# Pre-S proteins—a new marker for the hepatitis-B virus

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## INTRODUCTION

The hepatitis B virus (HBV) of man belongs to a group of viruses—HEPADNA viruses, the other members of which are the woodchuck (WHV), ground squirrel (GSHV) and duck hepatitis B virus (DHBV). They are hepatotropic and known to produce acute and chronic liver disease[1]. The introduction of molecular biology has considerably improved knowledge on the HBV and its replication. Recognition of a nucleic acid sequence preceding the S-gene coding for the major HBsAg polypeptide (i.e. at the pre-S region), its translation product (pre-S proteins) and the development of antibodies against pre-S proteins has resulted in new insights into the biology of HBV. A brief review of the structure of HBV-DNA and the biochemical features of its product is essential for a better understanding of the significance of pre-S proteins in HBV infection.

## STRUCTURE OF HBV-DNA AND BIOCHEMICAL FEATURES OF ITS PRODUCTS

The genetic structure of the virus has been characterised by molecular cloning and sequence analysis of the HBV genome[2]. HBV contains a DNA molecule as its genome. This HBV-DNA is unusual in the way that it is a small, circular, partly double stranded DNA molecule with a single stranded region of variable length. The long or (−) strand is linear and has a fixed length of 3200 nucleotides. The short or the (+) strand is of variable length ranging from 50 to 75% of the length of the (—) strand.

Four open reading frames of the HBV-DNA have been localised on the (−) strand of HBV-DNA (Fig. 1). They are named according to the proteins they produce viz. S, C, P, X. The region-S encloses proteins of the viral envelope and is subdivided into the gene-S and the pre-S gene. The pre-Sregion contains two initiation sites allowing it to be further subdivided into pre-S1 and pre-S2 regions. Region 'C' encodes the proteins of the nucleocapsid, and is further subdivided into pre-C and C-genes which are responsible for the formulation of HBeAg and HBcAg, respectively. Region 'P' is believed to encode for DNA-polymerase; however this gene has not been expressed in vitro. Region 'X' has recently been expressed and is believed to be the oncogene for HCC. However, more evidence needs to be obtained.

## MORPHOLOGY OF THE HBV ENVELOPE

Electrophoresis of the components of the HBV envelope has revealed 6 different proteins which

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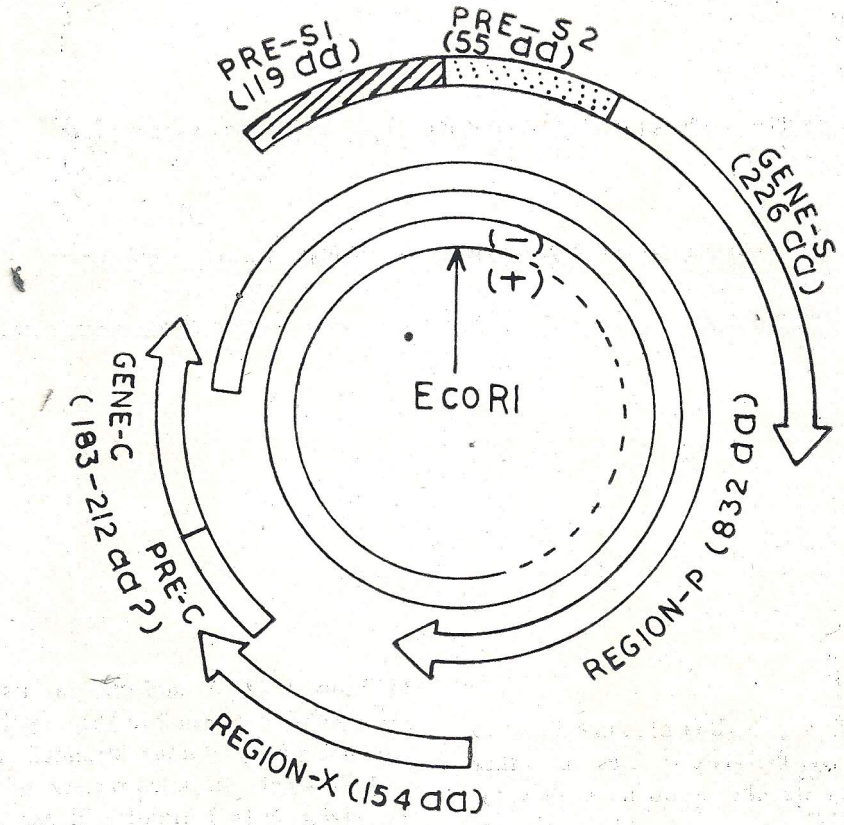
PRE-S1 (119 aa)
PRE-S2 (55 aa)
GENE-S (226 aa)
(-)
(+)
EcoRI
REGION-P (832 aa)
REGION-X (154 aa)
PRE-C
GENE-C (183-212 aa ?)

Fig. 1

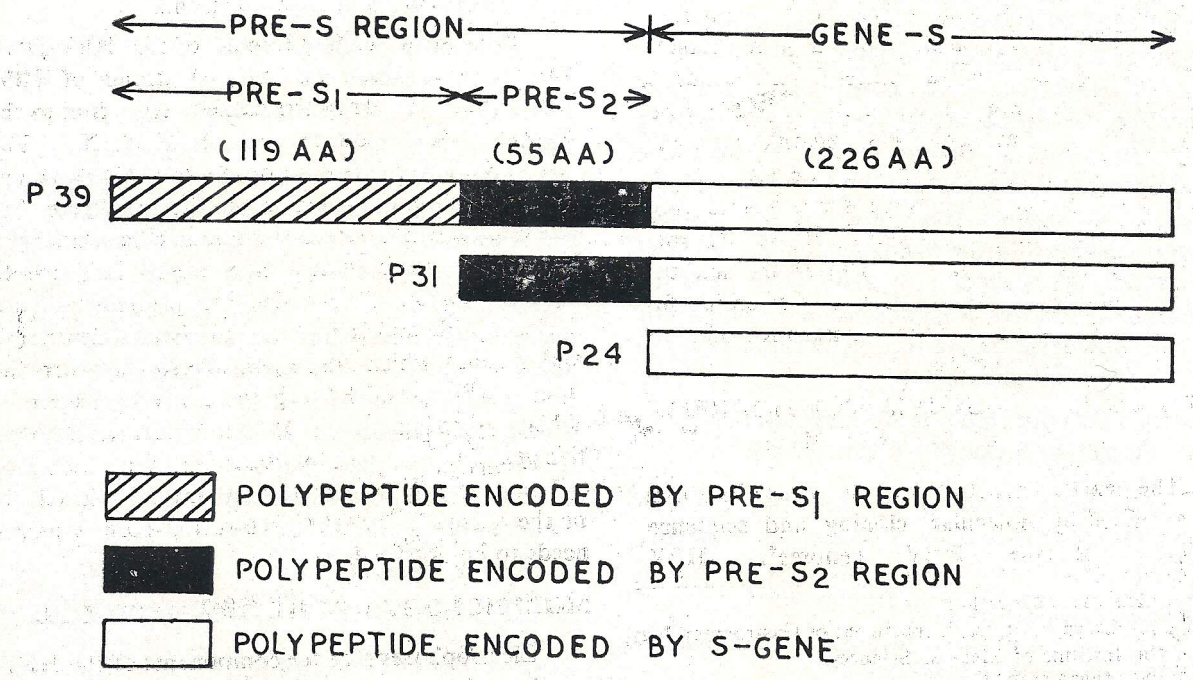
PRE-S REGION
GENE-S
PRE-S1
PRE-S2
(119 AA)
(55 AA)
(226 AA)
P 39
P 31
P 24
POLYPEPTIDE ENCODED BY PRE-S1 REGION
POLYPEPTIDE ENCODED BY PRE-S2 REGION
POLYPEPTIDE ENCODED BY S-GENE

Fig. 2

correspond to 3 polypeptide chains[3] (Fig.. 2). These are :

a) **Small protein**

The major viral envelope protein (HBsAg) is encoded by the S-gene. It has a sequence of 226 aminoacids (aa) and a molecular weight of 24000 daltons. This protein exists in nonglycosylated (P24) and glycosylated forms (GP27)[4].

b) **Intermediate protein**

The second type of protein isolated from the envelope is always glycosylated with a molecular weight varying from 33000 to 36000 daltons (GP33/GP36). This protein, besides containing a P24 sequence of 226 aa, also contains an amino-terminal extension of 55 aa specifically encoded by the pre-S2 region of HBV. Thus the aa sequence of GP33/GP36 protein is of 281 aa and is encoded both by the S and the pre-S2 region[5]. In vitro this polypeptide binds with polymerised human serum albumin (PHSA) prepared by glutaraldehyde treatment and is thought to act as a PHSA receptor[6].

c) **Large protein**

Recently a large surface protein (P36) and its glycosylated form (GP42) encoded by S, pre-S2 and pre-S1 genes collectively has been isolated. It contains an extra chain of 108 aa along with 226 aa of small protein and 55 aa of intermediate protein[7]. The possible functions of this protein are :

i) To act as a marker of viral replication

ii) To increase infectivity of HBV

iii) To help in the maturation of the total virus particle

We know that HBsAg present in the sera with HBV infections is in the form of a 42-nm complete Dane particle and 20-nm spherical and 20-nm filamentous particles. The spherical 20-nm particles from non-viraemic HBsAg carriers are composed exclusively of the major envelope protein P24/GP27. These particles, in purified form, are the major source of the conventional HBV vaccine. The purified 20-nm particle, especially the filamentous type from the blood of viraemic HBsAg carriers contains in addition to P24/GP27, the intermediate protein (GP33/GP36). This protein represents 5-15% of the total protein of these particles[3] and the purified complete HBV particles of 42-nm (Dane particle) and HBsAg filaments contain, in addition, the large surface protein P39/GP 42 which contribute 10-20% of the envelope of intact virus as well as that of HBsAg filaments[3].

## IMMUNOREAGENTS USED TO DETECT PRE-S PROTEINS

**Monoclonal antibodies against pre-S encoded proteins[3,7]**

a) A murine monoclonal antibody obtained after immunisation of mice with purified complete HBV virus (MA 18/7) has been found to bind specifically to the large polypeptide GP33/GP42 and the gene product of the pre-S1 region.

b) Another murine monoclonal antibody (Q 19/10) has been generated by immunising mice with purified filamentous 20nm particles derived from viraemic carriers. This antibody was found to be selective for the pre-S2 domain of the GP33/GP36 protein. It does not react with the pre-S domain of the GP39/GP42 protein.

**Poylclonal antibodies against pre-S proteins**

a) The genetic sequence of the (−) strand of HBV-DNA responsible for pre-S proteins is expressed in *E. coli* which results in a fusion protein. This protein is injected into a rabbit to develop a polyclonal antibody.[8]

b) Synthetic proteins identical to the pre-S gene product have also been produced and are used to induce antibodies in the rabbit[9].

c) Acute phase sera of hepatitis B have already been proved to contain anti-pre-S antibody, from which it has been isolated by extracting the IgG fraction used in the tests[10].

## METHODS TO DETECT PRE-S PROTEINS

**In serum**

*I. Immunoblot technique*[7] :— (*Western blot technique*)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is used to separate

the proteins of different molecular weights. These proteins are transferred to a nitro cellulose membrane and subjected to transversal electrophoresis. This membrane is treated with anti-pre-S monoclonal antibody followed by addition of either radiolabelled $^{125}I$ or peroxidase conjugated anti-human gammaglobulin. The specific reaction can be visualised by autoradiography or enzymatic staining. The disadvantage of the test is that it cannot be used for quantitative assessment.

II. *ELISA/RIA*[8-10]—These techniques involve the use of anti-pre-S monoclonal antibodies, or antibodies, purified from acute phase sera of hepatitis B patients, for the detection of pre-S proteins.

III. *RPHA*[11]—Sheep RBCs coated with monoclonal antibodies against pre-S proteins are used to develop haemagglutination tests for pre-S proteins.

**In tissues**

*Detection of pre-S proteins in liver tissues.*[12] Using appropriate fluorescent and enzyme linked monoclonal or polyclonal antibodies, pre-S1 and pre-S2 encoded proteins, have been localised in liver sections from biopsies.

## METHODS TO DETECT ANTI-PRE S ANTIBODIES

These antibodies can be detected in the sera by assessing their inhibitory capability towards the binding between HBsAg having the pre-S epitopes and either PHSA or monoclonal antibody against pre-S proteins labelled with a radioisotope/enzyme tracer.

## BIOLOGICAL IMPLICATIONS OF PRE-S PROTEINS

The pre-S region of the HBV genome has been found to be the most divergent but still conserved region amongst all the HEPADNA viruses. This suggests that pre-S is functional but the actual aminoacid sequence of this region is conserved to a lower degree than in any other region of the virus. This raises the possibility of species specificity of the product of the pre-S region e.g. the PHSA-receptor (pre-S2 product) can be attached to albumin polymers from the human and chimpanzee alone and hence can attach to these animals only.

Studies on pre-S proteins have helped in understanding the biological behaviour of HBV and we are discussing this under the following heads

a) PHSA receptors and HBV infection

b) Markers of viral replication

c) Immune clearance of HBV—the role of anti-pre-S antibodies.

d) Immunogenicity and its role in improving the HBV vaccine.

### a) PHSA receptors

In recent years several investigators[14-16] have demonstrated binding between PHSA and HBsAg/Dane particles. Under the electron microscope the Dane particles and HBsAg were seen to aggregate after the addition of PHSA. It was further reported that the PHSA binding activity in HBV infected patients was higher in HBeAg positive sera than in HBeAg negative or anti-HBe positive sera[14]. A receptor for PHSA was assumed to be present on Dane particles and HBsAg particles—this was later confirmed by Machida et al[17]. They showed that the PHSA receptor on the HBV particle corresponded to the 55 amino acid sequence encoded by the pre-S gene of HBV[6]. Further studies confirmed it to be specially encoded by the pre-S2 region of the HBV genome[3-5]. The presence of the PHSA receptor on Dane particles and HBsAg particles derived from HBeAg positive sera has been further confirmed by using a monoclonal antibody against the pre-S2 encoded protein[10,13].

In the last two years several studies on PHSA receptors have revealed the following facts :

I. A pre-S2 encoded protein corresponding to the GP33/GP36 intermediate protein of the Dane particle/HBsAg particle contains the binding site for PHSA[3-6,10-14,17].

II. The PHSA receptor is protein in nature and is digested by pronase and trypsin[14].

III. The receptors are ligand specific i e., they only bind to polyalbumin formed by glutaraldehyde treatment and ageing and not to heat aggregated albumin polymers and polymers formed by other chemical processes[14].

IV. They are also species specific i.e. PHSA receptors only bind to polyalbumin from human sources and chimpanzees and not to albumin derived from other animal sources[14,17].

PHSA was postulated to be the linker molecule between the virus and the hepatocyte[15]. Based on this hypothesis it was assumed that a PHSA receptor should also be present on hepatocytes to bind the HBV carrying polyalbumin to the hepatocyte plasma membrane. Such PHSA receptors were first demonstrated on rabbit hepatocytes[18] and subsequently on human hepatocytes[19] by scanning electron microscopy. These hepatocyte PHSA receptors were found to have the following properties[14]:

I They were not species specific or ligand specific.

II They were organ specific i.e. they were not present in other organs. This may explain hepatotrophism of HBV.

Further studies have shown binding of polyalbumin to the rat hepatocyte plasma membrane and cultured hepatocytes from human hepatocellular carcinoma cells[20].

## MECHANISM OF HBV ENTRY INTO THE HEPATOCYTE

According to a recent postulant of HBV entry into the hepatocyte the HBV bound to PHSA reaches the liver cell and gets attached to the PHSA receptor on it. HBV gains entry into the hepatocyte by either endocytosis of the liver cell or by fusion of the plasma membranes of the HBV and the hepatocyte. This allows the entry of the HBV nucleocapsid into the hepatocyte. Small amounts of circulating PHSA formed by the process of ageing may also get bound to HBV after its entry into the circulation. PHSA receptors in liver cells are present normally to take up the aging polyalbumin for catabolisation[18]. The HBV-polyalbumin complex formed gets attached to the hepatocyte PHSA receptor and then HBV enters the hepatocyte.

Immunohistochemical and immunofluorescence studies using monoclonal and polyclonal antibodies[12,14] have revealed the presence of both pre-S1 and pre-S2 (PHSA) proteins in the hepatocyte cytoplasm of chronically HBV infected liver cells. Further, the PHSA receptors (pre-S2) have been shown to be present in large numbers of these infected hepatocytes compared with uninfected hepatocytes.

In order to substantiate the PHSA mediated hepatotrophism of HBV, naturally occurring PHSA would have to be tested, for its binding capacity with HBV particles as in PHSA aggregated by glutaraldehyde. Yu et al[21] reported that albumin polymers isolated from fresh or long stored albumin solutions were much less reactive with HBV particles than PHSA.

## MARKER FOR VIRAL REPLICATION

Both pre-S1 and pre-S2 encoded proteins have been recently implicated as markers of viral replication[3,22].

The presence of pre-S2 proteins (PHSA binding activity) in HBeAg positive sera has been found to be in significantly higher proportions than in HBeAg negative and in anti-HBe positive sera[14,16]. However, recent studies[10,13] have shown that viral multiplication continues in HBeAg negative sera in chronic HBV infection. Hadziyannis et al[24] and Budkowska[10] showed that PHSA binding activity and pre-S2 proteins estimated by monoclonal antibodies, are detected in a substantial number of patients who have chronic HBV infection with a positive anti-HBe reaction. Further Budkowska[10] has correlated pre-S2 coded determinants on HBV particles with HBV-DNA polymerase positivity. Over and above this, pre-S2 positivity in patients with acute hepatitis B detected only during the early phase and never during the recovery phase of disease, further confirms the above facts[10,13].

In a recent study[22], pre-S1 proteins were correlated with the presence of HBV-DNA, both, in the sera and liver of patients with acute as well

as chronic hepatitis B. None of the patients with hepatocellular carcinoma (HCC) positive for HBsAg but negative for HBV DNA revealed any pre-S proteins in the sera or liver tissues suggesting that the presence of pre-S1 proteins could be taken as a definite marker of viral multiplication.

## IMMUNE CLEARANCE OF HBV & PRE-S PROTEINS

Recent reports[10,13,25] on the serological profile of anti-pre-S2 antibody in patients with acute hepatitis B, chronic hepatitis B, HBV-carriers, HBV-vaccine recipients & blood donors revealed that

I) Pre-S proteins could be detected during the first 2 weeks of ocute hepatitis B after which seroconversion occurred to anti-pre S (2nd month); followed by HBsAg clearance and recovery. None of the patients in the studies who developed chronic sequelae developed anti pre-S. Pre-S proteins persisted in them with other markers of viral multiplication.

II) Anti-pre-S in these patients were found to be present upto 12 months with two peaks—probably corresponding to IgM and IgG antibodies (to be confirmed).

III) Except in Okamoto's study[13], vaccine recipients developed anti-pre-S antibodies earlier than anti-HBs (the vaccine used in Okamoto's study might have been HBsAg without pre-S proteins, which may have been absent or lost during preparation) indicating their protective role.

IV) Only 1-2% of carriers showed anti-pre-S antibodies.

V) Blood donors positive for anti-HBs were also positive for anti-pre-S.

Similar results have been obtained for anti-pre-S antibodies in different groups of patients infected with HBV at our centre. The results are shown in Table-I.

Pre-S proteins seem to have considerable prognostic implications. Their persistence indicates continued viral multiplication with progression to chronic liver disease whereas seroconversion to anti-pre-S indicates recovery. Anti-pre-S has been thought to either block the PHSA receptor on the HBV particle, thereby not allowing PHSA to bind to it and interupt its entry into the hepatocyte[3] or from immune complexes with Dane particles which are cleared from the circulation by immunological reactions.

## IMMUNOGENICITY OF PRE-S PROTEINS

In mouse experiments Milich et al[26] demonstrated that the proteins encoded by the S, pre-S2 and pre-S1 regions have been found to be highly immunogenic (pre S1 > pre S2 > S). The same authors have also shown that the immune response to all the three polypeptides are independent of one another, i.e. even though they are coded by the

TABLE—I ANTI-PRE-S ANTIBODY IN HBV INFECTED PERSONS (AIIMS)

| Category | No. | $HBsAg^+$ | IgM $Anti\text{-}HBc^+$ | $Anti\text{-}HBs^+$ | $Anti\text{-}pre\text{-}S^+$ |
|---|---|---|---|---|---|
| Vaccine recipient | | | | | |
| IInd dose (1 month) | 12 | 0 | 0 | 4(33%) | 9(75%) |
| IIIrd dose (6 month) | 12 | 0 | 0 | 10(83%) | 12(100%) |
| FHF | 18 | 18 | 8 | 1(5.5%) | 6(33.3%) |
| SAHF | 10 | 10 | 4 | 5(50%) | 0(nil) |
| CAH | 10 | 10 | — | 6(60%) | 1(10%) |
| HBV Carrier | 105 | 105 | — | 1(1%) | 0 |

same gene, their immune response is regulated by different sets of histocompatibility complex linked immune response genes (Ir.). The envelope polypeptides of the HBV present an array of T cell determinants that the host can recognise and the specificity of the recognition process is determined by histocompatibility linked genes[26]. Pre-S1 and pre-S2 have been shown to be immunogenic at both T and B cell levels. Mice, non-responders to the S-region, (determined genetically by the H2 linked Ir gene) on immunisation with pre-S2 region protein circumvented non response to the S-region. Similarly immunisation with pre-S1 protein in non responders to pre-S2 and S-proteins showed circumvented response to these proteins. The mechanism of the circumvention seems to be induction of specific T helper cells by pre-S proteins which induce B cell activity and start producing antibody against the previously non-responding polypeptide S-proteins. Thus inclusion of pre-S proteins in the preparation of HB-vaccine may make the vaccine more effective and reduce the nonresponder rate.

Recent studies[27,28] have revealed that, the efficacy of heat inactivated HB vaccine in patients on maintenance haemodialysis was 77 to 94% which was much higher than the efficacy of the conventional vaccine (50%) prepared by the treatment of proteolytic enzymes in a similar group of proteins. These proteases digest the pre-S proteins, whereas in the former type of vaccine, heat does not destroy the pre-S proteins. This, further confirms the effectivity of pre-S protein in inducing protection against HBV infection. A synthetic vaccine prepared recently from the pre-S2 region of HBV-DNA has been shown to be highly effective in chimpanzees[29].

## CONCLUSIONS

1. Newly characterised pre-S1 and pre-S2 proteins of the HBV envelope are present in patients with continued viral replication and hence can be a measure of infectivity.

2. They have a prognostic significance.

3. Anti-pre-S antibodies may have a role in HBV clearance.

4. They provide a putative mechanism of for HBV entry into the hepatocyte and species specificity of the HBV and its hepatotrophism may be explained.

5. These proteins have provided an avenue for developing a better HB-vaccine particularly for immunocompromised patients.

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