

HEPATITIS B VIRUS VACCINES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application Serial No. 61/785,838, filed March 14, 2013. The disclosure of the prior application is considered part of (and is incorporated by reference in) the disclosure of this application.

BACKGROUND

1. Technical Field

This document relates to methods and materials for producing immune responses against hepatitis B viruses. For example, this document provides vaccines (e.g., nucleic acid vaccines, virus-like particle vaccines, and polypeptide vaccines) capable of being administered to mammals (e.g., humans) under conditions that induce production of immune responses against hepatitis B viruses.

2. Background Information

Hepatitis B virus (HBV) is a hepadnavirus that causes an inflammatory illness of the liver. About a third of the world population is infected at one point in their lives, and about 350 million people are chronic carriers. The virus can be transmitted by exposure to infectious blood or body fluids. The acute illness causes liver inflammation, vomiting, jaundice, and, in rare cases, death. Chronic hepatitis B infections may cause cirrhosis and liver cancer.

SUMMARY

This document provides methods and materials for producing immune responses against hepatitis B viruses. For example, this document provides vaccines (e.g., nucleic acid vaccines, virus-like particle vaccines, and polypeptide vaccines) capable of being administered to mammals (e.g., humans) under conditions that induce production of immune responses against hepatitis B viruses as well as methods for producing immune responses against hepatitis B viruses within a mammal (e.g., a human).

As described herein, polypeptides having the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or a sequence at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to such as sequence) can be produced and formulated into a vaccine

preparation having the ability to produce an immune response against hepatitis B viruses when administered to a mammal (e.g., a human). Such vaccine preparations can be administered to a mammal prior to the mammal being exposed to hepatitis B viruses. In such cases, the administered vaccine preparation can provide increased protection against hepatitis B virus infection. In some cases, such vaccine preparations can be administered to a mammal after the mammal is infected with hepatitis B virus (e.g., an acutely hepatitis B virus infected or chronically hepatitis B virus infected mammal). In such cases, administration of the vaccine preparation can be used to treat the hepatitis B virus infection. For example, administration of a vaccine preparation provided herein to a mammal infected with hepatitis B virus can result in a reduction in hepatitis B viral load, a reduction in the severity of the symptoms of the hepatitis B virus infection, a reduction in the degree of liver cirrhosis, a reduction in the incidence of hepatocellular cancer, or a clearance of the hepatitis B virus from the liver.

In some cases, a polypeptide provided herein (e.g., a polypeptide having the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58, or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58) can be formulated into virus-like particles that can be used as vaccine preparations having the ability to produce an immune response against hepatitis B viruses when administered to a mammal (e.g., a human). The vaccine preparations containing such virus-like particles can be administered to a mammal prior to the mammal being exposed to hepatitis B viruses. In such cases, the administered vaccine preparation containing virus-like particles can provide increased protection against hepatitis B virus infection. In some cases, a vaccine preparation containing a virus-like particle provided herein can be administered to a mammal after the mammal is infected with hepatitis B virus (e.g., an acutely hepatitis B virus infected or chronically hepatitis B virus infected mammal). In such cases, administration of the vaccine preparation containing a virus-like particle can be used to treat the hepatitis B virus infection. For example, administration of a vaccine preparation containing a virus-like particle provided herein to a mammal infected with hepatitis B virus can result in a reduction in hepatitis B viral load, a reduction in the severity of the symptoms of the hepatitis B virus infection, a reduction in the degree of liver cirrhosis, a reduction in the incidence of hepatocellular cancer, or a clearance of the hepatitis B virus from the liver.

As also described herein, nucleic acid molecules encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or a sequence at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to such as sequence) can be
5 obtained and formulated into a nucleic acid vaccine preparation having the ability to express the encoded polypeptide and produce an immune response against hepatitis B viruses when administered to a mammal (e.g., a human). Such nucleic acid vaccine preparations can be administered to a mammal prior to the mammal being exposed to hepatitis B viruses. In such cases, the administered nucleic acid vaccine preparation can provide increased protection
10 against hepatitis B virus infection. In some cases, such nucleic acid vaccine preparations can be administered to a mammal after the mammal is infected with hepatitis B virus (e.g., an acutely hepatitis B virus infected or chronically hepatitis B virus infected mammal). In such cases, administration of the nucleic acid vaccine preparation can be used to treat the hepatitis B virus infection. For example, administration of a nucleic acid vaccine preparation provided
15 herein to a mammal infected with hepatitis B virus can result in a reduction in hepatitis B viral load, a reduction in the severity of the symptoms of the hepatitis B virus infection, a reduction in the degree of liver cirrhosis, a reduction in the incidence of hepatocellular cancer, or a clearance of the hepatitis B virus from the liver.

Having the ability to use the vaccine preparations provided herein produce immune
20 responses against hepatitis B viruses can allow clinicians to provide their patients with increased protection against hepatitis B virus infections. In addition, the vaccine preparations provided herein can allow clinicians to treat patients previously infected with hepatitis B virus.

In general, one aspect of this document features a polypeptide comprising an amino
25 acid sequence that is at least 97 percent identical (e.g., at least 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:10.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:12.

In another aspect, this document features a polypeptide comprising an amino acid
30 sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:14.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 95 percent identical (e.g., at least 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:16.

5 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 97 percent identical (e.g., at least 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:18.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:20.

10 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 97 percent identical (e.g., at least 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:22.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 89 percent identical (e.g., at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 15 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:24.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 89 percent identical (e.g., at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:26.

In another aspect, this document features a polypeptide comprising an amino acid 20 sequence that is at least 95 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:28.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 94 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:30.

25 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:32.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) 30 to the amino acid sequence set forth in SEQ ID NO:34.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:36.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:38.

5 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 97 percent identical (e.g., at least 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:40.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:42.

10 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:44.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 95 percent identical (e.g., at least 96, 97, 98, 99, or 100 percent
15 identical) to the amino acid sequence set forth in SEQ ID NO:46.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 96 percent identical (e.g., at least 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:48.

In another aspect, this document features a polypeptide comprising an amino acid
20 sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:50.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 93 percent identical (e.g., at least 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:52.

25 In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 92 percent identical (e.g., at least 93, 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:54.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 86 percent identical (e.g., at least 87, 88, 89, 90, 91, 92, 93, 94, 95,
30 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:56.

In another aspect, this document features a polypeptide comprising an amino acid sequence that is at least 89 percent identical (e.g., at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical) to the amino acid sequence set forth in SEQ ID NO:58.

Any one of the polypeptides described in the above 25 paragraphs can comprise the ability to induce an immune response against a hepatitis B virus when the polypeptide is administered to a mammal. In some cases, any one of the polypeptides described in the above 25 paragraphs can be a fusion polypeptide comprising a second amino acid sequence.

5 The second amino acid sequence can encode a tag selected from the group consisting of GST, FLAG, GFP, and c-myc. The second amino acid sequence can encode a cytokine selected from the group consisting of GM-CSF, IL-2, and IL-12. The polypeptide can be substantially pure. The polypeptide can be an isolated polypeptide.

10 In another aspect, this document features a virus-like particle comprising one or more polypeptides selected from the group consisting of the polypeptides described in the above said 25 paragraphs. The virus-like particle can comprise one polypeptide selected from the group. The virus-like particle can comprise two, three, four, or five different polypeptides selected from the group. The virus-like particle can comprise the ability to induce an immune response against a hepatitis B virus when the particle is administered to a mammal.

15 In another aspect, this document features a vaccine preparation comprising one or more polypeptides selected from the group consisting of the polypeptides described in the above said 25 paragraphs. The vaccine preparation can comprise one polypeptide selected from the group. The vaccine preparation can comprise two, three, four, or five different polypeptides selected from the group. The vaccine preparation can comprise the ability to induce an immune response against a hepatitis B virus when the vaccine preparation is administered to a mammal. The vaccine preparation can comprise an adjuvant. The adjuvant can be selected from the group consisting of aluminum-based compounds, Montanide ISA 51, Montanide ISA 720, and CpG oligodeoxynucleotides. The adjuvant can comprise alum or Al_2O_3 .

25 In another aspect, this document features a nucleic acid molecule comprising a nucleic acid sequence encoding a polypeptide selected from the group consisting of the polypeptides described in the above said 25 paragraphs. The nucleic acid molecule can comprise a promoter sequence operably linked to the nucleic acid sequence. The nucleic acid molecule can be a vector. The vector can be a plasmid. The vector can be a viral vector. The viral vector can be selected from the group consisting of adenoviral vectors, adeno-associated virus vectors, and vaccinia viral vectors.

In another aspect, this document features a vaccine preparation comprising one or more nucleic acid molecules. The nucleic acid molecule comprises a nucleic acid sequence encoding a polypeptide selected from the group consisting of the polypeptides described in

the above said 25 paragraphs. The nucleic acid molecule can comprise a promoter sequence operably linked to the nucleic acid sequence. The nucleic acid molecule can be a vector. The vector can be a plasmid. The vector can be a viral vector. The viral vector can be selected from the group consisting of adenoviral vectors, adeno-associated virus vectors, and vaccinia viral vectors. The vaccine preparation can comprise a nucleic acid molecule comprising a nucleic acid sequence encoding one polypeptide selected from the group. The vaccine preparation can comprise one or more nucleic acid molecules comprising a nucleic acid sequence encoding two, three, four, or five different polypeptides selected from the group. The vaccine preparation can comprise the ability to induce an immune response against a hepatitis B virus when the vaccine preparation is administered to a mammal and the polypeptide is expressed within the mammal. The vaccine preparation can comprise an adjuvant. The adjuvant can be selected from the group consisting of aluminum-based compounds, Montanide ISA 51, Montanide ISA 720, and CpG oligodeoxynucleotides. The adjuvant can comprise alum or Al_2O_3 .

In another aspect, this document features a method for inducing an immune response against a hepatitis B virus within a mammal. The method comprises administering a vaccine preparation to the mammal. The vaccine preparation comprises (a) one or more polypeptides selected from the group consisting of the polypeptides described in the above said 25 paragraphs, (b) one or more virus-like particles comprising one or more polypeptides selected from the group consisting of the polypeptides described in the above said 25 paragraphs, or (c) one or more nucleic acid molecules comprising a nucleic acid sequence encoding a polypeptide selected from the group consisting of the polypeptides described in the above said 25 paragraphs. The mammal can be a human. The mammal can be a human not previously infected with a hepatitis B virus. The mammal can be a human acutely infected with a hepatitis B virus. The mammal can be a human chronically infected with a hepatitis B virus. The immune response can comprise the production of anti-hepatitis B virus antibodies. The method can comprise administering a second vaccine preparation to the mammal after the step of administering the vaccine preparation to the mammal, wherein the second vaccine preparation comprises an hepatitis B virus antigen. The method can comprise administering a second vaccine preparation to the mammal after the step of administering the vaccine preparation to the mammal, wherein the second vaccine preparation comprises (a) one or more polypeptides selected from the group consisting of the polypeptides described in the above said 25 paragraphs, (b) one or more virus-like particles comprising one or more polypeptides selected from the group consisting of the polypeptides described in the above

said 25 paragraphs, or (c) one or more nucleic acid molecules comprising a nucleic acid sequence encoding a polypeptide selected from the group consisting of the polypeptides described in the above said 25 paragraphs.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic of the strategy used in designing the strain-specific oligonucleotide primers used to keep the 'a' epitope loop constrained during the DNA shuffling reaction. Thirty nucleotides of the HBV (strain ayw) 'a' loop sequence were held constant and flanked by fifteen nucleotides on each side derived from the related HBs genes of chimpanzee/gibbon, woodchuck, and woolly monkey. A restriction endonuclease BsrB I site sequence (CCGCTC) was introduced in the sequence encoding the HBV "a" loop.

Figure 2 shows the positive (+) strain oligonucleotide sequences used to hold the HBV "a" loop sequence constant during DNA shuffling. A restriction endonuclease BsrB I site sequence (CCGCTC) was introduced, and a BspM I site sequence (ACCTGCN4) was knocked out by the two conservative changes shown in lower case (t and c). These altered restriction sites were engineered into the oligonucleotides to enable a rapid evaluation of oligo incorporation by restriction analysis. The conserved 30 nucleotides from the AYW 'a' epitope loop are shown above the dashed line and underlined in the sequences below the dashed line. All primers are positive sense as indicated by (+). Primers shown: chibbon (+) primer (chimpanzee/gibbon); WM (+) primer (woolly monkey); WD (+) primer (woodchuck).

Figure 3 shows representative ELISA data from primary screening of a first shuffled library are shown. Anti-Hepatitis B antibody levels present in the sera of mice injected with

clones from the first shuffled library were measured by ELISA and expressed as milli-
International Units per mL. A reference level typically used is the highest antibody level
induced in mice by a positive-control DNA vaccine (measured as mIU/mL using a
commercial anti-HBsAg detection kit). The striped bar indicates antibody levels induced by
5 a wild-type clone. The black bars indicate antibody levels above reference value induced by
shuffled clones.

Figure 4 contains a listing of the nucleic acid sequence and amino acid sequence for
one of the human parental clone used in the initial shuffling.

Figure 5 contains a listing of the nucleic acid sequence and amino acid sequence for
10 one of the woolly monkey parental clone used in the initial shuffling.

Figure 6 contains a listing of the nucleic acid sequence and amino acid sequence for
one of the human/woodchuck parental clone used in the initial shuffling.

Figure 7 contains a listing of the nucleic acid sequence and amino acid sequence for
one of the composite chimpanzee-gibbon parental clone used in the initial shuffling.

Figure 8 contains a listing of the nucleic acid sequence and amino acid sequence for
15 clone 6101 obtained from the initial shuffling.

Figure 9 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6102 obtained from the initial shuffling.

Figure 10 contains a listing of the nucleic acid sequence and amino acid sequence for
20 clone 6103 obtained from the initial shuffling.

Figure 11 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6104 obtained from the initial shuffling.

Figure 12 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6105 obtained from the initial shuffling.

Figure 13 contains a listing of the nucleic acid sequence and amino acid sequence for
25 clone 6106 obtained from the initial shuffling.

Figure 14 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6107 obtained from the initial shuffling.

Figure 15 contains a listing of the nucleic acid sequence and amino acid sequence for
30 clone 6108 obtained from the initial shuffling.

Figure 16 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6109 obtained from the initial shuffling.

Figure 17 contains a listing of the nucleic acid sequence and amino acid sequence for
clone 6111 obtained from the initial shuffling.

Figure 18 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6201 obtained from a second shuffling.

Figure 19 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6202 obtained from a second shuffling.

5 Figure 20 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6203 obtained from a second shuffling.

Figure 21 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6204 obtained from a second shuffling.

10 Figure 22 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6205 obtained from a second shuffling.

Figure 23 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6206 obtained from a second shuffling.

Figure 24 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6207 obtained from a second shuffling.

15 Figure 25 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6208 obtained from a second shuffling.

Figure 26 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6209 obtained from a second shuffling.

20 Figure 27 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6210 obtained from a second shuffling.

Figure 28 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6211 obtained from a second shuffling.

Figure 29 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6212 obtained from a second shuffling.

25 Figure 30 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6213 obtained from a second shuffling.

Figure 31 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6214 obtained from a second shuffling.

30 Figure 32 contains a listing of the nucleic acid sequence and amino acid sequence for clone 6215 obtained from a second shuffling.

Figure 33 is a nucleic acid sequence alignment of the four parental clones encoding hepatitis surface antigen proteins obtained from HBV, woolly monkey hepatitis virus (WMHV), human/woodchuck hepatitis virus (HWHV, and chimpanzee-gibbon hepatitis virus (CGHV), and the indicated clones.

Figure 34 is an amino acid sequence alignment of the hepatitis surface antigen proteins encoded by the four parental clones from HBV, woolly monkey hepatitis virus (WMHV), human/woodchuck hepatitis virus (HWHV), and chimpanzee-gibbon hepatitis virus (CGHV), and the indicated clones.

5 Figure 35 contains a wild-type hepatitis B virus sequence with a summary of the differences observed in clones 6101-6109, 6111, and 6201-6215.

Figure 36 contains a diagram of a vector used to express HBsAg clones.

Figure 37. Geometric Mean Titers (GMT) of anti-HBsAg obtained with first round clones. Geometric Mean Titers $\pm$ SEM of anti-HBsAg were measured by ELISA in sera
10 obtained by immunizing test groups of mice each with one of ten improved clones selected as inducing the highest expression levels in the initial screening procedure. The clone names are shown on the X axis. HBV is a plasmid used as a control that wild-type hepatitis B envelope gene-containing plasmid used as a control.

Figure 38. Geometric Mean Titers of anti-HBsAg obtained with second round clones.
15 A second round library was obtained by shuffling the ten improved clones selected from the first round library. One of ten second round library clones selected based on their induction of anti-HBsAg levels, or the wild-type human hepatitis B envelope gene (HBV), was used to immunize each group of mice. Geometric Mean Titers of anti-HBsAg in the sera from the immunized mice were measured by ELISA. The clone names are shown on the X axis; each
20 bar represents one group of mice. The Y-axis shows the GMT $\pm$ SEM of anti-HBsAg for each group in International units.

Figure 39 is a bar graph plotting the GMT (mIU/mL) for the indicated clones when administered to 30 six-week old C57BL/6 mice.

Figure 40 is a bar graph plotting the GMT (mIU/mL) for the indicated clones when
25 administered to mice alone (DNA prime; gray) or when administered to mice prior to the mice receiving is a boost with wild-type protein (Protein Boost; black). The bars labeled "Engerix" are from groups of mice that received one protein injection (gray bar) or the initial priming protein injection followed by the boosting injection (black bar).

Figure 41 is a bar graph plotting the GMT (mIU/mL) for the indicated clones when
30 administered to outbred mice.

Figure 42 is a bar graph plotting the GMT (mIU/mL) for the indicated clones when administered to non-responder mice.

Figure 43 is a dot-plot analysis of the CD8⁺ T-cell immune responses to stimulation with genotype D HBsAg peptides in naive mice and mice vaccinated with a vaccine

preparation (Vaccine ID No. 9106). The Y-axis of the dot plot shows the percentage of CD8⁺ T cells staining positively for interferon-gamma.

Figure 44 is a dot-plot analysis of the CD8⁺ T-cell immune responses to stimulation with genotype A HBsAg peptides in naive mice and mice vaccinated with a vaccine preparation (Vaccine ID No. 9106). The Y-axis of the dot plot shows the percentage of CD8⁺ T cells staining positively for interferon-gamma.

Figure 45 is a dot-plot analysis of the CD8⁺ T-cell immune responses to stimulation with genotype D HBsAg peptides in naive mice and mice vaccinated with a vaccine preparation (Vaccine ID No. 9106) via two DNA injections along with electroporation.

Figure 46 is a dot-plot analysis of the CD8⁺ T-cell immune responses to stimulation with genotype A HBsAg peptides in naive mice and mice vaccinated with a vaccine preparation (Vaccine ID No. 9106) via two DNA injections along with electroporation.

Figure 47 is a dot-plot analysis of the CD4⁺ T-cell immune responses to stimulation with genotype D HBsAg peptides in naive mice and mice vaccinated with a vaccine preparation (Vaccine ID No. 9106) via two DNA injections along with electroporation.

Figure 48 is a dot-plot analysis of the CD4⁺ T-cell immune responses to stimulation with genotype A HBsAg peptides in naive mice and mice vaccinated with a vaccine preparation (Vaccine ID No. 9106) via two DNA injections along with electroporation.

DETAILED DESCRIPTION

This document provides methods and materials for producing immune responses against hepatitis B viruses. For example, this document provides polypeptides, nucleic acid molecules encoding such polypeptides, virus-like particles containing such polypeptides, vaccine preparations containing one or more polypeptides provided herein, vaccine preparations containing one or more nucleic acid molecules provided herein, vaccine preparations containing one or more virus-like particles provided herein, and methods for inducing immune responses against hepatitis B viruses within mammals (e.g., humans).

As described herein, a polypeptide provided herein can be designed to include the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). Such polypeptides can be used to induce an immune response against hepatitis B viruses within a mammal. In some cases, a polypeptide

provided herein can have the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 and can have the identity to SEQ ID NO:2, 4, 6, and 8 as indicated in Table 1.

5 Table 1. Percentages of amino acid residues of the indicated clones that are identical to those of the indicated parental clones.

Clone No.	SEQ ID NO: ^a	SEQ ID NO:2 ^b	SEQ ID NO:4 ^c	SEQ ID NO:6 ^d	SEQ ID NO:8 ^e
6101	10	87.6	96.9	59.7	78.5
6102	12	95.6	88.1	60.6	82.9
6103	14	95.6	88.1	62.8	80.3
6104	16	95.1	92.0	60.2	78.5
6105	18	96.5	88.1	60.6	82.9
6106	20	92.9	90.7	62.8	78.5
6107	22	96.9	87.6	61.5	80.3
6108	24	88.1	84.1	65.6	79.4
6109	26	88.1	84.1	65.6	79.4
6111	28	94.7	92.5	61.9	77.6
6201	30	93.4	91.2	60.2	79.4
6202	32	95.6	87.2	61.1	83.3
6203	34	95.1	92.0	60.2	78.5
6204	36	95.1	92.0	60.2	78.5
6205	38	92.5	91.2	60.6	79.4
6206	40	96.0	89.4	61.5	82.0
6207	42	92.5	90.3	62.4	78.1
6208	44	92.9	86.3	62.8	81.6
6209	46	94.2	87.6	61.1	84.2
6210	48	95.1	92.5	61.1	78.5
6211	50	92.5	91.2	60.6	79.4
6212	52	92.0	91.6	60.2	78.9
6213	54	91.6	91.2	61.1	82.9
6214	56	84.5	85.0	65.6	72.4
6215	58	88.9	87.6	67.0	77.6

a: provided SEQ ID NO: is for the amino acid sequence of the indicated clone number.

- b: SEQ ID NO:2 is the amino acid sequence for the human parental clone.
- c: SEQ ID NO:4 is the amino acid sequence for the woolly monkey parental clone.
- d: SEQ ID NO:6 is the amino acid sequence for the human/woodchuck parental clone.
- e: SEQ ID NO:8 is the amino acid sequence for the chimpanzee/gibbon parental clone.

5

In some cases, a polypeptide provided herein can be an isolated polypeptide. The term “isolated” as used herein with reference to polypeptides refers to (a) a polypeptide that is not associated with proteins that such polypeptide is normally associated with in nature or (b) a polypeptide that does not occur or exist in nature.

10

In some cases, a polypeptide provided herein can be a substantially pure polypeptide. The term “substantially pure” as used herein with reference to polypeptides refers to a polypeptide preparation that is substantially free of other polypeptides, lipids, carbohydrates, and nucleic acid. In some cases, a substantially pure polypeptide can be a polypeptide that is at least 60 percent pure or is a chemically synthesized polypeptide. A substantially pure polypeptide can be at least about 60, 65, 70, 75, 80, 85, 90, 95, or 99 percent pure. Typically, a substantially pure polypeptide will yield a single major band on a non-reducing polyacrylamide gel.

15

In some cases, a polypeptide provided herein can include the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 except that the amino acid sequence includes a variation (e.g., a substitution, addition, or deletion) at one or more positions (e.g., one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, 13, 14, 15, 16, 17, 18, 19, 20, or more positions). Such variant sequences, e.g., those having one or more amino acid substitutions relative to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58, can be prepared and modified as described herein. For example, a polypeptide provided herein can include the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 except that the amino acid sequence includes a conservative or non-conservative amino acid substitution at one or more positions (e.g., one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, 13, 14, 15, 16, 17, 18, 19, 20, or more positions). In some cases, an amino acid substitution can be made by selecting substitutions that do not differ significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, (b) the charge or hydrophobicity of the molecule at a particular site, or (c) the bulk of the side chain. For example, naturally occurring residues can be divided into groups based on side-chain properties: (1) hydrophobic

20

25

30

35

amino acids (methionine, alanine, valine, leucine, and isoleucine); (2) neutral hydrophilic amino acids (cysteine, serine, and threonine); (3) acidic amino acids (aspartic acid and glutamic acid); (4) basic amino acids (asparagine, glutamine, histidine, lysine, and arginine); (5) amino acids that influence chain orientation (glycine and proline); and (6) aromatic amino acids (tryptophan, tyrosine, and phenylalanine). Substitutions made within these groups can be considered conservative substitutions. Non-limiting examples of substitutions include, without limitation, substitution of valine for alanine, lysine for arginine, glutamine for asparagine, glutamic acid for aspartic acid, serine for cysteine, asparagine for glutamine, aspartic acid for glutamic acid, proline for glycine, arginine for histidine, leucine for isoleucine, isoleucine for leucine, arginine for lysine, leucine for methionine, leucine for phenylalanine, glycine for proline, threonine for serine, serine for threonine, tyrosine for tryptophan, phenylalanine for tyrosine, and/or leucine for valine.

Further examples of conservative substitutions that can be made at any position within the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 include, without limitation, those set forth in Table 2.

Table 2. Examples of conservative amino acid substitutions.

Original Residue	Exemplary substitutions
Ala	Val, Leu, Ile
Arg	Lys, Gln, Asn
Asn	Gln, His, Lys, Arg
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln, Lys, Arg
Ile	Leu, Val, Met, Ala, Phe
Leu	Ile, Val, Met, Ala, Phe
Lys	Arg, Gln, Asn
Met	Leu, Phe, Ile
Phe	Leu, Val, Ile, Ala
Pro	Gly
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe, Thr, Ser
Val	Ile, Leu, Met, Phe, Ala

In some case, an amino acid sequence used to make a polypeptide provided herein can include one or more non-conservative substitutions. Non-conservative substitutions typically entail exchanging a member of one of the classes described above for a member of another class.

5 In some cases, a polypeptide provided herein can have an amino acid sequence with at least 90% (e.g., at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100%) sequence identity to a reference sequence (e.g., SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). Percent sequence identity is calculated by determining the number of matched positions in aligned amino acid sequences
10 (target amino acid sequence aligned to an identified amino acid sequence), dividing the number of matched positions by the number of amino acids of the identified amino acid sequence (e.g., SEQ ID NO:10), and multiplying by 100. A matched position refers to a position in which identical amino acids occur at the same position in aligned amino acid sequences. Percent sequence identity also can be determined for nucleic acid sequences.

15 Percent sequence identity is determined by comparing a target amino acid sequence to the identified amino acid sequence (e.g., SEQ ID NO:10) using the ClustalW alignment tool provided in the Geneious software platform (version 6.05, Biomatters Ltd, Auckland, New Zealand).

Any appropriate method can be used to obtain a polypeptide provided herein. For
20 example, common polypeptide purification techniques such as affinity chromatography and HPLC as well as polypeptide synthesis techniques can be used. In addition, any appropriate material can be used as a source to obtain a polypeptide provided herein. For example, cultured cells engineered to over-express a particular polypeptide provided herein (e.g., a cell line designed to include a nucleic acid molecule provided herein) can be used to produce a
25 polypeptide provided herein. Such cells can be prokaryotic cells (e.g., bacterial cells such as *E. coli*, *Bacillus subtilis*, or *Pseudomonas* cells) or eukaryotic cells (e.g., yeast cells such as *Saccharomyces cerevisiae*, *Hansenula polymorpha* or *Pichia pastoris* cells, insect cells such as *Drosophila melanogaster* (e.g., Schneider 2 or Schneider 3 cells), *Spodoptera frugiperda*, or *Trichoplusia ni* cells, or mammalian cells such as CHO, HEK 293, MRC, or PER-C6
30 cells). In some cases, a polypeptide provided herein can be designed to contain an amino acid sequence that allows the polypeptide to be captured onto an affinity matrix. For example, a tag such as c-myc, hemagglutinin, polyhistidine, Flag™ tag (Kodak), Strep-Tag, V5, or VSV-G can be used to aid polypeptide purification. Such tags can be inserted anywhere along a polypeptide including at either the carboxyl or amino termini.

In some cases, a polypeptide provided herein can be a fusion polypeptide. Such a fusion polypeptide can include one or more additional amino acid sequences in addition to the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). The additional amino acid sequences can be the amino acid sequence of a tag as described above, a marker such as GFP or Luciferase, an enzyme such as alkaline phosphatase or GST, or a cytokine such as GM-CSF, IL-2, or IL-12, or a chemokine such as IP-10, MCP-3, or RANTES.

This document also provides nucleic acid molecules that encode a polypeptide provided herein. The term “nucleic acid” as used herein encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid can be double-stranded or single-stranded. Where single-stranded, the nucleic acid can be the sense strand or the antisense strand. In addition, nucleic acid can be circular or linear.

The term “isolated” as used herein with reference to nucleic acid refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally-occurring genome of the organism or virus from which it is derived. For example, an isolated nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, an isolated nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

The term “isolated” as used herein with reference to nucleic acid also includes any non-naturally-occurring nucleic acid since non-naturally-occurring nucleic acid sequences are not found in nature and do not have immediately contiguous sequences in a naturally occurring genome. For example, non-naturally-occurring nucleic acid such as an engineered

nucleic acid is considered to be isolated nucleic acid. Engineered nucleic acid can be made using common molecular cloning or chemical nucleic acid synthesis techniques. Isolated non-naturally-occurring nucleic acid can be independent of other sequences, or incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or the genomic DNA of a prokaryote or eukaryote. In addition, a non-naturally-occurring nucleic acid can include a nucleic acid molecule that is part of a hybrid or fusion nucleic acid sequence.

It will be apparent to those of skill in the art that a nucleic acid existing among hundreds to millions of other nucleic acid molecules within, for example, cDNA or genomic libraries, or gel slices containing a genomic DNA restriction digest is not to be considered an isolated nucleic acid.

A nucleic acid molecule provided herein (e.g., an isolated nucleic acid molecule) can encode any of the polypeptides provided herein. For example, a nucleic acid molecule provided herein can encode a polypeptide having the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). Examples of such nucleic acid molecules include, without limitation, nucleic acid molecules that have the nucleic acid sequence set forth in SEQ ID NO:9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, or 57. In some cases, nucleic acid molecule provided herein can be codon-optimized to express the encoded polypeptide in cells of a particular species (e.g., codon-optimized for expression in bacterial cells, yeast cells, insect cells, fungal cells, algal cells, mammalian cells, or human cells). For example, a nucleic acid molecule provided herein encoding a polypeptide having the amino acid sequence set forth in SEQ ID NO:10 can include a codon-optimized version of the nucleic acid sequence set for in SEQ ID NO:9.

In some cases, a nucleic acid molecule provided herein can be a vector. A vector can be is a replicon, such as a plasmid, phage, virus, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. An “expression vector” is a vector that includes one or more expression control sequences. An “expression control sequence” is a sequence (e.g., a DNA sequence) that controls or regulates the transcription and/or translation of another sequence (e.g., another DNA sequence).

In expression vectors, a nucleic acid molecule provided herein (e.g., a nucleic acid encoding a polypeptide provided herein) can be operably linked to one or more expression control sequences. As used herein, “operably linked” means incorporated into a genetic construct so that expression control sequences effectively control expression of a coding sequence of interest. Examples of expression control sequences include promoters, enhancers, and transcription terminating regions. A promoter is an expression control sequence composed of a region of a DNA molecule, typically within 100 to 500 nucleotides upstream of the point at which transcription starts (generally near the initiation site for RNA polymerase II). To bring a coding sequence under the control of a promoter, the translation initiation site of the translational reading frame of the polypeptide can be positioned between one and about fifty nucleotides downstream of the promoter. Enhancers provide expression specificity in terms of time, location, and level. Unlike promoters, enhancers can function when located at various distances from the transcription site. An enhancer also can be located downstream from the transcription initiation site. A coding sequence is “operably linked” and “under the control” of expression control sequences in a cell when RNA polymerase is able to transcribe the coding sequence into mRNA, which then can be translated into the polypeptide encoded by the coding sequence.

Suitable expression vectors include, without limitation, plasmids and viral vectors derived from, for example, bacteriophage, baculoviruses, tobacco mosaic virus, herpes viruses, cytomegalovirus, retroviruses, vaccinia viruses, adenoviruses, and adeno-associated viruses. Numerous vectors and expression systems are commercially available from such corporations as Invitrogen/Life Technologies (Carlsbad, CA).

An expression vector can include a tag sequence designed to facilitate subsequent manipulation of the expressed nucleic acid sequence (e.g., purification or localization). Tag sequences, such as green fluorescent protein (GFP), glutathione S-transferase (GST), polyhistidine, c-myc, hemagglutinin, or Flag™ tag (Kodak, New Haven, CT) sequences typically are expressed as a fusion with the encoded polypeptide. Such tags can be inserted anywhere within the polypeptide including at either the carboxyl or amino terminus.

A nucleic acid molecule provided herein can be obtained using any appropriate method including, without limitation, common molecular cloning and chemical nucleic acid synthesis techniques. For example, PCR can be used to obtain a nucleic acid containing a nucleic acid sequence sharing similarity to a nucleic acid molecule provided herein, and common mutagenesis techniques can be used to introduce desired nucleotide additions, deletions, substitutions, or combinations thereof into the obtained nucleic acid. PCR refers to

a procedure or technique in which target nucleic acid is amplified in a manner similar to that described in U.S. Patent No. 4,683,195, and subsequent modifications of the procedure described therein. Generally, sequence information from the ends of the region of interest or beyond are used to design oligonucleotide primers that are identical or similar in sequence to opposite strands of a potential template to be amplified. Using PCR, a nucleic acid sequence can be amplified from RNA or DNA. For example, a nucleic acid sequence can be isolated by PCR amplification from total cellular RNA, total genomic DNA, and cDNA as well as from bacteriophage sequences, plasmid sequences, viral sequences, and the like. When using RNA as a source of template, reverse transcriptase can be used to synthesize complimentary DNA strands.

This document also provides virus-like particles that include one or more of the polypeptides provided herein. For example, a virus-like particle provided herein can be designed to include a polypeptide having the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). In some cases, a single virus-like particle can include more than one different polypeptide provided herein. For example, a single virus-like particle provided herein can include both a polypeptide having the amino acid sequence as set forth in SEQ ID NO:16 and a polypeptide having the amino acid sequence as set forth in SEQ ID NO:24. Other combinations of polypeptides that can be used to make virus-like particles having a collection of different polypeptides include, without limitation, those set forth in Table 3.

Table 3. Combinations of polypeptides for virus-like particles (VLPs).

VLP ID No.	Sequence identifiers of polypeptides to be included in the VLP
7101	14, 16, 18
7102	22, 24, 28
7103	14, 16, 18, 22, 24
7104	36, 38
7105	32, 52, 58
7106	32, 36, 38, 52, 58
7107	14, 16, 36, 38

7108	22, 24, 28, 32, 52, 58
7109	16, 18, 24, 32, 36, 38, 52, 58

Any appropriate method can be used to make virus-like particles provided herein. For example, a virus-like particle provided herein can be made using a method described elsewhere. See, e.g., U.S. Patent No. 4,803,164, which describes a method to produce HBs-containing VLPs in yeast cells; U.S. Patent No. 6,551,820, which describes a method to produce HBs-containing VLPs in transgenic plants; and European Patent Application No. EP0241021 A2, which describes a method to produce HBs-containing VLPs in Chinese Hamster Ovary cells or normal liver cells.

This document also provides vaccine preparations for inducing an immune response within a mammal against a hepatitis B virus. In some cases, a vaccine preparation provided herein can include one or more of the polypeptides provided herein. For example, a vaccine preparation provided herein can be designed to include a polypeptide having the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). In some cases, a single vaccine preparation can include more than one different polypeptide provided herein. For example, a single vaccine preparation provided herein can include both a polypeptide having the amino acid sequence as set forth in SEQ ID NO:16 and a polypeptide having the amino acid sequence as set forth in SEQ ID NO:24. Other combinations of polypeptides that can be used to make a vaccine preparation having a collection of different polypeptides include, without limitation, those set forth in Table 4.

Table 4. Combinations of polypeptides for vaccine preparations.

Vaccine ID No.	Sequence identifiers of polypeptides to be included in the vaccine preparation
8101	14, 16, 18
8102	22, 24, 28
8103	14, 16, 18, 22, 24
8104	36, 38

8105	32, 52, 58
8106	32, 36, 38, 52, 58
8107	14, 16, 36, 38
8108	22, 24, 28, 32, 52, 58
8109	16, 18, 24, 32, 36, 38, 52, 58

In some cases, a vaccine preparation provided herein can include one or more of the nucleic acid molecules provided herein. For example, a vaccine preparation provided herein can be designed to include a nucleic acid vector having a promoter operably linked to a

5 nucleic acid sequence that encodes a polypeptide having the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50,

10 52, 54, 56, or 58). In some cases, a single vaccine preparation can include more than one different nucleic acid molecule provided herein. For example, a single vaccine preparation provided herein can include both a nucleic acid vector designed to express a polypeptide having the amino acid sequence as set forth in SEQ ID NO:16 and a nucleic acid vector designed to express a polypeptide having the amino acid sequence as set forth in SEQ ID
15 NO:24. Other combinations of vectors that can be used to make a vaccine preparation having a collection of different vectors include, without limitation, those set forth in Table 5. In some cases, a single nucleic acid vector can encode two or more polypeptides provided herein. For example, a single nucleic acid vector can be designed to encode a combination of polypeptides as set forth in Table 4.

20

Table 5. Combinations of vectors for vaccine preparations.

Vaccine ID No.	Sequence identifiers of polypeptides to be encoded by individual vectors included in the vaccine preparation
9101	14, 16, 18
9102	22, 24, 28
9103	14, 16, 18, 22, 24
9104	36, 38
9105	32, 52, 58

9106	32, 36, 38, 52, 58
9107	14, 16, 36, 38
9108	22, 24, 28, 32, 52, 58
9109	16, 18, 24, 32, 36, 38, 52, 58

In some cases, a vaccine preparation provided herein can include one or more of the virus-like particles provided herein. For example, a vaccine preparation provided herein can be designed to include a virus-like particle that includes a polypeptide having the amino acid sequence as set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58 (or an amino acid sequence that is at least about 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 percent identical to the amino acid sequence set forth in SEQ ID NO:10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, or 58). In some cases, a single vaccine preparation can include more than one different virus-like particles provided herein. For example, a single vaccine preparation provided herein can include both a virus-like particle that includes a polypeptide having the amino acid sequence as set forth in SEQ ID NO:16 and a virus-like particle that includes a polypeptide having the amino acid sequence as set forth in SEQ ID NO:24. Other combinations of virus-like particles that can be used to make a vaccine preparation having a collection of different virus-like particles include, without limitation, those set forth in Table 6.

Table 6. Combinations of VLPs for vaccine preparations.

Vaccine ID No.	Specific VLPs to be included in the vaccine preparation
10101	VLP ID#7101, VLP ID#7102
10102	VLP ID#7104, VLP ID#7105
10103	VLP ID#7101, VLP ID#7104
10104	VLP ID#7102, VLP ID#7105
10105	VLP ID#7101, VLP ID#7102, VLP ID#7104, VLP ID#7105
10106	VLP ID#7103, VLP ID#7106
10107	VLP ID#7101, VLP ID#7104, VLP ID#7105

Any appropriate method can be used to formulate a vaccine preparation provided herein (e.g., a polypeptide vaccine, nucleic acid vaccine, or VLP vaccine provided herein). For example, a polypeptide vaccine provided herein, a nucleic acid vaccine provided herein,

or a VLP vaccine provided herein can be formulated to include one or more adjuvants. An adjuvant can be an immunological compound that can enhance an immune response against a particular antigen such as a polypeptide provided herein. Suitable adjuvants include, without limitation, alum as well as other aluminum-based compounds (e.g., Al_2O_3) that can be
5 obtained from various commercial suppliers. For example, REHYDRAGEL[®] adjuvants can be obtained from Reheis Inc. (Berkeley Heights, NJ). REHYDRAGEL[®] adjuvants are based on crystalline aluminum oxyhydroxide, and are hydrated gels containing crystalline particles with a large surface area (about 525 m²/g). Their Al_2O_3 content typically ranges from about 2 percent to about 10 percent. Rehydragel LG, for example, has an Al_2O_3 content of about 6
10 percent, and flows readily upon slight agitation. Rehydragel LG also has a protein binding capacity of 1.58 (i.e., 1.58 mg of bovine serum albumin bound per 1 mg of Al_2O_3), a sodium content of 0.02 percent, a chloride content of 0.28 percent, undetectable sulphate, an arsenic level less than 3 ppm, a heavy metal content less than 15 ppm, a pH of 6.5, and a viscosity of 1090 cp. Rehydragel LG can be combined with a polypeptide solution (e.g., a polypeptide in
15 PBS) to yield $\text{Al}(\text{OH})_3$. In some cases, ALHYDROGEL[™], an aluminum hydroxy gel adjuvant (Alhydrogel 1.3%, Alhydrogel 2.0%, or Alhydrogel “85”) obtained from Brenntag Stinnes Logistics, can be used.

In some cases, Montanide ISA 51 can be included in a vaccine preparation provided herein. MN51 (MONTANIDE[®] Incomplete SEPPIC Adjuvant (ISA) 51) as well as MN720
20 are available from Seppic (Paris, France). MN51 contains mannide oleate (MONTANIDE[®] 80, also known as anhydro mannitol octadecenoate) in mineral oil solution (Drakeol 6 VR). MONTANIDE[®] 80 is a limpid liquid with a maximum acid value of 1, a saponification value of 164-172, a hydroxyl value of 89-100, an iodine value of 67-75, a maximum peroxide value of 2, a heavy metal value less than 20 ppm, a maximum water content of 0.35%, a maximum
25 color value of 9, and a viscosity at 25°C of about 300 mPas. MONTANIDE[®] associated with oil (e.g., mineral oil, vegetable oil, squalane, squalene, or esters) is known as MONTANIDE[®] ISA. Drakeol 6 VR is a pharmaceutical grade mineral oil. Drakeol 6 VR contains no unsaturated or aromatic hydrocarbons, and has an A.P.I. gravity of 36.2-36.8, a specific gravity at 25°C of 0.834-0.838, a viscosity at 100°F of 59-61 SSU or 10.0-10.6 centistokes, a
30 refractive index at 25°C of 1.458-1.463, a better than minimum acid test, is negative for fluorescence at 360 nm, is negative for visible suspended matter, has an ASTM pour test value of 0-15°F, has a minimum ASTM flash point of 295°F, and complies with all RN requirements for light mineral oil and ultraviolet absorption. MN51 contains about 8 to 12 percent anhydro mannitol octadecenoate and about 88 to 92 percent mineral oil.

Other immunostimulatory components that can be used include, without limitation, plant extracts derived from the Soap bark tree (*Quillaja* species) containing members of a family of plant-based compounds called saponins.

Other adjuvants that can be included in a vaccine preparation provided herein include, without limitation, immuno-stimulating complexes (ISCOMs) that can contain such components as cholesterol and saponins. Examples include, without limitation, ISCOMATRIX™ and MATRIX-M™. ISCOM matrices can be prepared and conjugated to Cu²⁺. Adjuvants such as FCA, FIA, MN51, MN720, and Al(OH)₃ are commercially available from companies such as Seppic, Difco Laboratories (Detroit, MI), and Superfos Biosector A/S (Vedbeek, Denmark).

Other immunostimulatory components include, without limitation, muramyl dipeptide (e.g., N-acetylmuramyl-L-alanyl-D-isoglutamine; MDP), monophosphoryl-lipid A (MPL), formyl-methionine containing tripeptides such as N-formyl-Met-Leu-Phe, or a bacterial lipopolysaccharide. Such compounds are commercially available from Sigma Chemical Co. (St. Louis, MO) and RIBI ImmunoChem Research, Inc. (Hamilton, MT), for example. In some cases, an adjuvant can be Complete Freund's Adjuvant or Incomplete Freund's Adjuvant.

In some cases, a nucleic acid vaccine preparation provided herein can be formulated to lack an adjuvant. For example, a nucleic acid vaccine preparation provided herein can be designed to include a nucleic acid molecule that encodes a polypeptide provided herein without including any adjuvant.

In some cases, a polypeptide vaccine provided herein, a nucleic acid vaccine provided herein, or a VLP vaccine provided herein can be formulated to include other components such as cytokines, chemokines, monoclonal antibodies, or co-stimulatory molecules such as B7.

This document also provides methods for preparing a vaccine preparation provided herein. Such methods can involve suspending an amount of a polypeptide provided herein, a VLP provided herein, or a nucleic acid vector provided herein in a suitable amount of a physiological buffer (e.g., PBS). The polypeptides, VLPs, or nucleic acid vectors then can optionally be combined with a suitable amount of an adjuvant/immunostimulatory compound. The combining step can be achieved by any appropriate method, including, for example, stirring, shaking, vortexing, or passing back and forth through a needle attached to a syringe.

A vaccine preparation provided herein can be prepared in batch, such that enough unit doses are obtained for multiple injections (e.g., injections into multiple mammals or multiple injections into the same mammal). A “unit dose” of a vaccine preparation provided herein refers to the amount of a vaccine preparation administered to a mammal at one time. A unit dose of a vaccine preparation provided herein can contain an amount of polypeptides, VLPs, or nucleic acid molecules effective to induce an immune response against a hepatitis B virus. For example, a unit dose of a vaccine preparation provided herein can contain between about 0.1 µg and about 1 g (e.g., 1 µg, 10 µg, 15 µg, 25 µg, 30 µg, 50 µg, 100 µg, 250 µg, 280 µg, 300 µg, 500 µg, 750 µg, 1 mg, 10 mg, 15 mg, 25 mg, 30 mg, 50 mg, 100 mg, 250 mg, 280 mg, 300 mg, 500 mg, 750 mg, or more) of polypeptides, VLPs, or nucleic acid molecules. In the case of vaccine preparations containing viral vectors, a unit dose of a vaccine preparation can have a titer between about 10^3 to 10^{10} (e.g., 10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , or 10^{10}) viral particles or plaque forming units.

This document also provides methods for inducing an immune response within a mammal (e.g., a human) against a hepatitis B virus. For example, one or more polypeptide vaccines provided herein, one or more nucleic acid vaccines provided herein, one or more VLP vaccine provided herein, or combinations thereof can be administered to a mammal (e.g., a human) to induce an immune response against hepatitis B viruses. In some cases, an immune response against hepatitis B viruses can be induced by administering one or more nucleic acid vaccines provided herein followed by a vaccine that includes a hepatitis B virus antigen or followed by one or more polypeptide vaccines provided herein. For example, Vaccine ID No. 9101 can be administered to a human, and 1 to 30 days later, Vaccine ID No. 8105 can be administered to that human. In some cases, Vaccine ID No. 9101 can be administered followed 20 to 60 days later by Vaccine ID No. 7101. In some cases, Vaccine ID No. 9109 can be administered followed 30 to 120 days later by Vaccine ID No. 7106.

In some cases, a vaccine preparation provided herein can be delivered as a prophylactic vaccine to increase a mammal's resistance to a hepatitis B virus infection. For example, one or more polypeptide vaccines provided herein, one or more nucleic acid vaccines provided herein, one or more VLP vaccine provided herein, or combinations thereof can be administered to a human who has not been infected with a hepatitis B virus.

In some cases, a vaccine preparation provided herein can be used to treat a mammal after the mammal is infected with hepatitis B virus (e.g., an acutely hepatitis B virus infected or chronically hepatitis B virus infected mammal). For example, one or more polypeptide vaccines provided herein, one or more nucleic acid vaccines provided herein, one or more

VLP vaccine provided herein, or combinations thereof can be administered to a human acutely or chronically infected with hepatitis B virus. Administration of a vaccine preparation provided herein to a mammal infected with hepatitis B virus can result in a reduction in hepatitis B viral load, a reduction in the severity of the symptoms of the hepatitis B virus infection, a reduction in the degree of liver cirrhosis, a reduction in the incidence of hepatocellular cancer, or a clearance of the hepatitis B virus from the liver.

This document also provides methods for priming a mammal (e.g., a human) to receive a vaccine containing a hepatitis B virus antigen. For example, one or more polypeptide vaccines provided herein, one or more nucleic acid vaccines provided herein, one or more viral vaccines provided herein, one or more VLP vaccine provided herein, or combinations thereof can be administered to a mammal (e.g., a human) to prime that mammal for generating an enhanced immune response against hepatitis B viruses. Once primed, the mammal can be treated with a vaccine containing a hepatitis B virus antigen (e.g., Engerix) or a vaccine preparation provided herein.

A vaccine preparation provided herein can be administered using any appropriate method. For example, the administration can be, for example, topical (e.g., transdermal or intranasal), pulmonary (e.g., by inhalation or insufflation of powders or aerosols), oral, or parenteral (e.g., by intradermal, subcutaneous, intramuscular, or intraperitoneal injection, or by intravenous drip). Administration can be rapid (e.g., by injection) or can occur over a period of time (e.g., by slow infusion or administration of slow release formulations). In some cases, a nucleic acid vaccine can be delivered intramuscularly followed 1-90 days (e.g., between 1 and 90, between 1 and 80, between 1 and 70, between 1 and 60, between 1 and 50, between 5 and 90, between 10 and 90, between 20 and 90, between 5 and 75, between 10 and 75, between 10 and 50, between 20 and 50, between 25 and 50, or between 30 and 60 days) later by a VLP vaccine containing adjuvant.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 – Producing Shuffled Hepatitis B Surface Antigen

The parental DNA sequences encoding the preS2 and S regions of the hepatitis envelope protein were derived from the human, chimpanzee, gibbon, woolly monkey, and woodchuck hepadnaviruses and were obtained as follows.

The preS2+S envelope sequence of the human HBV corresponding to that of the ayw subtype (GenBank[®] Accession No. J02203; GI No. 329640) was amplified by PCR from a plasmid vector called pCAG-M-Kan. The nucleic acid sequence for this obtained DNA is set forth in SEQ ID NO:1 and encodes a hepatitis B surface antigen polypeptide having the amino acid sequence set forth in SEQ ID NO:2.

The woolly monkey envelope (preS2+S) sequence (GenBank[®] Accession No. AF046996; GI No. 3150070) was synthesized by oligonucleotide gene assembly (Stemmer *et al.*, *Gene*, 164(1):49-53 (1995)). The nucleic acid sequence for this obtained DNA is set forth in SEQ ID NO:3 and encodes a hepatitis B surface antigen polypeptide having the amino acid sequence set forth in SEQ ID NO:4.

A hybrid envelope gene was designed by combining the preS2 sequence of the human HBV (adw2 subtype; GenBank[®] Accession No. X02763; GI No. 59418) and the S sequence of the woodchuck hepatitis virus (strain WHV8; GenBank[®] Accession No. J04514; GI No. 336146). The gene was synthesized by oligonucleotide gene assembly. The nucleic acid sequence for this obtained DNA is set forth in SEQ ID NO:5 and encodes a hepatitis B surface antigen polypeptide having the amino acid sequence set forth in SEQ ID NO:6.

The amino acid sequence differences between the gibbon (GenBank[®] Accession No. U46935; GI No. 1814218) and the chimpanzee hepatitis (GenBank[®] Accession No. D00220; GI No. 163838595) preS2+S envelope proteins were minimal. A single composite parent gene was therefore synthesized by oligonucleotide gene assembly that contained the sum of all the amino acid changes in the chimpanzee and gibbon sequences relative to the corresponding human sequence. During the course of the synthesis of this composite chimp-gibbon sequence, a mutation occurred which led to the introduction of isoleucine at amino acid number 197 in place of the methionine found in the wild-type chimpanzee and gibbon sequences. The nucleic acid sequence for this obtained DNA is set forth in SEQ ID NO:7 and encodes a hepatitis B surface antigen polypeptide having the amino acid sequence set forth in SEQ ID NO:8.

The workflow for the hepatitis B surface antigen (HBsAg) library was as follows. The original library was generated using DNase treatment of the four parental sequences described above (SEQ ID NOs:1, 3, 5, and 7). The shuffling of four parental sequences was achieved by assembling DNase I-digested gene fragments. Basically, each parental gene fragment was made from plasmids in PCR reactions using recombinant *Thermus thermophilus* with two vector primers (Pre-for-1 (+):GCA GCT CCT TGC TCC TAA CAG (SEQ ID NO:64); and Pre-rev-2(-):GTA TCA CGA GGC CCT TTC GTC (SEQ ID NO:65)).

The PCR was performed at 30 cycles of 1 minute at 95°C, 1 minute at 60°C and 2 minutes at 72°C. The amplified products were purified in gel electrophoresis and digested by DNase I into fragments ranging from 25 bp to 100 bp, which were further purified in gel. These fragments and the three “a”-epitope constrained primers were mixed and assembled with
5 Taq/Pfu (9:1) polymerase in two rounds of 25 cycle PCR reactions (30 seconds at 96°C, 30 seconds at 40°C and 30 seconds at 72°C). In the assembly reactions, the percentage of “a” sequence incorporation was controlled by adjusting the ratio of the constrained “a” oligos to DNase I-digested gene fragments based on their molecular weights (Figure 1). Assembled HBs recombinants were rescued in 30 cycles of PCR by Taq polymerase (Qiagen) with two
10 nested primers (V1521+: GCT GAC AGA CTA ACA GAC TG (SEQ ID NO:66); and V2453 (-): AAC AGA TGG CTG GCA ACT AG (SEQ ID NO:67); 30 seconds at 95°C, 30 seconds at 55°C, and 1 minute at 72°C). Subsequently, the shuffled HBs fragments were digested with EcoR I/Asp718, purified in gel, and cloned into EcoR I/Asp718-cut pMKan vector using a standard cloning procedure.

15 The HBs “a” loop is a major neutralizing epitope and induces protective neutralizing antibodies against hepatitis B virus infection. To preserve this sequence, “a” epitope-constrained libraries were generated. In the assembly reactions, the individual “a” epitope-constrained primer was mixed with the DNase I-digested parental HBs fragments at three concentrations, 0.5:1, 1:1, and 2:1. The incorporation of ‘a’ epitope primers in shuffling
20 reactions were identified by BsrB I restriction enzyme digestion of the assembled HBs fragments. The rate was further evaluated with BsrB I-digested plasmid DNA samples. The ratio of 0.5:1 exhibited about 30% incorporation, and both 1:1 and 2:1 ratios exhibited 60-65% incorporation. Sequencing analyses of plasmid clones indicated that the three constrained ‘a’ epitopes were equally incorporated.

25 *‘a’ loop-constrained primers*

The thirty nucleotides of the human ‘a’ loop 1 sequence were held constant and flanked by fifteen nucleotides on each side derived from the related HBs genes of Chimpanzee/Gibbon (chibbon), Woolly Monkey (WM), or Woodchuck (WD) (Figure 2). The
30 primers were designed for human HBs ‘a’ constrained epitopes in the chimpanzee/gibbon hybrid (SEQ ID NO:7), Woolly monkey (SEQ ID NO:3), and human/woodchuck hybrid (SEQ ID NO:5).

Screening

Clones from the library were screened for protein expression using an immunofluorescence assay (IFA) for expression. Briefly, DNA prepared by BioRobot 9600 was transfected to Cos-7 cells (ATCC #CRL-1651) using SuperFect™ transfection reagent in 96-well format as described by the manufacturer (Qiagen). At 48-hour post-transfection, the cells were fixed with paraformaldehyde and stained with goat anti-HBs antibody (Dako #B0560), and then FITC-conjugated rabbit anti-goat immunoglobulins (Dako #P0449). The FITC-positive cells were visualized under fluorescent microscopy. Clones that produced positive IFA signals were then screened in mice by direct intramuscular injection of the plasmid DNA, injecting one shuffled clone in a single mouse. Antibody responses were measured using validated clinical kits (either the Monolisa anti-HBs 3.0 ELISA from Sanofi Diagnostics or the AUSAB EIA ELISA from Abbott Laboratories) and by standard ELISA assays using commercial preparations of the HBsAg obtained from (Seradyn recombinant HBsAg #ABH0705) or the wild-type ayw or adw2 preS2 peptides (custom synthesized by Seradyn) (Figure 3).

The most immunogenic clones were further analyzed in larger groups of mice (Figure 37). Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 100 µg/mL in sterile PBS. For each test group, 10 six-week old C57BL/6 mice were injected intramuscularly with 50 µL DNA solution in the tibialis anterior muscle of each leg muscle (10 µg DNA total per mouse). Sera were analyzed 4 weeks after treatment for the level of anti-HBsAg as measured by the Sanofi Monolisa anti-HBS kit (expressed above as the GMT ± SEM for each group in mIU per mL). Titers were calculated for mice that received clones 6101, 6102, 6103, 6104, 6105, 6106, 6107, 6108, 6109, or 6111, or the wild-type human hepatitis B envelope gene (HBV). Mice injected with clones 6103, 6104, 6105, 6107, or 6111 from the first round library developed titers that were significantly higher than those in mice that were injected with the wild-type hepatitis B envelope gene containing plasmid control (HBV) (Figure 37).

Based on these results, ten clones were selected (clones 6101, 6102, 6103, 6104, 6105, 6106, 6107, 6108, 6109, and 6111) and then shuffled to generate a second round library. This library was screened as before (IFA and mouse immunization), and immunogenic clones were selected and analyzed further in larger groups of animals (Figure 39). Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 100 µg/mL in sterile PBS. For each test group, 10 six-week old C57BL/6 mice were anesthetized and injected intramuscularly with 50 µL DNA solution in the tibialis anterior

muscle of each leg muscle (10 µg DNA total per mouse). Sera was analyzed 4 weeks after treatment for the level of anti-hepatitis B antibody as measured by the Sanofi Monolisa anti-HBS kit (expressed above as the GMT ± SEM for each group in International Units). Titers were calculated for mice that received clones 6201, 6202, 6204, 6205, 6208, 6209, 6210, 5 6212, 6213, or 6215, or the wild-type human hepatitis B envelope gene (HBV). Mice injected with clones 6201, 6202, 6204, 6205, 6208, 6209, 6210, 6212, 6213, or 6215 developed titers that were significantly higher than those in mice that were injected with the wild-type hepatitis B envelope gene containing plasmid control (HBV) (Figure 38).

The nucleic acid and amino acid sequences of the original four parents (Figures 4-7), 10 the ten clones selected from the first round of shuffling (Figures 8-17), and the fifteen clones selected from the second round of shuffling (Figures 18-32) are presented in Figures 4-32. An alignment of the parental and shuffled DNA and protein sequences were obtained using the Clustal W algorithm within the AlignX component of Vector NTI ver 6.0 and are shown in Figures 33 and 34, respectively. A summary of the amino acid changes for the clones 15 shown in Figures 8-32 is shown in Figure 35, along with an indication of the various regions of the protein. The nucleotide sequence and component locations of the vector used in this work are shown in Figure 36.

All but two of the clones (clones 6101 and 6111) were obtained from a shuffled library in which the main antigenic determinant (the so-called “a-loop”) of the human HBV 20 envelope protein was held constant during the shuffling reaction. This was accomplished by including specific oligonucleotides in the shuffling reaction to generate clones in which this sequence was held constant. Several obtained clones did not retain this conserved a-loop in its entirety due to a spontaneous mutation not found in any of the parents used in the shuffling reaction (proline at amino acid number 183 in the place of alanine in the wild-type 25 sequence).

To obtain additional reliable data, an experiment was conducted in which three first round clones (6104, 6105, and 6108) and five second round clones (6202, 6204, 6205, 6212, and 6215) were used to immunize groups of 30 mice per clone. In this experiment, four plasmids were used that express each of the parental envelope sequences used in the initial 30 shuffling reaction (designated HBV, WM, CH, and WD in Figure 39). Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 100 µg/mL in sterile PBS. For each test group, 30 six-week old C57BL/6 mice were anesthetized and injected intramuscularly with 50 µL DNA solution in the tibialis anterior muscle of each leg muscle (10 µg DNA total per mouse). Sera were analyzed four weeks after treatment for the level of

anti-hepatitis B antibody as measured by the Abbott AUSZYME ELISA (expressed above as the GMT $\pm$ SEM for each group in International Units). Titers were calculated for mice that received DNA clones from the first round library (6104, 6105, and 6108), the second round library (6202, 6204, 6205, 6212, and 6215), and the parents from the original shuffling reaction (wild-type human hepatitis B envelope gene (HBV), woolly monkey hepatitis envelope gene (WM), chimpanzee/gibbon hepatitis envelope gene (CH), and woodchuck hepatitis envelope (WD)). Another group of mice were injected with 1 μ g of the commercially available hepatitis B recombinant protein vaccine (Engerix-B, SmithKline Beecham), which was derived from a wild-type HBV gene sequence.

Mice injected with clones 6104, 6105, 6108, 6202, 6204, 6205, 6212, and 6215 developed titers that were significantly higher than mice that were injected with the original parental clones including the wild-type Hepatitis B envelope gene containing plasmid control (HBV) (Figure 39). The commercial protein vaccine containing purified protein from the wild-type human Hepatitis B envelope gene adjuvanted with aluminum elicited a superior titer compared to DNA injection of the gene by itself (compare "Engerix" to "HBV"). The titer obtained with the commercial vaccine (Engerix) was surpassed by clones 6104, 6105, 6202, 6204, 6205, 6212, and 6215.

Clones 6104, 6105, 6108, 6202, 6204, 6205, 6212, and 6215 were further analyzed for their ability to prime the immune system to respond to wild-type hepatitis B surface antigen. Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 100 μ g/mL in sterile PBS. For each test group, 20 six-week old C57BL/6 mice were anesthetized and injected intramuscularly with 50 μ L DNA solution in the tibialis anterior muscle of each leg muscle (10 μ g DNA total per mouse). Three weeks after the initial DNA immunization, 10 mice in each group were injected intraperitoneally with 1 μ g of the commercially available hepatitis B recombinant protein vaccine (Engerix-B, SmithKline Beecham) diluted four fold in endotoxin free PBS (5 μ g/mL final). Two weeks after the protein injection sera from all mice was analyzed for the level of anti-hepatitis B antibody as measured by the Abbott AUSZYME ELISA (expressed above as the GMT for each group in International Units). Titers were calculated for mice that received DNA clones 6104, 6105, 6108, 6202, 6204, 6205, 6212, or 6215, one of the four parental clones, or the backbone DNA plasmid containing an irrelevant gene sequence.

Clones 6202, 6204, 6205, 6212, and 6215 were superior to the wild-type hepatitis B envelope protein gene for priming the immune system to respond to the hepatitis B protein (Figure 40). This improved priming was exemplified by the significantly larger memory

responses observed after the protein injection in mice that received the shuffled clones as compared to mice that received the wild-type clone (compare black bars of 6104, 6105, 6108, 6202, 6204, 6205, 6212, and 6215 with black bar of HBV in Figure 40). The relative titers between groups that only received the DNA prime (grey bars) were very similar to the relationships seen in previous experiments (see Figure 37), but the post boost titers did not maintain this relationship. This change of profile indicated that the high memory response was not solely due to a simple boosting of the higher titer produced by primary injection but rather may indicate that there are aspects of the shuffled genes that enable them to enhance the immune system's ability to respond to a second exposure (compare grey and black bars of 6202 to those of 6205).

In another experiment, clones 6102, 6103, 6104, 6105, 6108, 6205, 6212, and 6213 were tested in a population of outbred mice. Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 1000 µg/mL in sterile PBS. For each test group, 10 six-week old outbred Swiss Webster mice were anesthetized and injected intramuscularly with 50 µL DNA solution in the tibialis anterior muscle of each leg muscle (100 µg DNA total per mouse). Sera were analyzed four weeks after treatment for the level of anti-hepatitis B antibody as measured by Abbott AUSZYME ELISA. Titers were calculated for groups of ten mice each that received clones 6102, 6103, 6104, 6105, 6108, 6205, 6212, or 6213, or for two groups of ten mice each that received the wild-type human hepatitis B envelope gene (HBV). Groups of mice injected with clones 6103, 6105, 6108, 6205, 6212, and 6213 exhibited a higher percentage of seroconverting mice and/or higher anti-HBsAg titers than groups of mice that were injected with the wild-type hepatitis B envelope gene containing plasmid control (HBV) (Figure 41). This population of outbred mice more accurately reflects the heterogeneity seen in natural populations. The ability of shuffled clones to outperform the wild-type clone in this environment suggests that the shuffled clones may outperform the wild-type sequences in other outbred populations such as the human population.

In another experiment, clones 6104 and 6105 were tested in a population of non-responder mice. Briefly, purified DNA (Aldevron LLC, Fargo, ND) was diluted to a final concentration of 1000 µg/mL in sterile PBS. For each test group, 10 six-week old B10M mice were anesthetized and injected intramuscularly with 50 µL DNA solution in the tibialis anterior muscle of each leg muscle (100 µg DNA total per mouse). Sera were collected by retro-orbital methods four weeks after treatment, and the mice were then given a second dose of DNA. Three weeks after the second treatment, sera was collected. The level of anti-hepatitis B antibody as measured by the Abbott AUSZYME ELISA (expressed as the GMT

± SEM for each group in International Units) was determined for both the sera after a single treatment (grey bars in Figure 42) and the sera after two doses (black bars in Figure 42). Titers were calculated for mice that received DNA clones 6104 and 6105 and wild-type human hepatitis B envelope gene (HBV) (Figure 42).

5 Shuffled clones 6104 and 6105 were able to stimulate strong immune responses in a mouse strain that typically does not respond to hepatitis B antigens. These results were particularly interesting because the B10M non-responder strain of mice may be similar to a subset of chronically infected humans who also do not respond to HBsAg. The ability of the shuffled clones to overcome this lack of response demonstrates that the shuffled clones
10 provided herein can be used as a therapeutic against chronic hepatitis B.

In another experiment, five clones (6202, 6204, 6205, 6212 and 6215) were mixed in equal amounts and diluted to a final concentration of 1 mg/mL DNA in sterile PBS to form a vaccine preparation (Vaccine ID No. 9106). Five 6-week old C57BL/6 mice were anesthetized, and 50 µL of the DNA solution were injected into each tibialis anterior muscle
15 for a total of 100 µg DNA per mouse. The injections were repeated three additional times at 3-week intervals. In some cases, only two DNA injections were used with electroporation. The mice were killed six days after the last injection, the spleens were harvested, and single-cell suspensions were prepared. A peptide pool covering the sequence of the genotype A or D HBsAg was used to stimulate the cells. The numbers of CD8⁺ T cells expressing
20 interferon-gamma and the numbers of CD4⁺ T cells expressing interferon-gamma were determined by intracellular cytokine staining and flow cytometry. Spleen cells were isolated from an aged-matched group of untreated mice and submitted to the same stimulation and analysis protocol.

The mice vaccinated with Vaccine ID No. 9106 exhibited increased numbers of CD8⁺
25 T cells expressing interferon-gamma and increased numbers of CD4⁺ T cells expressing interferon-gamma following stimulation with genotype A or D HBsAg peptides as compared to the number of cells detected in naïve mice (Figures 43-48).

OTHER EMBODIMENTS

30 It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 97 percent identical to the amino acid sequence set forth in SEQ ID NO:10.
- 5 2. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:12.
3. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:14.
- 10 4. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 95 percent identical to the amino acid sequence set forth in SEQ ID NO:16.
5. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 97 percent identical to the amino acid sequence set forth in SEQ ID NO:18.
- 15 6. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:20.
- 20 7. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 97 percent identical to the amino acid sequence set forth in SEQ ID NO:22.
8. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 89 percent identical to the amino acid sequence set forth in SEQ ID NO:24.
- 25 9. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 89 percent identical to the amino acid sequence set forth in SEQ ID NO:26.
10. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 95 percent identical to the amino acid sequence set forth in SEQ ID NO:28.
- 30 11. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 94 percent identical to the amino acid sequence set forth in SEQ ID NO:30.

12. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:32.

5 13. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:34.

14. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:36.

10

15. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:38.

15 16. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 97 percent identical to the amino acid sequence set forth in SEQ ID NO:40.

17. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:42.

20 18. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:44.

19. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 95 percent identical to the amino acid sequence set forth in SEQ ID NO:46.

25

20. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 96 percent identical to the amino acid sequence set forth in SEQ ID NO:48.

21. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:50.

30

22. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 93 percent identical to the amino acid sequence set forth in SEQ ID NO:52.

23. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 92 percent identical to the amino acid sequence set forth in SEQ ID NO:54.

24. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence
5 is at least 86 percent identical to the amino acid sequence set forth in SEQ ID NO:56.

25. A polypeptide comprising an amino acid sequence, wherein said amino acid sequence is at least 89 percent identical to the amino acid sequence set forth in SEQ ID NO:58.

10 26. The polypeptide of any one of claims 1-25, wherein said polypeptide comprises the ability to induce an immune response against a hepatitis B virus when said polypeptide is administered to a mammal.

27. The polypeptide of any one of claims 1-26, wherein said polypeptide is a fusion
15 polypeptide comprising a second amino acid sequence.

28. The polypeptide of claim 27, wherein said second amino acid sequence encodes a tag selected from the group consisting of GST, FLAG, GFP, and c-myc.

20 29. The polypeptide of claim 27, wherein said second amino acid sequence encodes a cytokine selected from the group consisting of GM-CSF, IL-2, and IL-12.

30. The polypeptide of any one of claims 1-29, wherein said polypeptide is substantially
25 pure.

31. The polypeptide of any one of claims 1-29, wherein said polypeptide is an isolated polypeptide.

30 32. A virus-like particle comprising one or more polypeptides selected from the group consisting of the polypeptides of claims 1-29.

33. The virus-like particle of claim 32, wherein said particle comprises one polypeptide selected from said group.

34. The virus-like particle of claim 32, wherein said particle comprises two, three, four, or five different polypeptides selected from said group.

5 35. The virus-like particle of claim 32, wherein said particle comprises the ability to induce an immune response against a hepatitis B virus when said particle is administered to a mammal.

36. A vaccine preparation comprising one or more polypeptides selected from the group
10 of polypeptides consisting of the polypeptides of claims 1-29.

37. The vaccine preparation of claim 36, wherein said vaccine preparation comprises one polypeptide selected from said group.

15 38. The vaccine preparation of claim 36, wherein said vaccine preparation comprises two, three, four, or five different polypeptides selected from said group.

39. The vaccine preparation of claim 36, wherein said vaccine preparation comprises the ability to induce an immune response against a hepatitis B virus when said vaccine
20 preparation is administered to a mammal.

40. The vaccine preparation of claim 36, wherein said vaccine preparation comprises an adjuvant.

25 41. The vaccine preparation of claim 40, wherein said adjuvant is selected from the group consisting of aluminum-based compounds, Montanide ISA 51, Montanide ISA 720, and CpG oligodeoxynucleotides.

42. The vaccine preparation of claim 41, wherein said adjuvant comprises alum or Al_2O_3 .
30

43. A nucleic acid molecule comprising a nucleic acid sequence encoding a polypeptide selected from the group consisting of the polypeptides of claims 1-29.

44. The nucleic acid molecule of claim 43, wherein said molecule comprises a promoter sequence operably linked to said nucleic acid sequence.

45. The nucleic acid molecule of claim 43, wherein said molecule is a vector.

5

46. The nucleic acid molecule of claim 45, wherein said vector is a plasmid.

47. The nucleic acid molecule of claim 45, wherein said vector is a viral vector.

10 48. The nucleic acid molecule of claim 45, wherein said viral vector is selected from the group consisting of adenoviral vectors, adeno-associated virus vectors, and vaccinia viral vectors.

49. A vaccine preparation comprising one or more nucleic acid molecules selected from
15 the group consisting of the nucleic acid molecules of claims 43-48.

50. The vaccine preparation of claim 49, wherein said vaccine preparation comprises one nucleic acid molecule selected from said group.

20 51. The vaccine preparation of claim 49, wherein said vaccine preparation comprises two, three, four, or five different nucleic acid molecules selected from said group.

52. The vaccine preparation of claim 49, wherein said vaccine preparation comprises the ability to induce an immune response against a hepatitis B virus when said vaccine
25 preparation is administered to a mammal and said polypeptide is expressed within said mammal.

53. The vaccine preparation of claim 49, wherein said vaccine preparation comprises an adjuvant.

30

54. The vaccine preparation of claim 53, wherein said adjuvant is selected from the group consisting of aluminum-based compounds, Montanide ISA 51, Montanide ISA 720, and CpG oligodeoxynucleotides.

55. The vaccine preparation of claim 54, wherein said adjuvant comprises alum or Al₂O₃.

56. A method for inducing an immune response against a hepatitis B virus within a mammal, wherein said method comprises administering a vaccine preparation to said mammal, wherein said vaccine preparation comprises:

(a) one or more polypeptides selected from the group consisting of the polypeptides of claims 1-29,

(b) one or more virus-like particles selected from the group consisting of the virus-like particles of claims 32-35, or

10 (c) one or more nucleic acid molecules selected from the group consisting of the nucleic acid molecules of claims 43-48.

57. The method of claim 56, wherein said mammal is a human.

15 58. The method of claim 56, wherein said mammal is a human not previously infected with a hepatitis B virus.

59. The method of claim 56, wherein said mammal is a human acutely infected with a hepatitis B virus.

20 60. The method of claim 56, wherein said mammal is a human chronically infected with a hepatitis B virus.

61. The method of claim 56, wherein said immune response comprises the production of anti-hepatitis B virus antibodies.

25 62. The method of claim 56, wherein said method comprises administering a second vaccine preparation to said mammal after the step of administering said vaccine preparation to said mammal, wherein said second vaccine preparation comprises an hepatitis B virus antigen.

30 63. The method of claim 56, wherein said method comprises administering a second vaccine preparation to said mammal after the step of administering said vaccine preparation to said mammal, wherein said second vaccine preparation comprises:

(a) one or more polypeptides selected from the group consisting of the polypeptides of claims 1-29,

(b) one or more virus-like particles selected from the group consisting of the virus-like particles of claims 32-35, or

5 (c) one or more nucleic acid molecules selected from the group consisting of the nucleic acid molecules of claims 43-48.