Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 7540–7544, November 1985
Biochemistry

Expression in *Escherichia coli* of a cloned DNA sequence encoding the pre-S2 region of hepatitis B virus

(recombinant DNA/β-galactosidase fusion protein/affinity chromatography/antigenic determinants of the hepatitis B virus envelope)

WOLF OFFENSPERGER*, SILKE WAHL*, A. ROBERT NEURATH†, PETER PRICE*‡, NATHAN STRICK†, STEPHEN B. H. KENT§, JUDITH K. CHRISTMAN*‡, AND GEORGE ACS*‡

Departments of *Biochemistry and ‡Pediatrics, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029; †The Lindsley F. Kimball Research Institute of The New York Blood Center, 310 East 67th Street, New York, NY 10021; and §The California Institute of Technology, Pasadena, CA 91125

Communicated by Hans Popper, July 18, 1985

ABSTRACT **A DNA sequence encoding the entire pre-S2 region (amino acids 120–174; serotype ayw) of human hepatitis B virus envelope protein has been inserted into the *lacZ* gene of the plasmid pSKS105 yielding a recombinant, pWS3. Lac$^+$ colonies of the *Escherichia coli* M182 Δ(lacIOPZYA), isolated after transformation with pWS3, produced a pre-S2 peptide–β-galactosidase fusion protein. This fusion protein, which comprised as much as 3% of the total bacterial protein, was purified to >90% homogeneity by affinity chromatography on *p*-aminophenyl-β-D-thiogalactoside-Sepharose. It is immunoprecipitable with rabbit antibodies to a synthetic peptide corresponding to amino acids 120–145 of the pre-S2 region of serotype adw [pre-S(120–145)] or with antibodies to hepatitis B virus. pre-S(120–145) completely blocked the binding of either antibody to the pre-S2 peptide–β-galactosidase fusion protein. These results indicate that there are antigenic determinants on the fusion protein that are closely related to, if not identical to, determinants on synthetic pre-S(120–145) and on pre-S2 sequences of native hepatitis B virus particles. Thus, bacteria transformed with pWS3 can provide an abundant source of pre-S2–β-galactosidase fusion protein, which may prove useful either as a diagnostic reagent possessing marker enzyme activity suitable for ELISA tests or as an immunogen with potential to contribute to active prophylaxis of hepatitis B.**

Four open reading frames have been identified on the transcript of the L strand of the hepatitis B virus (HBV) genome—S, P, C, and X (reviewed in ref. 1). Region C codes for the major protein of the virus nucleocapsid [hepatitis B core antigen (2, 3)], region P is assumed to code for the viral polymerase, and region X codes for a protein that has not yet been detected in mature virus particles but has been detected in infected tissues (4). The region S codes for the surface proteins of HBV (HBsAg). Although this region has the potential to direct the synthesis of a protein 389–400 amino acids long, depending on serotype (5), the predominant protein component found in the HBV envelope or in 22-nm subviral particles contains only 226 amino acids and is the product of gene *S*, which starts within the S region with the methionine codon at nucleotide 155 (1). This methionine codon is preceded in the S region by two other methionine codons at nucleotides 3172 and 2848,¶ which define the limits of the pre-S2 and pre-S1 regions and encode sequences of 55 and 108–119 amino acids, respectively. Thus, transcripts of the entire S region could direct synthesis of three distinct proteins that share the same 226 COOH-terminal amino acids. Glycosylation of these proteins results in the multitude of components detected in earlier studies on the protein composition of the HBV envelope and of HBsAg.

Although the pre-S1 and pre-S2 sequences have a relatively low abundance in HBV or HBsAg isolated from the serum of HBV carriers, their biological role has already been explored by using appropriate polyclonal or monoclonal antibodies against HBV (7, 8), synthetic peptides (9), and proteins synthesized in cells transfected with expression vector containing the total S region or large portions of pre-S (10, 11). For example, the amino acid sequences encoded in the pre-S1 and pre-S2 regions have been shown to be involved in specific binding of HBV (HBsAg) to the surface of human hepatocytes (12). The amino acid sequence of pre-S2 has also been found to bind human and chimpanzee glutaraldehyde cross-linked polyalbumin (11, 13, 14). In addition, it has been shown that the 26 NH_2-terminal amino acids (120–145) of the pre-S2 region [pre-S(120–145)] correspond to a dominant sequential antigenic determinant of HBV and HBsAg (9). However, as yet, a convenient source for the large amounts of pure full-length pre-S sequences essential for further elucidation of the function of the pre-S regions has not been available. In this report, we describe the production, purification, and immunochemical characterization of a pre-S2–β-galactosidase fusion protein (pre-S2–Gal) synthesized as a major protein component in transformed *Escherichia coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. JM83 *ara*Δ*(lac pro) strA thi (φ80dlacI^q, ZΔM15)* (15) and pUC8 (16) were obtained from Bethesda Research Laboratories, and M182 Δ*(lac IPOZYA)-X74 galU galK strA^r* (17) and pSKS105 (18) were the kind gifts of M. J. Casadaban. pTHBV-1, a recombinant plasmid containing a head-to-tail dimer of a HBV genome (subtype ayw), was constructed as described in ref. 6.

Isolation, Detection, and Purification of β-Galactosidase and pre-S2–Gal. For preparation of lysates, bacteria from late-logarithmic phase or stationary cultures were pelleted and suspended in 1/20th of the culture volume in buffer containing 0.5 mg of lysozyme per ml, 0.1 M Tris·HCl (pH 8), 0.1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA. After three cycles of freeze–thawing, the suspension was centrifuged at 10,000 × *g* for 10 min and the supernatant was assayed for β-galactosidase activity (19, 20). Proteins with β-galactosidase activity were purified by affinity chromatography on *p*-aminophenyl-β-D-thiogalactoside-Sepharose prepared as described by Ullmann (21). The proteins present in various

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; pre-S2–Gal, fusion protein of pre-S2 peptide (amino acids 120–174 plus Met 175) with β-galactosidase.

¶Numbering of bases in the HBV sequences of pTHBV-1 has been changed from that used in ref. 6 to that used in ref. 1.

fractions were analyzed by $NaDodSO_4$/polyacrylamide slab gel electrophoresis (22).

Immunological Detection of pre-S2–Gal. pre-S2–Gal was detected by an immunoassay analogous to that described for the reaction between rabbit anti-pre-S(120–145) and pre-S (120–145) conjugated with β-galactosidase (9). Briefly, serial dilutions of bacterial lysates (50 μl in 0.14 M NaCl/0.01 M Tris·HCl, pH 7.2/0.02% NaN_3/10 mg of bovine serum albumin per ml) were mixed with 450 μl of rabbit anti-pre-S(120–145) diluted 1:180 in the same buffer. The mixture was incubated for 30 min at 37°C; 100 μl of a 10% suspension of *Staphylococcus aureus* bearing protein A (The Enzyme Center, Boston) was added and, after 30 min at room temperature, the bacteria were pelleted by centrifugation and washed twice. β-Galactosidase activity in the immune complex was determined fluorometrically (20), and results were corrected for nonspecific adsorption of β-galactosidase in the presence of diluted normal rabbit serum.

Enzymes and Chemicals. Restriction endonucleases, linkers, T4 ligase, and Klenow fragment of DNA *Pol* I were purchased from New England Biolabs and were used according to the manufacturer's recommendations. *o*-Nitrophenyl-β-D-galactopyranoside, 4-methylumbelliferyl-β-D-galactoside, and β-galactosidase were obtained from Sigma.

RESULTS

Construction of Plasmids (Fig. 1). The plasmid pUC8 (16) was linearized by digestion at its unique *Acc* I site. The staggered ends were filled in by polymerization using the Klenow fragment of *E. coli* polymerase I. The plasmid was then recircularized by ligation with T4 ligase and recloned. The resulting plasmid (pUC8a) was digested with *Pst* I, which cuts within the open reading frame for the *lacZ'* gene, and it was blunt-ended by using the 3′ → 5′ exonuclease of the Klenow fragment. *Sph* I linkers were added and the plasmid was recircularized by ligation after digestion with *Sph* I. This construction (pUC8b) resulted in restoration of the correct reading frame for the *lacZ'* gene and provided an additional ATG sequence in phase with and 39 bases downstream from the ATG sequence that marks the start of the *Z'* gene reading frame. A 165-base-pair *Nla* III restriction fragment (bases 3174–157)[¶] from pTHBV-1 (6) containing the entire sequence of the pre-S2 region was inserted into the *Sph* I site of pUC8b. Two plasmids were isolated: pWS1, which contained the insert in the proper orientation for the production of a fusion protein of pre-S2 (amino acids 120–174 plus the first methionine of the *S* gene) and β-galactosidase; and pWS2, which contained the insert in reverse orientation. The plasmid pWS3 was constructed by ligating the 187-base-pair *Bam*HI/*Hin*dIII fragment of pWS1 that contained the pre-S2 sequences with *Bam*HI- and *Hin*dIII-cleaved pSKS105 (18). The structure of all recombinant plasmids was confirmed by restriction analysis (not shown).

Expression of β-Galactosidase and pre-S2–Gal in JM83 Cells Transfected with pWS1 and pWS2. pUC8 directs synthesis of an NH_2-terminal fragment of β-galactosidase that complements the COOH-terminal fragment of β-galactosidase produced by JM83 cells. Thus, colonies of JM83 cells transformed with this plasmid can be readily distinguished from untransformed cells by their ability to form blue rather than white colonies on agar containing the chromogenic β-galactosidase substrate, 5-bromo-4-chloro-3-indolyl β-D-galactoside (23). JM83 cells transformed with either pWS1 or pWS2 gave rise to blue colonies with approximately the same frequency as those transformed with pUC8 (data not shown). Quantitation of active β-galactosidase in lysates of cultures grown out from two representative colonies, one transformed with pWS1, the other with pWS2, demonstrated that insertion of pre-S2 sequences into the *lacZ'* gene of pUC8 in either orientation still allowed efficient complementation of the *lacZΔM15* gene product of JM83 cells, although the levels of active enzyme were lower in pWS2 transformed cells than in pWS1 and pUC8 transformants (Table 1). However, when proteins from the transformed cells were precipitated with anti-pre-S(120–145) and *Staphylococcus* protein A, β-galactosidase activity was detectable only in precipitates from JM83 cells carrying pWS1. This demonstrated (*i*) that the anti-pre-S(120–145) antibodies did not recognize β-galactosidase and (*ii*) that pre-S2 sequences must be properly oriented with respect to *lacZ'* sequences for production of a fusion protein that can be recognized by antibodies specific for pre-S(120–145).

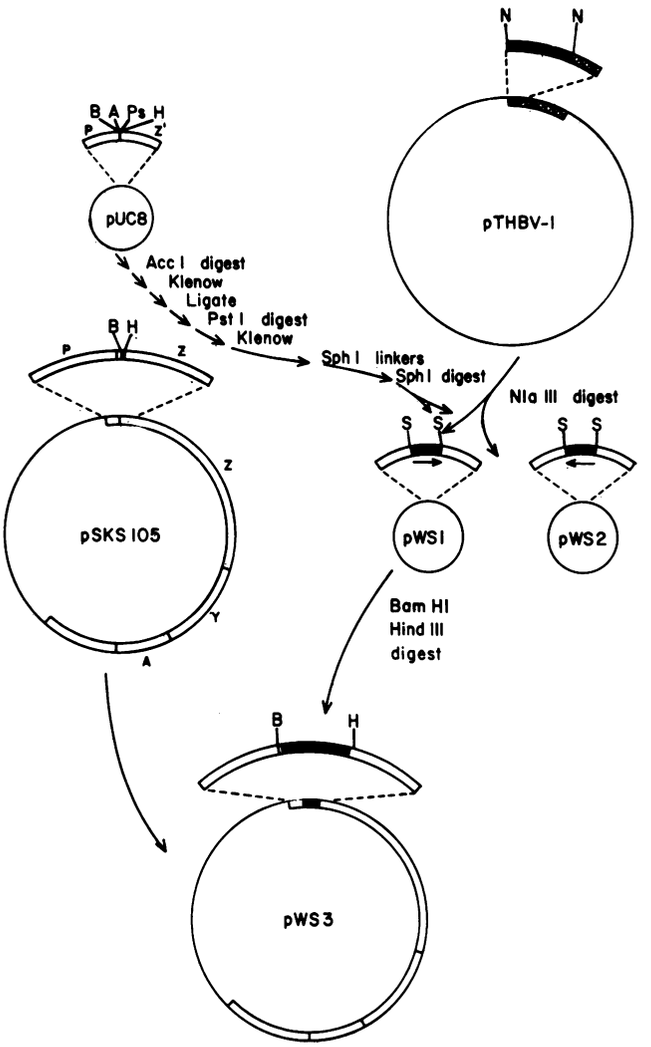

FIG. 1. Construction of pWS1, pWS2, and pWS3. Sequences from the *lacZ* gene are indicated by an open box; sequences corresponding to the pre-S2 sequence of the HBV S region by a solid box; the HBV *S* gene by a cross-hatched box, and pBR322 sequences by a line. Arrows indicate the direction of transcription of HBV S sequences and *lacZ* sequences. Restriction endonuclease cleavage sites are abbreviated as follows: B, *Bam*HI; A, *Acc* I; Ps, *Pst* I; H, *Hin*dIII; N, *Nla* III; S, *Sph* I.

Immunochemical Characterization and Quantitation of the pre-S2–Gal. Both anti-pre-S(120–145) and antiserum against HBV particles from which detectable antibodies against the S protein had been removed (7) recognized pre-S2–Gal produced by JM83 cells transformed with pWS1 but failed to react with proteins from JM83 cells transformed with pWS2 (Fig. 2). The reaction between the fusion protein and anti-pre-S(120–145) (Fig. 3*A*) and the reaction between the fusion protein and antibodies to the pre-S epitopes of native HBV

Table 1. Expression of β-galactosidase and pre-S2–Gal in *E. coli*

Strain	Total β-galactosidase, units per ml of lysate*	pre-S2–Gal,† fluorescence units per μl of lysate
JM83	>0.3	0.03
JM83/pUC8	135	0.04
JM83/pWS1	92	2.8
JM83/pWS2	30	0.026

Values shown are from a typical experiment. Levels of β-galactosidase and pre-S2–Gal activity found in six to seven different lysates from the same bacterial strains did not vary by more than 15%.

*Activity in lysate was determined directly as described in ref. 19. One unit of β-galactosidase is defined as the amount capable of hydrolyzing 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside per min at 28°C. The protein content of the lysate estimated from A_{280} nm was 5 mg/ml.

†Activity in lysate proteins precipitated with anti-pre-S(120–145) and *Staphylococcus* protein A measured as described in ref. 20 using 4-methyl-umbelliferyl-β-D-galactoside as substrate. Arbitrary fluorescence units = 100 × (fluorometer digital readout/fluorometer sensitivity range). Fluorescence units per ml of lysate precipitated with normal rabbit serum and *Staphylococcus* protein A were pWS1 = 0.033, pWS2 = 0.020.

envelope proteins (Fig. 3*B*) were both efficiently and completely inhibited by synthetic pre-S(120–145) and by HBV particles. This indicated a close immunological relationship, if not an identity, of epitopes present on the sequence of the 26 NH_2-terminal amino acids of pre-S2, regardless of whether they were located within a native envelope protein, pre-S2–Gal, or the synthetic peptide itself. Furthermore, the finding that synthetic pre-S(120–145) completely inhibited the reaction between pre-S2–Gal and antibodies to the pre-S region of HBV envelope protein suggested that the NH_2-terminal half of pre-S2 is immunologically dominant and that the contribution of the COOH-terminal half of pre-S2 to the antigenicity and immunogenicity of the HBV envelope proteins is comparatively small.

On the assumption that the binding capacity of anti-pre-S(120–145) was equivalent for synthetic pre-S(120–145) and

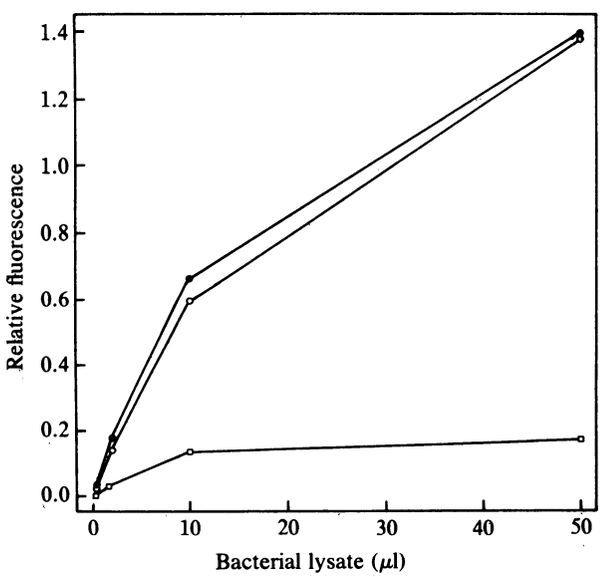

FIG. 2. Immunoassay of pre-S2–Gal precipitated with rabbit antisera to synthetic pre-S(120–145) (●) antisera to the pre-S determinant(s) of HBV particles (○), rabbit, or control rabbit serum (□). Dilution of each serum was 1:200. Assay details are given in *Materials and Methods*.

for pre-S2 sequences in a fusion protein, the concentration of pre-S2–Gal in lysates of bacteria transformed with pWS1 was estimated by determining the concentration of synthetic peptide required for 50% inhibition of antibody binding by pre-S2–Gal (Fig. 3*A*). The sample tested in that experiment contained ≈10 ng per 20 μl of synthetic pre-S(120–145). Since pre-S(120–145) has a molecular weight of 2945, it could be calculated that the concentration of pre-S2–Gal in the bacterial lysate was 0.17 μM, which indicates that a late-logarithmic phase culture of JM83/pWS1 cells contains ≈40 μg of pre-S2 per liter.

High Yield Production and Purification of a Pre-S2–Gal. Although the experiments described above clearly demonstrated the feasibility of producing a pre-S2 fusion protein with the antigenic properties of native pre-S2 in *E. coli*, the low yield of pre-S2–Gal from JM83/pWS1 cells prompted us to construct a recombinant between the pre-S2 sequences and pSKS105, a plasmid known to lead to highly efficient production of β-galactosidase in the Lac⁻ *E. coli*, M182 (18). To ensure preservation of the orientation of pre-S2 sequences with respect to the *lacZ* gene, a 187-base-pair *Bam*HI/*Hin*dIII fragment of pWS1 containing the entire pre-S2 sequence along with flanking *lacZ* sequences was substituted for the corresponding *Bam*HI/*Hin*dIII flanked *lacZ* sequences of pSKS105 (pWS3; Fig. 1). M182 cells transformed with pWS3 were identified by their ability to form blue rather than white colonies on 5-bromo-4-chloro-3-indolyl β-D-galactoside plates. Cell cultures derived from these colonies were tested for content of active β-galactosidase and for production of pre-S2–Gal. A typical culture of M182/pWS3 cells in late-logarithmic or stationary phase yielded ≈7,000,000 units or 200 mg of β-galactosidase per liter. [Calculated from the activity of a standard β-galactosidase preparation of known concentration assayed under identical conditions (19). This calculation was not made for JM83/pWS1 cells on the assumption that it would lead to an underestimation of content of the β-galactosidase formed by complementation in JM83.] Since a fusion protein between β-galactosidase (M_r, 116,000) and pre-S2 (M_r, ≈6000) is 5% by weight pre-S2, the cells have produced an estimated 10 mg of pre-S2 peptide per liter. This value agrees quite well with the content of pre-S2 estimated from a determination of the concentration of pre-S(120–145) required for 50% inhibition of anti-pre-S(120–145) binding by M182/pWS3 pre-S2–Gal (6 mg per liter of culture; data not shown).

Affinity Purification of Pre-S2–Gal. Pre-S2–Gal proved to be a major component of unfractionated lysate from M182/pWS3 cells. It was readily detectable as a Coomassie blue-stained protein species absent from extracts of untransformed M182 cells (Fig. 4*a*). The identity between this new protein species and the pre-S2–Gal was confirmed by immunoprecipitation with anti-pre-S(120–145) and *Staphylococcus* protein A (Fig. 4*b*, lane 1). With the appropriate concentration of anti-pre-S(120–145), pre-S2–Gal could be quantitatively precipitated (data not shown). However, no protein with the electrophoretic mobility of either pre-S2–Gal or β-galactosidase was detected in immunoprecipitates of lysates from M182 cells carrying pSKS105 without the pre-S2 insert (Fig. 4*b*, lane 2).

To purify pre-S2–Gal, the lysate from M182/pWS3 cells was passed over a column packed with *p*-aminophenyl-β-D-thiogalactoside-Sepharose. The β-galactosidase activity was quantitatively bound to the column and could be eluted with buffer containing 100 mM sodium borate (pH 10), 10 mM 2-mercaptoethanol (Table 2). This one-step procedure gave a 75-fold purification of the fusion protein with no loss of enzyme activity. The purified pre-S2–Gal ran as a single band with slightly slower electrophoretic mobility than β-galactosidase on analytical $NaDodSO_4$/polyacrylamide gels. It

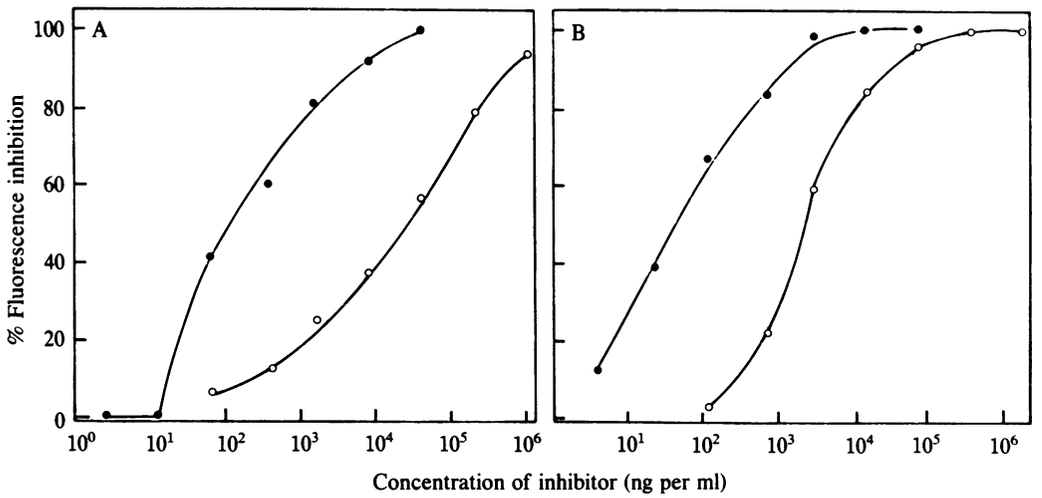

FIG. 3. (*A*) Inhibition of binding of anti-pre-S(120–145) to pre-S2–Gal by synthetic pre-S(120–145) (●) or by HBV particles (○). Twenty microliters of lysate from JM83/pWS1 cells was used per test and the dilution of the anti-pre-S(120–145) antiserum was 1:200. (*B*) Inhibition of binding of antibodies to the pre-S determinants of HBV to pre-S2–Gal by synthetic pre-S(120–145) (●) or by HBV particles (○). Twenty microliters of lysate from JM83/pWS1 cells was used per test and the dilution of the antiserum was 1:300.

was virtually the only protein detectable by Coomassie blue staining (Fig. 4*c*, lane 1).

DISCUSSION

Although the amino acid sequence encoded by the pre-S region of the HBV genome is underrepresented in proteins of the viral envelope, studies with appropriate synthetic peptide analogs have made it clear that domains within the pre-S region mediate the binding of HBV to hepatocytes and carry dominant sequential antigenic determinants involved in eliciting an immune response to HBV (9, 12). Thus, production of envelope proteins that include amino acid sequences of the pre-S region may play an essential role in the life cycle of HBV.

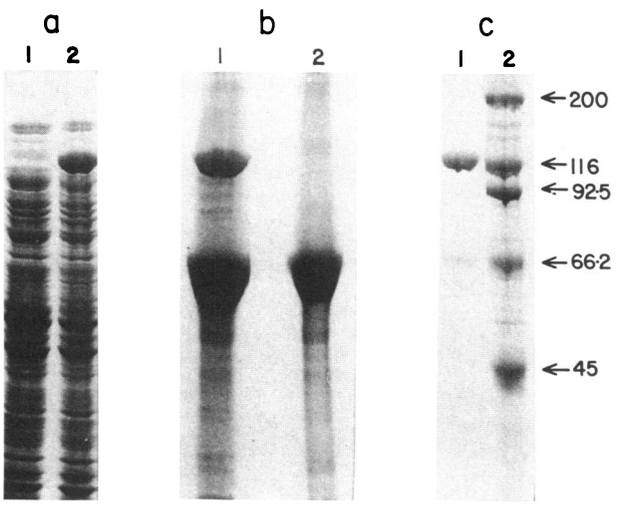

FIG. 4. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis pre-S2–Gal from M182/pWS3 cells. (*a*) Lane 1, lysate from M182 cells; lane 2, lysate from equivalent volume of M182/pWS3 cells containing 175 units of β-galactosidase activity. (*b*) Lane 1, anti-pre-S(120–145) immunoprecipitate of lysate from M182/pWS3 cells (1750 units of β-galactosidase activity); lane 2, anti-pre-S(120–145) immunoprecipitate of lysate from M182/pSKS105 cells (1750 units of β-galactosidase activity); (*c*) lane 1, affinity purified pre-S2–Gal fusion protein from M182/pWS3 cells (120 units of β-galactosidase activity from the purification presented in Table 3); lane 2, molecular weight markers ($\times 10^{-3}$).

Pre-S2–Gal produced in *E. coli* is recognized both by antibodies raised against the pre-S2 region of HBV and against synthetic pre-S(120–145) peptide. Furthermore, binding of these antibodies to the fusion protein is completely inhibited by an appropriate concentration of either pre-S(120–145) or HBV particles. These results clearly demonstrate that the same or similar antigenic determinants are present in the 26 amino acid NH_2-terminal sequence of pre-S2 regardless of whether it is in the form of a free peptide, comprises the NH_2 terminus of a native viral envelope protein or is embedded in the NH_2-terminal region of a functional β-galactosidase tetramer. They also suggest that the NH_2-terminal half of pre-S2 is immunologically dominant—i.e., that the contribution of the COOH-terminal half of pre-S2 (amino acids 146–174) to the antigenicity and immunogenicity of the HBV envelope proteins is comparatively small. However, antibodies to a synthetic peptide with the amino acid sequence of the COOH-terminal half of pre-S2 (amino acids 153–171) recognize antigenic determinants present in native HBsAg (24), and the possibility remains that the pre-S2 region of native viral envelope proteins contains

Table 2. Affinity purification of pre-S2–Gal

Fraction	Protein, mg	β-Galactosidase, units*	Specific activity, units/mg
Load	517	300,000	580
Wash	450	>0.3	0
Borate eluate	6.9	300,000	43,000

Data shown are from a representative experiment in which a lysate containing 300,000 units of β-galactosidase diluted in buffer containing 1.6 M NaCl, 20 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, and 10 mM 2-mercaptoethanol was passed over a 6-ml column packed with *p*-aminophenyl-β-D-thiogalactoside-Sepharose equilibrated with the same buffer. The column was washed with this buffer until no more protein was eluted and was then eluted with 100 mM sodium borate, pH 10/10 mM 2-mercaptoethanol (21). The values given for total enzyme activity and protein content were calculated from assays of β-galactosidase activity in aliquots of the fractions and from their absorbance at 280 nm. In four separate experiments, recovery of total protein ranged from 85% to 95% and recovery of pre-S2–Gal ranged from 95% to 100%. Recovered purified pre-S2–Gal comprised 1%–3% of the total protein in the lysates.

*A unit of β-galactosidase is defined as the amount capable of hydrolyzing 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside per min at 28°C.

additional antigenic determinants that are not represented in either pre-S(120–145) or in pre-S2–Gal.

It should be noted that the synthetic pre-S(120–145) used in these studies has the amino acid sequence of an adw serotype. Nevertheless, antibodies to this peptide bind equally well with the pre-S2 amino acid sequence encoded in the genome of a virus with serotype ayw. This conclusion is supported by the observation that estimations of the concentrations of pre-S2–Gal in lysates of M182/pWS3 cells are approximately the same whether based on the assumption that anti-pre-S(120–145) has an equivalent binding capacity for pre-S(120–145) and the corresponding region of pre-S2–Gal or are calculated from the β-galactosidase activity of the fusion protein. Similarly, if one considers that pre-S(120–145) sequences constitute ≈1% of the mass of HBV envelope proteins (8), the inhibition studies shown in Fig. 3*B* indicate that the binding capacity of antibodies to the pre-S region of native HBV envelope proteins is equivalent in molar terms for pre-S(120–145), pre-S2–Gal, and HBV.

The results presented here demonstrate that pre-S2, which shares antigenic determinants with pre-S2 in native HBV envelope proteins, can be synthesized in large amounts in *E. coli*. They also indicate the feasibility of using expression vectors to direct bacterial synthesis of fusion proteins containing both an antigenic determinant and a marker enzyme suitable for use in ELISA tests. It seems highly likely that other preselected sequences of the pre-S region can be synthesized as fusion proteins through use of the same or similar expression vectors. The availability of these proteins will certainly lead to unique reagents for diagnosis of HBV infection. However, if pre-S fusion proteins also prove to be immunogenic and/or to directly affect interaction of HBV with hepatocytes, they have the potential for contributing to a further definition of the role of the minor HBV envelope proteins carrying pre-S region domains, and they may aid in the development of new or improved modalities for active prophylaxis of HBV.

W.O. and S.W. are Fellows of the Deutsche Forschungsgemeinschaft. This investigation was partially supported by Public Health Service Grant RO1-CA34818 awarded by the National Cancer Institute.

1. Tiollais, P., Charnay, P. & Vyas, G. N. (1981) *Science* **213**, 406–411.
2. Pasek, M., Goto, T., Gilbert, W., Zink, B., Schaller, H., MacKay, P., Leadbetter, G. & Murray, K. (1979) *Nature (London)* **282**, 575–579.
3. Burrell, C. J., MacKay, P., Greenway, P. J., Hofschneider, P. H. & Murray, K. (1979) *Nature (London)* **279**, 43–47.
4. Moriarty, A. M., Alexander, H., Lerner, R. A. & Thornton, G. B. (1985) *Science* **227**, 429–432.
5. Neurath, A. R. & Kent, S. B. H. (1985) in *Immunochemistry of Viruses*, eds. Neurath, A. R. & van Regenmortel, M. H. V. (Elsevier, Amsterdam), in press.
6. Christman, J. K., Gerber, M., Price, P., Flordellis, C., Edelman, J. & Acs, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 1815–1819.
7. Neurath, A. R., Trepo, C., Chen, M. & Prince, A. M. (1976) *J. Gen. Virol.* **30**, 277–285.
8. Heermann, K. H., Goldmann, V., Schwartz, W., Seyffarth, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52**, 396–402.
9. Neurath, A. R., Kent, S. B. H. & Strick, N. (1984) *Science* **224**, 392–395.
10. Wong, D. T., Nath, N. & Sninsky, J. (1985) *J. Virol.* **55**, 223–231.
11. Michel, M. L., Pontisso, P., Sobczak, E., Malpiece, Y., Streeck, R. E. & Tiollais, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7708–7712.
12. Neurath, A. R., Kent, S. B. H., Strick, N., Taylor, P. & Stevens, C. E. (1985) *Nature (London)* **315**, 154–156.
13. Machida, A., Kishimoto, S., Ohnuma, H., Miyamoto, H., Baba, K., Oda, K., Nakamura, T., Miyakawa, Y. & Mayumi, M. (1984) *Gastroenterology* **85**, 268–274.
14. Persing, D. H., Varmus, H. E. & Ganem, D. (1984) in *Viral Hepatitis and Liver Disease*, eds. Vyas, G. N., Dienstag, Y. L. & Hoofnagle, J. H. (Grune & Stratton, New York), p. 687.
15. Messing, J. (1979) *Recomb. DNA Tech. Bull.* **2**, 43–48.
16. Vieira, J. & Messing, J. (1982) *Gene* **19**, 259–268.
17. Casadaban, M., Chou, J. & Cohen, S. N. (1980) *J. Bacteriol.* **143**, 971–980.
18. Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. & Chou, J. (1983) *Methods Enzymol.* **100**, 293–308.
19. Pardee, A. B., Jakob, F. & Monod, J. (1959) *J. Mol. Biol.* **1**, 165–178.
20. Neurath, A. R. & Strick, N. (1981) *J. Virol. Methods* **3**, 155–165.
21. Ullmann, A. (1984) *Gene* **29**, 27–31.
22. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
23. Miller, J. M. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
24. Neurath, A. R., Kent, S. B. H. & Strick, N. (1985) in *Proceedings of the Symposium on Synthetic Peptides in Biology and Medicine*, ed. Pettit, G. R. (Elsevier/North-Holland, New York), in press.