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Immunology

Immunogenicity of peptide fusions to hepatitis B virus core antigen

(recombinant DNA/serology/animal virus/human immunodeficiency virus/vaccine)

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ABSTRACT **Several gene fusions have been constructed in which coding sequences for antigenic regions of the pre-S sequences of hepatitis B virus, hepatitis B surface antigen, and the envelope protein of human immunodeficiency virus were linked to the 3′ end of that for the first 144 residues of hepatitis B core antigen. The sequences were expressed efficiently in *Escherichia coli* to give stable products that assembled to form particles morphologically similar to hepatitis B core antigen itself. The products exhibited the antigenic and immunogenic characteristics of both the hepatitis B core antigen epitopes and the epitopes carried by the additional sequences, thus illustrating the value of such proteins as immunological reagents and potential vaccines.**

The nucleocapsid protein, or core antigen (HBcAg), of hepatitis B virus (HBV) can be readily synthesized in *Escherichia coli* (1), where it assembles into nucleocapsid particles. When viewed in the electron microscope, these particles are indistinguishable from native HBV nucleocapsid particles isolated from infected hepatocytes (2). The C terminus of HBcAg (residues 150–183) is arginine-rich and homologous with protamines (3); the inference is that the C-terminal region binds the HBV DNA within the nucleocapsid particles, whereas the rest of the protein participates in other structural roles. A form of HBcAg lacking the C terminus and possessing different antigenic properties, hepatitis B e antigen (HBeAg), is found in the serum of HBV carriers (4). Although serum HBeAg is found mainly as a soluble dimer of 17-kDa subunits, the truncated form of HBcAg produced in *E. coli* also forms nucleocapsid-like particles (S.S. and P. Wingfield, unpublished data) indicating that the argininine-rich C-terminal region is not critical for particle assembly. It may, therefore, be possible to replace the C terminus of HBcAg with other epitope structures without losing the ability to form particles. Antigens presented in a particulate structure may have enhanced immunogenicity, and parts of HBcAg have recently been shown to activate the immune system (5).

To investigate the potential of HBcAg as a generic epitope carrier, we have constructed a series of expression plasmids similar to those described earlier, such as pR1-11 (6), but in which the coding sequence for the C-terminal region of HBcAg has been replaced with sequences coding for different epitopes from HBV surface and presurface proteins and from the envelope protein of human immunodeficiency virus (HIV-1). These fusion proteins are produced in high yields in *E. coli*, assemble into core-like particles, and are good immunogens.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strain RB791 (7) was obtained from R. Brent (Harvard University). Strain RB791 is a derivative of *E. coli* K-12 W3110 (8), which harbors an *F′lac* plasmid having a *lacI*^{Q} gene for overproduction of the *lac* repressor. Plasmid pR1-11, which directs the synthesis of HBcAg, has been described (6). Plasmid pBR1-Sa, which consists of the *Hpa* II 570-base-pair (bp) restriction fragment containing the *trp* promoter–operator–attenuator region (9) cloned between the *Eco*RI and *Sal* I restriction sites of pBR322, was the gift of K. Bertrand (Stanford University). The plasmid pHBV130 contains coding regions of HBsAg and also has been described (10).

Cloning Reagents and Techniques. Restriction endonuclease digestions and ligations (11) and transformations (12) were carried out essentially as described. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, and DNA polymerase I (Klenow fragment) was purchased from Boehringer Mannheim. DNA sequences were determined using the chain-termination method (13). Oligonucleotides were synthesized by the solid-phase phosphotriester method (14) and were provided by E. Kawashima (Biogen, Geneva).

Plasmid Constructions. To take advantage of controlled expression from the powerful *tac* promoter (45) plasmids expressing fusion proteins were based upon ptacHpaII, which was constructed from elements of the plasmids pR1-11 (6) and pBR1-Sa. Digestion of pBR1-Sa with *Eco*RI and *Taq* I released a 305-bp DNA fragment carrying the −35 region of the *trp* promoter that was inserted in pBR322 between its *Eco*RI and *Cla* I sites to give plasmid ptrp10. A 509-bp fragment carrying part of the *lac* UV5 promoter (including the −10 region) and the coding sequence for the first 144 residues of HBcAg was removed from pBR1-11 by digestion with *Hpa* II and inserted at the *Cla* I site of ptrp10 (adjacent to the −35 region) to form plasmid ptacHpaII. DNA fragments carrying the coding sequences for parts of HBsAg, pre-S1, and pre-S2 (derived from pHBV 130; ref. 10) and synthetic sequences coding for parts of HIV envelope were inserted at the *Hin*dIII site of ptacHpaII for production of the fusion proteins shown in Fig. 1.

Purification of HBcAg Fusion Proteins. Cultures of *E. coli* strain RB791 harboring the various plasmids were grown to an OD_{600} of 1.0 in L-broth and induced by the addition of isopropyl β-D-thiogalactopyranoside to 0.5 mM. After 18 hr of shaking at 37°C, the bacteria were harvested and lysed by sonication, and the cellular debris was removed by centrifugation at 15,000 × *g* for 1 hr. The fusion protein particles were collected by centrifugation at 100,000 × *g* for 1 hr, resuspended in 0.1 M $NaHCO_3$, pH 7.0, and chromatographed on Sepharose 4B-CL in the same solvent. Fractions containing the fusion protein were pooled, and the product

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Abbreviations: HBV, hepatitis B virus; HIV, human immunodeficiency virus; HBcAg, hepatitis B core antigen; anti-HBc, antibody against HBcAg; HBeAg, hepatitis B e antigen; anti-HBe, antibody against HBeAg; HBsAg, hepatitis B surface antigen; anti-HBs, antibody against HBsAg; HRP, horseradish peroxidase.

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was collected by ultracentrifugation as before. The particles were converted into the dimeric subunit form by suspension in 5 M urea/0.1 M $NaHCO_3$, pH 9.6 (P. Wingfield and S.S., unpublished data). The material was then fractionated on Sephacryl S-200 in 0.1 M $NaHCO_3$, pH 9.6, fractions containing the fusion protein were pooled, and the pH was adjusted to 7.0 within 1.0 M HCl. At this pH the dimeric subunits reassemble into core-like particles and were collected by ultracentrifugation. This procedure involving gel-filtration chromatography with the desired product at two different molecular weights provides an effective method for purifying the fusion proteins, for low-molecular-weight impurities are removed with the first gel filtration and high-molecular-weight impurities are eliminated with the second filtration. Yields of the core fusion particles ranged from 2 to 30 mg per liter of shake-flask cultures.

Antigenic Specificity Assays. The antigenic specificity of the HBcAg fusion proteins was evaluated by using commercial diagnostic kits. HBcAg activity was determined with the Corzyme kit (Abbott), which is designed to detect the antibody against HBcAg (anti-HBc), but its components can be used for an HBcAg assay. Samples to be tested for HBcAg were mixed with anti-HBc conjugated to horseradish peroxidase (HRP). A polystyrene bead coated with HBcAg was added to the mixture and incubated for 2 hr at 42°C. The bead was then washed, and the amount of anti-HBc–HRP conjugate bound was determined by measuring A_{492} 30 min after addition of the chromogenic substrate (*o*-phenylenediamine) for HRP; HBcAg present in the sample inhibits binding of the anti-HBc–HRP conjugate to the bead. HBeAg activity was measured using the Sorin Biomedica HBeAg/antibody against HBeAg (anti-HBe) kit, and hepatitis B surface antigen (HBsAg) activity was assessed with the Auszyme II diagnostic kit (Abbott).

Inoculation of Rabbits with HBcAg Fusion Proteins. Three injections of the purified fusion proteins were given intramuscularly at 4-week intervals; the sample for the first injection (0.2 mg) was mixed with Freund's complete adjuvant, and subsequent ones (0.1 mg) were mixed with incomplete adjuvant. Serum samples were taken before the injections and 2 weeks after each injection.

Measurement of *in vivo* Antibody Production. Rabbit sera were assayed for anti-HBc with the Corzyme kit from Abbott Laboratories and for anti-HBe with the Sorin Biomedica HBeAg/anti-HBe kit. Rabbit sera were evaluated for antibodies to HBsAg in two different solid-phase ELISA systems. In one of the these systems, wells of microtiter plates were coated with HBsAG (12 μg per well) or synthetic peptides (4 μg per well) at pH 9.6; serum samples were incubated in the wells (2 hr at 40°C), and the bound antibody was measured by incubation with HRP-labeled swine anti-rabbit immunoglobulin (Dako) diluted 1:100 followed by addition of the chromogenic substrate for HRP. In the other system, the ability of antiserum on the solid phase to bind HBsAg from the liquid phase was evaluated by coating microtiter wells with 20% serum in 0.1 M $NaHCO_3$, pH 9.0, followed by incubation with HBsAg solution for 2 hr at 37°C; bound HBsAg was measured by incubation (2 hr) at 40°C with HRP-labeled monoclonal antibody against HBsAg (anti-HBs) (Abbott) and addition of the chromogenic substrate for HRP. Serum HBsAg (*adw*) was the gift of W. Gerlich (University of Göttingen) and HBsAg (*ayw*) produced in yeast was the gift of P. Wingfield (Biogen, Geneva). Peptides that included the pre-S2 or main (*a* determinant) epitope of HBsAg were the gift of D. Milich (Scripps Institute, La Jolla), and the peptide of the pre-S1 epitope was synthesized for us by R. Ramage (University of Edinburgh).

RESULTS

Plasmids That Express HBcAg Fusion Proteins. The plasmids expressing fusion proteins were all derived from ptacHpaII, which carries a *Hin*dIII target a little downstream of the *Hpa* II target at nucleotide 431 in HBV DNA (equivalent to amino acid 144 of HBcAg). The coding sequences inserted include those for the pre-S1 and pre-S2 epitopes of HBV, which are regions from the large (p39 and gp42) and middle (p31, gp33, and gp36) forms of presurface antigen (15–17), and the dominant epitope of HBsAg (the *a* determinant), which is believed to lie between amino acids 122 and 149 (18–23). In addition, we inserted three different synthetic coding sequences for fragments of the transmembrane region (gp41) of HIV-1 envelope protein, which are postulated to be antigenic epitopes (24). The predicted primary structures of these fusion proteins are presented in Fig. 1.

Purification and Physical Characterization of HBcAg Fusion Proteins. *E. coli* strain RB791 (W3110-I^q) harboring these plasmids was grown in shake flasks, and the *tac* promoter was induced with isopropyl β-D-thiogalactopyranoside. Polyacrylamide gel analysis of the pre- and postinduction cultures show that all the expected fusion proteins are produced in good yields in the form of core-like particles and are readily purified on the basis of this characteristic. Electron microscopic analysis of the HBc–pre-S1(1–20) fusion product shows that this peptide is, indeed, in the form of particles similar to those comprising full-length HBcAg, although the fusion particles are slightly larger (Fig. 2).

Antigenic and Immunogenic Properties of HBcAg Fusion Protein Particles. Antigenic reactivities of HBcAg and the fusion derivatives were analyzed by ELISA or radioimmune assays. HBcAg and HBeAg reactivities of full-length HBcAg, HBcAg lacking the C terminus (HBc144), and HBcAg with the C terminus replaced with amino acids from the pre-S1 region of HBV [HBc–pre-S1(1–20)] are presented in Fig. 3. All three proteins display similar HBcAg reactivity. Full-length HBcAg has very low HBeAg activity, whereas both HBc144 and HBc–pre-S1(1–20) have high HBeAg reactivity.

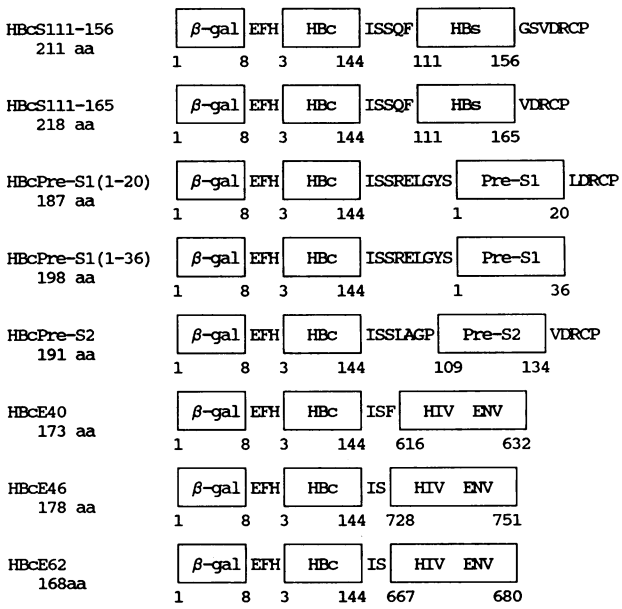

FIG. 1. Predicted amino acid sequences in the single-letter code of HBcAg fusion proteins. Names and total number of amino acids (aa) of the proteins are at left. Numbers below boxes indicate the positions of the amino acids in the original protein. HIV ENV, HIV envelope protein (gp160); β-gal, β-galactosidase.

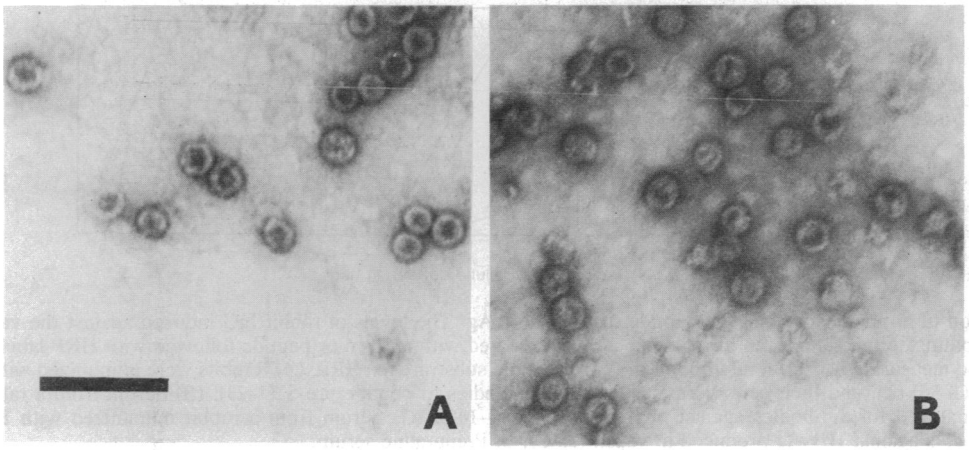

FIG. 2. Electron micrographs of HBcAg (*A*) and HBc–pre-S1(1–20) (*B*). (Bar = 100 nm.)

Neither of the fusion proteins containing amino acid sequences from the main epitope of HBsAg, HBc–S(111–156), and HBc–S(111–165) displayed significant HBsAg reactivity in the Abbott Auszyme assay. This result is not inconsistent with the conformation dependence of the principal antigenic activity of HBsAg.

Rabbits inoculated with the fusion proteins HBc–S(111–156), HBc–pre-S1(1–20), HBc–pre-S1(1–36), and HBc–pre-S2 mounted an immune response against the protein with which they were inoculated, with serum titers in the range of 1/30,000. Analysis of the specificity of the immune response showed that the sera contained anti-HBc and anti-HBe, as well as a high titer of antibodies to the peptide fused to the core particles. For example, Fig. 4 shows that serum from a rabbit inoculated with HBc–pre-S1(1–20) was strongly positive for both anti-HBc and anti-HBe. Antibodies against the fusion peptide were measured against the appropriate peptide on the solid phase as described. Fig. 5*A* shows the activity of sera from the rabbits inoculated with HBc–pre-S1(1–20) or HBc–pre-S1(1–36) against a peptide containing amino acids 1–23 of the HBsAg pre-S1 region, and Fig. 5*B* shows the corresponding activity of sera from the rabbits immunized with HBc–pre-S2 against a pre-S2 peptide composed of amino acids 120–145 of the presurface region.

The induction of anti-HBs by HBc–S(111–156) was evaluated with solid-phase assays by using HBsAg (subtype *ayw*) produced in yeast and serum HBsAg (subtype *adw*). Fig. 5*C* shows that the serum had a high anti-HBs titer when assayed against HBsAg (*ayw*), derived from yeast, and a similar titer was displayed against the serum (*adw*) antigen (data not shown). To show that these antibodies could react also with HBsAg in the soluble, native form, we set up a "sandwich" type immunoassay in which wells in microtiter plates were coated with anti-HBc-S(111–156) sera and then incubated with various concentrations of HBsAg (produced in yeast) in the liquid phase. Fig. 6 shows that the rabbit antiserum against HBc–S(111–156) can bind HBsAg particles, as does human antiserum to HBsAg produced in yeast.

Fusions of HBcAg with Epitopes of the HIV-1 Envelope Protein. Three different possible epitopes of the gp41 region of HIV-1 envelope protein (gp160) were examined in a further series of constructions expressing HBcAg fusions. All of these fusion proteins (see Fig. 1) were produced in good yield, formed HBcAg-like particles, and were readily purified. After these experiments were initiated, it was shown that antibodies raised against a peptide containing amino acids 735–752 of HIV-1 envelope protein recognized native gp160 (by immunoprecipitation, ref. 25) and neutralized infectivity of HIV-1 isolates (26, 27). BALB/c mice inoculated with HBcE46, which contains amino acids 728–751 of HIV-1 envelope protein, raised an immune response against both HBcAg and the HIV-1 peptide.

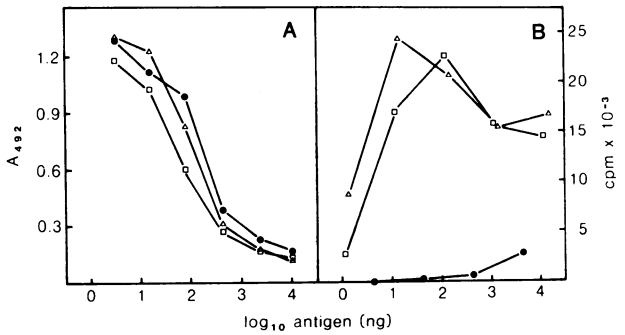

FIG. 3. Antigenic reactivities of HBcAg fusion proteins. (*A*) HBcAg reactivity was measured in a competition assay by mixing samples with anti-HBc conjugated to HRP and incubating this mixture with a polystyrene bead coated with HBcAg. The amount of anti-HBc–HRP conjugate bound to the bead was determined by measuring A_{492} after addition of the chromogenic substrate for HRP. (*B*) HBeAg reactivity was determined by monitoring the formation of a complex between anti-HBe-HRP conjugate and the antigen, which was then taken up by antibody on the solid phase and quantitated by measuring A_{492} after adding the chomogenic substrate for HRP. ●, HBcAg; △, HBc144; and □, HBc–pre-S1(1–20).

DISCUSSION

In this paper we describe several examples of the fusion of polypeptide sequences to the major segment of HBcAg,

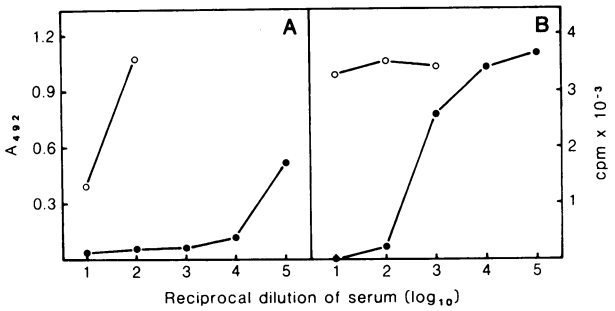

FIG. 4. Induction of anti-HBc (*A*) and anti-HBe (*B*) in rabbits inoculated with HBc–pre-S1(1–20). Inhibition by the test serum of the absorption of antibody–HRP conjugate by antigen on the solid phase was measured by determination of A_{492} after addition of the chromogenic substrate for HRP. ○, Preimmune sera; ●, sera after immunization.

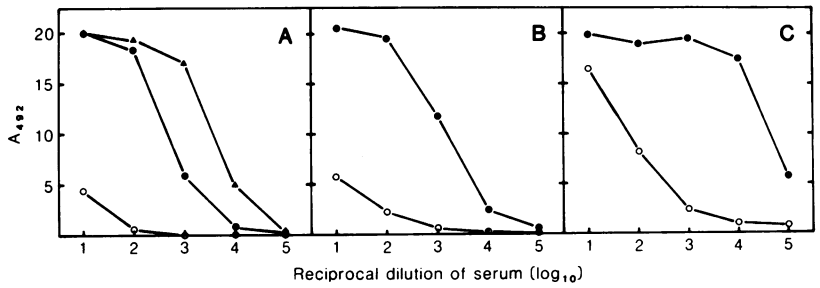

FIG. 5. Induction of antibodies against the peptide fused to HBcAg. The levels of rabbit IgG induced against the various peptides were monitored by incubating serum samples in microtiter plate wells coated with antigen or peptide followed with HRP-labeled swine anti-rabbit immunoglobulin and measuring A_{492} after addition of the chromogenic substrate for HRP. (*A*) Rabbits were immunized with HBc–pre-S1(1–20) (●) or HBc–pre-S1(1–36) (▲), and their sera were analyzed for antibodies to peptide pre-S1(1–23). (*B*) Serum from a rabbit immunized with HBc–pre-S2 (●), analyzed for antibodies against peptide pre-S2(120–145). (*C*) Serum from a rabbit immunized with HBc–S(111–156) (●), analyzed for antibodies against HBsAg produced in yeast. (A–C), ○, Preimmune serum.

resulting in the formation of particles that are morphologically very similar to HBcAg itself (Fig. 2) and, like HBcAg, are strongly immunogenic. Such derivatives of HBcAg, produced by expression of appropriate genetic constructions or possibly by chemical cross-linking of peptides to HBcAg particles, are of interest both as reagents for a wide range of immunological studies and as potential vaccines.

In all fusions described here the polypeptides derived from other viral coding sequences were attached at the C end of residue 144 of HBcAg via a small number (2–9) of amino acid residues resulting from translation of nucleotides linking the elements of the genetic constructions. The largest of them, HBc–S(111–165), has 65 amino acids attached at this position and has 218 residues in all compared with 183 of the normal mature HBcAg polypeptide; we do not know the limit to the size of polypeptide extension that can be accommodated in HBcAg-like particles, but one carrying an addition of 115 residues has been made. Because replacement of the first 2 amino acids of HBcAg with the first 8 residues of the β-galactosidase of *E. coli* plus a tripeptide linker sequence does not impair assembly of the core-like particles (6), attachment of additional antigenic sequences at the N-terminal instead of, or perhaps in addition to, the C-terminal region of HBcAg may be expected to exhibit somewhat similar physical and immunological properties. A product of this type has, in fact, been described (28) and contained a peptide sequence from the envelope of foot and mouth disease virus linked through a heptapeptide sequence into 6 residues of the HBV pre-core sequence followed by the full HBcAg sequence. The protein was unstable in *E. coli*,

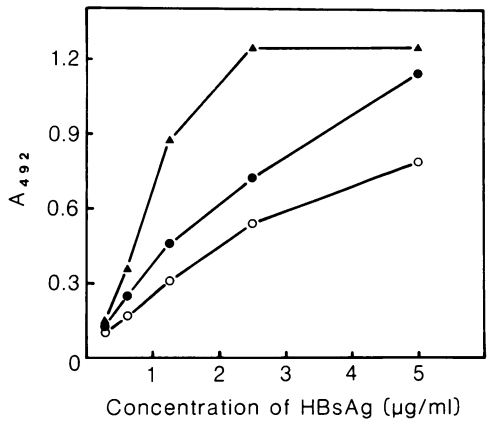

FIG. 6. Binding of liquid-phase HBsAg by solid-phase anti-HBc–S(111–156). The level of bound HBsAg was determined by incubation with anti-HBs–HRP conjugate and measuring A_{492} after addition of the chromogenic substrate for HRP. Microtiter wells were coated with preimmune serum (○), rabbit antiserum to HBc–S(111–156) (●), or human antiserum to yeast-produced HBsAg (△).

however, and the genetic construct was expressed in a vaccinia vector system to give a particulate product that was highly immunogenic.

Fusion of additional segments of natural gene products to HBcAg thus appears to offer a useful general approach to the location and mapping of both antigenic and immunogenic epitopes within a particular protein and may be of particular value with large protein structures. Other systems that hold similar potential are the particles formed by HBsAg, which although unstable in *E. coli* (29) are produced effectively in yeast (30), and the so-called Ty virus-like particles of yeast. The HBV pre-S sequences (31), segments of herpes simplex virus 1 glycoprotein D (32), and a malarial parasite antigen (33) have been fused to HBsAg and expressed in yeast as particulate products. HIV envelope segments have also been fused to HBsAg for expression in animal cells (34) and to Ty particles for expression in yeast (35). Fusions of these and other types that can assemble to form well-defined particulate structures are clearly candidates for development as vaccines. The HBcAg molecule is especially attractive because it is both a T-cell and a B-cell (T-cell independent) immunogen and can prime the T-cell response in a manner dependent upon the histocompatibility of the recipient animal (5). Immunization of chimpanzees with HBcAg and HBeAg has been shown to confer substantial protection against infection by the virus (36, 37).

That HBcAg is a potent stimulator of B lymphocytes is apparent from the early appearance of anti-HBc following infection with HBV (38) and from studies of the immunogenicity of HBcAg synthesized in *E. coli* (3, 6). The core antigen, and not HBsAg, is displayed, presumably as fragments associated with class I major histocompatibility complex antigen, upon the surface of hepatocytes in patients chronically infected with HBV, and it is this antigen to which T lymphocytes are sensitized (39), implying a role for HBcAg in cell-mediated immunity. Furthermore, HBeAg, which can be derived from HBcAg (4, 40), has been observed as a surface component of HBV particles (41) and is an important serological marker that frequently persists in chronic infections. More recently, Milich and his colleagues (42) have shown that HBcAg contains epitopes that stimulate helper T cells in mice and that animals primed with HBcAg or particular peptides within this sequence when challenged with HBV (which cannot replicate in the mouse) produced antibodies to HBsAg, including the pre-S epitopes, as well as HBcAg (5). This result may well explain the rapid appearance of high levels of anti-HBs and elevated levels of anti-HBe in chimpanzees that had been immunized with HBcAg or HBcAg and HBeAg and then challenged with HBV (36, 37, 43).

The fusions carrying the major antigen region of HBsAg were particularly interesting because although stable, particulate products were obtained they did not show the typical

antigenic response of HBsAg in a standard radioimmune or ELISA test. HBc-S(111–156) did, however, elicit an antibody response in a manner reminiscent of products resulting from the (very inefficient) direct expression of HBsAg sequences in *E. coli* (29), and the rabbit sera displayed high titers of antibodies against HBsAg either on the solid phase or in solution (Figs. 5C and 6).

The present study has been confined to observations of antibody response to the various fusion proteins, and all of them elicited high titers of antibodies against the additional epitopes, but examination of the more complex cellular immune reactions is required before the potential of fusions to HBcAg for vaccine development can be properly evaluated. In this context the fusions carrying HBV pre-S sequences are of interest because a synthetic peptide of 19 residues from the pre-S2 region of HBV has been shown to induce protection against the virus in the chimpanzee (44). Furthermore, a fusion carrying a foot-and-mouth disease virus epitope at the N-terminal region of HBcAg induced antibody titers in guinea pigs comparable with those elicited by inactivated virus particles (28). Collectively these observations suggest that HBcAg could serve as a carrier and adjuvant for many other antigens and thus provide a basis for multipurpose vaccines. By virtue of its capacity to prime helper T cells, HBcAg itself or as a fusion carrying pre-S epitopes may also be of value in those cases, including immunodeficient patients, who do not respond to vaccines based upon HBsAg. Similarly the fusion carrying the HIV envelope peptide could be a useful immunogen for eliciting a response against HIV in individuals with diminished T-cell levels.

We thank D. Milich for peptides from the pre-S2 and main epitope regions of HBsAg, W. Gerlich for serum HBsAg (*adw*) and monoclonal anti-HBs(pre-S1) and anti-HBs(pre-S2), R. Ramage for the peptide from the pre-S1 region of HBsAg, and P. Wingfield for HBsAg (*ayw*) produced in yeast. We are indebted to Elisabeth Allet for expert technical assistance, to D. Semon for DNA sequence analysis, and to P. Chisholm for the immunogenic evaluation of the HBcAg–HIV envelope fusions.

1. Burrell, C. J., MacKay, P., Greenaway, P. J., Hofschneider, P.-H. & Murray, K. (1979) *Nature (London)* **279,** 43–47.
2. Cohen, B. J. & Richmond, J. E. (1982) *Nature (London)* **296,** 677–678.
3. Pasek, M., Goto, T., Gilbert, W., Zink, B., Schaller, H., MacKay, P., Leadbetter, G. & Murray, K. (1979) *Nature (London)* **282,** 575–579.
4. Takahashi, K., Machida, A., Funatsu, G., Nomura, M., Usuda, A., Aoyagi, S., Tachibana, K., Miyamoto, H., Imai, M. & Nakamura, T. (1983) *J. Immunol.* **130,** 2903–2907.
5. Milich, D. R., McLachlan, A., Thornton, G. B. & Hughes, J. L. (1987) *Nature (London)* **329,** 547–549.
6. Stahl, S., MacKay, P., Magazin, M., Bruce, S. A. & Murray, K. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1606–1610.
7. Amman, E., Brosius, J. & Ptashne, M. (1983) *Gene* **25,** 167–178.
8. Bachmann, B. J. (1972) *Bacteriol. Rev.* **36,** 525–557.
9. Lee, F., Bertrand, K., Bennett, G. & Yanofsky, C. (1978) *J. Mol. Biol.* **121,** 193–217.
10. Gough, N. M. & Murray, K. (1982) *J. Mol. Biol.* **162,** 43–67.
11. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
12. Mandel, M. & Higa, A. (1970) *J. Mol. Biol.* **53,** 159–162.
13. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
14. Ito, H., Ike, Y., Ikuta, S. & Itakura, K. (1982) *Nucleic Acids Res.* **10,** 1755–1769.
15. Heermann, K. H., Goldmann, U., Schwartz, W., Seyffarth, T., Baumgarten, H. & Gerlich, W. H. (1984) *J. Virol.* **52,** 396–402.
16. Neurath, A. R., Kent, S. B. H. & Strick, N. (1984) *Science* **224,** 392–394.
17. Neurath, A. R., Kent, S. B. H., Strick, N., Taylor, P. & Stevens, C. E. (1985) *Nature (London)* **315,** 154–156.
18. Dreesman, G. R., Hollinger, R. B., MacCombs, R. M. & Melnick, J. L. (1973) *J. Gen. Virol.* **19,** 129–134.
19. Brown, S. E., Howard, C. R., Zuckerman, A. J. & Stewart, M. W. (1984) *J. Immunol. Methods* **72,** 41–48.
20. Hopp, T. P. & Woods, K. R. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3824–3828.
21. Bhatnager, P. K., Papas, E., Blum, H. E., Milich, D. R., Nitecki, D., Karels, M. J. & Vyas, G. N. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 4400–4404.
22. Prince, A. M., Ikram, H. & Hopp, T. P. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 579–582.
23. Antoni, B. A. & Petersen, D. L. (1988) in *Viral Hepatitis and Liver Disease*, ed. Zuckerman, A. J. (Liss, New York), pp. 313–317.
24. Ratner, L., Haseltine, W., Patarca, R., Livak, K. J., Starcich, B., Josephs, S. F., Doran, E. R., Rafalski, J. A., Whitehorn, E. A., Baumeister, K., Ivanoff, L., Petteway, S. R., Jr., Pearson, M. L., Lautenberger, J. A., Papas, T. S., Ghrayeb, J., Chang, N. T., Gallo, R. C. & Wong-Staal, F. (1985) *Nature (London)* **313,** 277–284.
25. Kennedy, R. C., Kenkel, R. D., Pauletti, D., Allan, J. S., Lee, T. H., Essex, M. & Dreesman, G. R. (1986) *Science* **231,** 1556–1559.
26. Chanh, T. C., Dreesman, G. R., Kanda, P., Linette, G. P., Sparrow, J. T., Ho, D. D. & Kennedy, R. C. (1986) *EMBO J.* **5,** 3065–3071.
27. Dalgleish, A. G., Chanh, T. C., Kennedy, R. C., Kanda, P., Clapham, P. R. & Weiss, R. A. (1988) *Virology* **165,** 209–215.
28. Clarke, B. E., Newton, S. E., Carroll, A. R., Francis, M. J., Appleyard, G., Syred, A. D., Highfield, P. E., Rowlands, D. J. & Brown, F. (1987) *Nature (London)* **330,** 381–383.
29. MacKay, P., Pasek, M., Magazin, M., Kovacic, R. T., Allet, B., Stahl, S., Gilbert, W., Schaller, H., Bruce, S. A. & Murray, K. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4510–4514.
30. Valenzuela, P., Medina, A., Rutter, W. J., Ammerer, G. & Hall, B. (1982) *Nature (London)* **298,** 347–350.
31. Valenzuela, P., Coit, D. & Kuo, C. H. (1985) *Bio/Technology* **3,** 317–320.
32. Valenzuela, P., Coit, D., Medina-Selby, M. A., Kuo, C. H., Van Nest, G., Burke, L., Bull, P., Urdea, M. S. & Graves, P. V. (1985) *Bio/Technology* **3,** 323–326.
33. Rutgers, T., Gordon, D., Gathoye, A. M., Hollingdale, M., Hockmeyer, W., Rosenberg, M. & De Wilde, M. (1988) *Biotechnology* **6,** 1065–1070.
34. Michel, M.-L., Mancini, M., Sobczak, E., Favier, V., Guetard, D., Bahraoui, E. M. & Tiollais, P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7957–7961.
35. Adams, S. E., Dawson, K. M., Gulf, K., Kingsman, S. M. & Kingsman, A. J. (1987) *Nature (London)* **329,** 68–70.
36. Murray, K., Bruce, S. A., Hinnen, A., Wingfield, P., van Erd, P. M. C. A., de Reus, A. & Schellekens, H. (1984) *EMBO J.* **3,** 645–650.
37. Murray, K., Bruce, S. A., Wingfield, P., van Eerd, P., de Reus, A. & Schellenkens, H. (1987) *J. Med. Virol.* **23,** 101–107.
38. Vyas, G. N., Cohen, S. N. & Schmid, R., eds. (1978) *Viral Hepatitis* (Franklin Inst., Press, Philadelphia)
39. Vento, S., Hegarty, J. E., Alberti, A., O'Brien, C. J., Alexander, G. J. M., Eddleston, A. L. W. F. & Williams, R. (1985) *Hepatology* **5,** 192–197.
40. MacKay, P., Lees, J. & Murray, K. (1981) *J. Med. Virol.* **8,** 237–241.
41. Ohori, H., Onodera, S. & Ishida, N. (1979) *J. Gen. Virol.* **43,** 423–427.
42. Milich, D. R., McLachlan, A. & Thornton, G. B. (1987) *J. Immunol.* **139,** 1223–1231.
43. Murray, K. (1988) *Vaccine* **6,** 164–174.
44. Itoh, Y., Takai, E., Ohnuma, H., Kitajima, K., Tsuda, F., Machida, A., Mishiro, S., Nakamura, T., Miyakawa, Y. & Mayumi, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9174–9178.
45. de Boer, H., Comstock, L. J. & Vasser, M. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 21–25.