성을 보여주었는데, 이와 같은 단일클론항체 H8의 *adr* 아형과 *adw*2아형에 대한 결합성의 차이는 preS2 부위의 아미노산 차이에 기인한다고 볼 수 있다.

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