Antigenic Determinant Mapping in preS2 Region of Hepatitis B Surface Antigen

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B 형 간염바이러스 표면항원 preS2 부위의 항원결정인자 규명

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ABSTRACT: A DNA sequence encoding the *adr* subtype preS2 region of hepatitis B virus envelope protein was fused to 5'end of *lacZ* gene yielding a plasmid pTSZ, in order to produce a preS2-β-galactosidase fusion protein. Serial deletions from 3' and 5'end of preS2 were constructed in plasmids, which were expressed and their antigenicities were examined with the monoclonal antibody H8. Deletions from amino and carboxy terminal to certain points did not affect the antigenicity, but the longer deletions destroyed the antigenicity. End points of deleted preS2 sequence were determined by DNA sequencing. As a result, each end of preS2 epitope was located in the region of amino acid residue 130-132 and 140-142, respectively. Residue 143 may be supplementary for antigenic epitope since the deletion from carboxy terminal to residue 143 revealed partial defect of antigenicity. In the interval of antigenic epitope the amino acid differences between *adr* and *adw*2 subtype occurred at residue 130, 132, and 141. This result indicated that one or more of the three residues are responsible for the binding specificity of monoclonal antibody H8 to *adr* subtype preS2 fusion protein.

KEY WORDS: antigenic determinant, preS2, HBsAg

The antigenic reactivity of proteins reside in restricted parts of the molecule known as antigenic determinants or epitopes. Antigenic determinants represent the accessible patches on the surface of a native protein that interact with the binding sites of antibody molecules, and have been classified as continuous or discontinuous. The continuous epitopes are composed of residues which are consecutive in the polypeptide sequence, while discontinuous determinants consist of residues from different parts of the polypeptides which are brought together in the native structure. The predictive methods of determining antigenic sites directly from protein sequences must rely on an accurate prediction of the surface contour of a given protein. The success in the predic-

tion of discontinuous epitope is remote because the mechanism of protein folding has not been completely elucidated. However, some predictive methods have been developed for continuous epitope based on some experimental observations.

Hopp and Woods (1981) suggested that antigenic epitopes can be located as those segments of primary structure that are markedly hydrophilic. In a similar vein, a surface probability plot has been developed (Emini *et al.*, 1985) based on side-chain solvent accessibility values of the individual amino acids (Janin *et al.*, 1978). Highly mobile protein segments (flexible regions) seemed to correlate well with known antigenic determinants (Tanier *et al.*, 1984; Westhof *et al.*, 1984). However, Novotony *et al.* (1986) proposed

that surface accessibility rather than segmental mobility correlates with the protein antigenicity. Integrating flexibility parameters with hydropathy/solvent accessibility values, Jameson and Wolf (1988) introduced algorithm to predict a linear surface contour profile of the protein, referred to as the 'antigenic index'. Nevertheless it is so far difficult to assign antigenicity with complete confidence to a predict.

The goal of present study is to identify and characterize the preS2 epitope of an *adr* subtype hepatitis B virus (HBV) that has been previously isolated from a Korean hepatitis patient (Kim and Kang, 1984). Immunity against viral infection is elicited by antigenic determinants exposed on the virus envelope. Though the preS2 region of HBV surface antigen (HBsAg) is a minor component of the virus particle, it has been known to carry an epitope which (i) elicits protective immunity in animals (Itoh *et al.*, 1986), (ii) is immunodominant (Milich *et al.*, 1985; Neurath *et al.*, 1984; Neurath *et al.*, 1986) (iii) represents conformation-independent, continuous determinant (Neurath *et al.*, 1985).

In the present study, in order to map the epitope region of an *adr* subtype preS2 precisely, a serial deletions of *adr* subtype preS2 were constructed and expressed in *E. coli* as β-galactosidase fusion protein. The effect of each deletion on the antigenicity of preS2 was examined with monoclonal antibody (Mab) H8 which carried *adr* subtype specificity.

MATERIALS AND METHODS

Enzymes and chemicals

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

Strains and plasmids

E. coli JM109 [*recA1, endA1, gyrA96, thi, hsd* R17, *supE44, relA1, Δ (lac-proAB), F'(traD36, pro* AB$_+^+$, *lacI^q, lacZ ΔM15)*] was used as a host for subcloning plasmids. 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. Plasmids pTBG and pTBG(H+) were

described previously (Lee *et al.*, 1990a) which encoded under the control of *tac* promoter free β-galactosidase and hybrid β-galactosidase protein fused with preS2 of *adw2* subtype, respectively.

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-strand plasmid DNA as a template (Chen and Seeburg, 1985).

Gel electrophoresis and western blotting

SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (1970). Antigenicity of preS2 was detected by Western blotting method (Towbin *et al.*, 1979) with preS2-specific Mab H8 which was described in the accompanying paper (Lee *et al.*, 1990b).

RESULTS

Expression of preS2-*lacZ* Fusion Vector, pTSZ

In order to express the *adr* subtype preS2 sequence of HBV in *E. coli*, we adopted β-galactosidase gene (*lacZ*) fusion system with the following reasons. First, without a fusion preS2 could not be expressed as a stable product. Secondly, the β-galactosidase fusion allows screening for the in-frame clones of partially-deleted preS2 sequence. Plasmid pTSZ was constructed in such a way that the preS2 sequence of an *adr* subtype was fused to 5'-end of *lacZ* and the expression of the fusion gene was under control of *tac* promoter. The 157-bp DdeI-AvaII fragment of preS2 (*adr*) sequence was isolated from pHBV-315 (Kim and Kang, 1984) and inserted into HincII site of pUC9 after the cohesive ends were filled with Klenow treatment. From this, 190-bp EcoRI-HindIII fragment encoding preS2 was isolated and substituted for preS2 (*adw2*) sequence in pTBG(H+) plasmid (Lee *et al.*, 1990a). Recombinant preS2-β-galactosidase fusion protein encoded by pTSZ retained the β-galactosidase activity and bound specifically to anti-preS2 monoclonal antibody, Mab H8. The Mab H8 appeared to be highly specific for the *adr* subtype, as shown in Fig. 1. Though the amount of preS2-β-galactosidase fusion protein encoded by pTBG (H+) was much greater than that of pTSZ (Fig. 1a, lane 3 vs. lane 2), the Mab H8 reacted with *adr* subtype preS2 encoded by pTSZ more specifically than *adw2* subtype encoded by pTBG(H+) (Fig. 1b, lane 2 vs. lane 3).
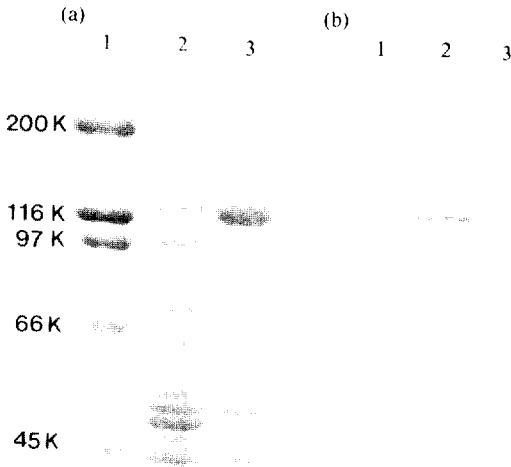
Fig. 1. *Subtype specificity of monoclonal antibody H8.*

(a) Recombinant *E. coli* JM109 cells harboring pTSZ (lane 2) and pTBG(H−) (lane 3) plasmids were grown at 37 °C and the synthesis of preS2-β-galactosidase fusion proteins were induced by adding 2 mM IPTG. Culture lysates were analyzed on 8% SDS-polyacrylamide gel electrophoresis. Protein bands were visualized by staining with Coomassie Brilliant Blue. Lane 1 represents the Bio-Rad high molecular weight standard marker. (b) Western blotting of (a). Immunoblotting was performed with monoclonal antibody H8 and peroxydase-conjugated anti-mouse IgG antibody.

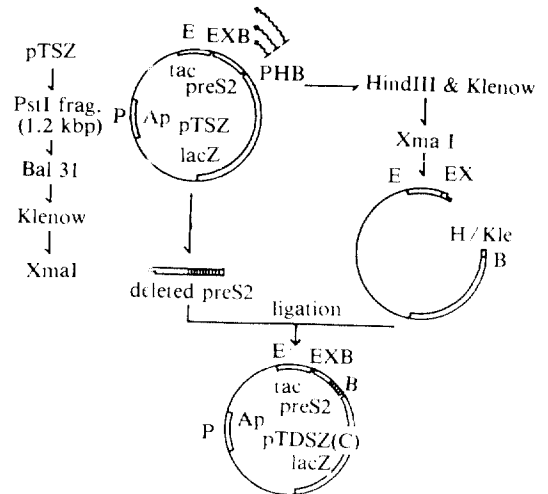## Construction of serial deletions for epitope mapping

In order to locate the antigenic epitope of preS2, serial deletions of preS2 were made which lacked variable sizes of amino-terminal portion of preS2 (Fig. 2). Plasmid pTSZ was digested with EcoRI and treated with Bal31. At various time points aliquots of samples were withdrawn and the reaction was stopped. All the reaction products were then pooled and further digested with HindIII. Partially deleted preS2 sequences of various lengths were isolated by 2.0% agarose gel electrophoresis, and substituted for preS2 sequence of pTBG(H+) plasmid to yield pTDSZ (ΔN). *E. coli* JM109 cells were transformed with PTDSZ(ΔN) and clones in which the preS2 was still fused with *lacZ* in-frame were screened on LB plate containing Xgal and IPTG. In order to select only the true in-frame clones, the concentration of IPTG added was 1 μM. An identical strategy was adopted to produce partial deletions of preS2 from carboxy terminal and the pTDSZ(ΔC) plasmids were obtained (Fig. 3).

Total 143 and 150 clones were selected for amino
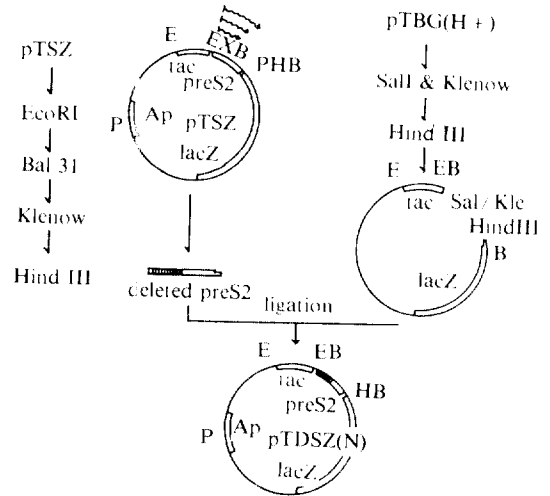
Fig. 2. *Construction of serial deletions at amino terminal portion of preS2.*

Plasmid pTSZ was digested with EcoRI, treated with Bal31, and then the protrude ends were filled with Klenow. The products were further digested with HindIII and the partially deleted preS2 fragments were isolated and inserted into pTBG (H+) vector from which the preS2 (*adw2*) sequence was removed by treatment with SalI, Klenow, and Hind III, successively.

Fig. 3. *Construction of serial deletions at carboxy terminal portion of preS2.*

Plasmid pTSZ was digested with PstI, treated with Bal31, and then the protrude ends were filled with Klenow. The products were further digested with XmaI and the partially deleted preS2 fragments were isolated and substituted for intact preS2 sequence of pTSZ vector.

Fig. 4. *Antigenic properties of deleted preS2.*

(a) Culture lysates of *E. coli* JM109 cells harboring each pTDSZ(△C) plasmid were analysed by SDS-polyacrylamide gel electrophoresis and protein bands were visualized by staining with Coomassie Brilliant Blue. (b) Western blot of (a). Experimental conditions were identical to those described in Fig. 1. From left to right were loaded culture lysates of the clones with increasing deletions from carboxy terminal of preS2 sequence.

and carboxy terminal deletion procedures, respectively. In order to select deletions of appropriate sizes, the plasmids prepared from screened clones were digested by BamHI and 8% polyacrylamide gel electrophoresis was performed (data not shown).

Antigenic properties of partially deleted preS2

For clones selected by size determination of preS2 fragment, the synthesis of the recombinant fusion proteins was analyzed by SDS-polyacrylamide gel

electrophoresis. Fig. 4a shows the recombinant fusion proteins with partial deletions in preS2 from carboxy terminal. The order of each sample was in such a way that the length of deletion increases as the number of lane increases. Most of carboxy terminal deletions of preS2 produced β-galactosidase fusion proteins as much as parental pTSZ carrying intact preS2. However, when Western blot was performed with the Mab H8 (Fig. 4b), some difference was noticed. Deletions from carboxy terminal to certain points (lanes 2-10) did not affect the binding affinity of preS2 to Mab H8, while the deletions of slightly longer length as in clones 113, 145, and 148 decreased the binding affinity (lanes 11-13) and the longer deletions completely destroyed the antibody binding of preS2 (lanes 14-17). Identical analyis was performed with amino terminal deletions and the results were consistent with that shorter deletions retained antigenicity while longer deletions did not (data not shown).

In order to determine the precise end points of deleted preS2 sequences which were at the junction of retaining antigenicity, DNA sequencing was performed by using reverse sequencing primer which was complementary to the downstream sequence adjacent to *tac* promoter region in pTDSZ plasmids. The end point of amino acid sequence in each deletion was deduced from determined nucleotide sequence. Fig. 5 summarized the results along with Western blot data. Since the clone 502 and 108 in which amino terminal eight and ten residues were

Fig. 5. *Schematic presentation of deletion end points along with Western blot result.*

Precise deletion end points of pTDSZ clones were determined by DNA sequencing. Dotted lines represent the deleted portions. For each clone, the position of terminal amino acid in remaining preS2 is indicated as residue number. Positive and negative antigenicities were also indicated at the right-most side of each clone. Amino acid sequence deduced from DNA sequence of *adr* preS2 is represented and differences in *adw*2 are shown in the parentheses.

deleted, respectively, still retained the preS2 antigenicity, amino terminal ten residues of the preS2 did not seem to be needed for the binding activity to the Mab H8. However when amino terminal portion was deleted up to residue 132 as in clone 332, the antigenicity was completely destroyed. Similarly deletions from 25th residue to carboxy terminal did not affect the antigenicity, whereas deletion of one more residue as in 113 caused partial affect in the antigenicity (Fig. 4, lane 11). Deletions in clone 145 and 148 (Fig. 4, lanes 12 and 13) were identical to that of clone 113, as revealed by DNA sequencing. Longer deletions as in clone 143 abolished the antigenicity of preS2.

DISCUSSION

Synthetic peptides have been frequently used to probe antigenic determinants of the proteins particularly for the continuous linear epitopes (Geyson et al., 1987; Moudallal et al., 1985; Dyson et al., 1985; Westhof et al., 1984). As an alternative, we introduced recombinant DNA technology in this study to locate antigenic determinant of preS2 region of HBV envelope protein. One advantage of gene manipulation is that various modifications can be easily made such as serial deletions and site-saturating substitutions. Immunological characterization of this pool may clarify the antigenic determinants of proteins.

In the present study, it was shown that deletions of preS2 antigen from amino and carboxy termini to certain points did not affect its antigenicity and that only the longer deletions destroyed the antigenicity (Fig. 5). This result indicated that the preS2 epitope is indeed a conformation-independent continuous epitope, as has been suggested previously (Neurath et

al., 1985). Determination of deletion end points revealed that amino and carboxy terminal ends of preS2 epitope were located in the region of residue 130-132 and 140-143, respectively. Thus, the antigenic determinant of preS2 to the Mab H8 was mapped at most in amino acid residues 130-143 and the amino acid sequence in this interval is of critical importance in representing antigenicity. The hydrophilicity plot (Hopp and Woods, 1981) of preS2 sequence showed that the amino acid sequence in this region contains the most hydrophilic region (residues 132-137) which was followed by rather hydrophobic sequence (residues 138-143). Our result corresponds not sufficiently but necessarily to the notion that the antigenic determinant of protein coincides with regions of high hydrophilicity (Hopp and Woods, 1981).

Using synthetic peptides and antibodies in sera from humans who recovered from hepatitis B or were vaccinated, Neurath and colleagues (1986) showed that the antigenic epitope of adw2 subtype preS2 resides between reidues 120 and 145. In the epitope region we mapped for the adr preS2 (residue 130-143), the variations in the amino acid sequences between adr and adw2 subtype preS2 occurred at residue 130, 132, and 141. Since the Mab H8 interacted specifically with adr subtype preS2 but had little cross-reactivity with adw2 subtype (Fig. 1), one or more of the subtype-variable residues may be responsible for the subtype specificity. The interval we mapped is long enough to carry a continuous epitope which is considered to reside in five to eight residues. In order to locate the preS2 epitope in a more defined region and identify critical residues for antigenicity, we are currently examining the effect of residue substitutions induced by site-directed mutagenesis.

적　요

adr 아형 B형 간염바이러스의 preS2 유전자 부위를 lacZ 유전자의 5'말단에 연결하여 preS2-β-galactosidase 융합단백질을 생성하는 플라스미드 pTSZ 를 건설하였다. 클론된 preS2 유전자의 3' 및 5'말단을 결손시켜 얻은 재조합 플라스미드를 발현시킨 후 결손된 preS2를 포함하는 융합단백질의 항원성을 단일클론항체 H8을 사용하여 비교하여 보았다. 양말단에서 일정부위까지의 결손은 항원성에 영향을 미치지 않았지만 그 이상의 결손에 의하여는 항원성이 소실됨을 볼 수 있었다. 이상의 항원성 전환부위를 DNA 염기 서열 분석에 의하여 결정할 수 있었다. 그 결과 항원결정인자의 양말단은 preS2 서열 중 아미노산 잔기 130-132와 140-142 사이에 각각 존재함을 알 수 있었고, 아미노산 143번의 결손은 항원성의 부분적인 감소를 초래하는 것으로 보아 항원성 결정에 보충적 역할을 한다고 생각된다. 한편 adr 과 adw2아형 간의 아미노산서열의 차이가 항원결정부위 중 130, 132 및 141번 위치에 존재하며 단일클론항체 H8이 adr 아형에만 특이하게 결합하는 것으로부터, 세 잔기 중 하나 혹은 그 이상이 아형특이성에 관여한다고 추정된다.

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