VIROLOGY 218, 61–70 (1996)
ARTICLE NO. 0166

Poliovirus Recombinants Expressing Hepatitis B Virus Antigens Elicited

View metadata, citation and similar papers at core.ac.uk
brought
provided by Elsevier

TAE-JIN YIM,*,† SHENBEI TANG,* and RAUL ANDINO*,[1]

*Department of Microbiology and Immunology, University of California, San Francisco, Box 0414, San Francisco, California 94143-0414; and †Program in Biological Sciences, Research Training Program, Biochemistry and Molecular Biology, University of California, San Francisco, Box 0448, San Francisco, California 94143

Received December 6, 1995; accepted January 22, 1996

Expression of foreign antigens in the context of poliovirus vectors may provide a plausible approach to vaccine development. Poliovirus recombinants were constructed by fusing preS surface or core HBV proteins to the poliovirus polyprotein as previously described (Andino *et al., Science,* 265, 1448–1451, 1994). All recombinant viruses replicated with near wild-type efficiency in tissue culture cells and stably expressed high levels of the HBV antigens. The kinetics of recombinant RNA synthesis were indistinguishable from that of wild-type poliovirus. Exogenous proteins were not incorporated into the poliovirus particles, but HBV core proteins self-assembled into 100S particles composed of free HBV core proteins and fusions with poliovirus capsid proteins. Mice susceptible to poliovirus infection were inoculated with recombinant virus and elicited humoral immune responses against the HBV antigens. © 1996 Academic Press, Inc.

A successful vaccine should be safe, effective, and prevent or limit initial replication of a pathogen. In addition, the vaccine should be easy to store and transport as well as capable of being administered in a cost effective manner. The latter attribute has the greatest impact for vaccination programs in the developing world, where most of the epidemic viral diseases occur and where vaccines could have the greatest impact on public health. The Sabin live attenuated poliovirus vaccine is easily administered by the oral route, has a low distribution cost, induces both serum antibodies and intestinal mucosal resistance, confers long lasting immunity, and has greatly facilitated the control of widespread paralytic poliomyelitis epidemic (Dowdle *et al.,* 1994).

In contrast, despite the existence of an effective vaccine, HBV continues to be a major cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Hollinger, 1990). Worldwide, there are now more than 250 million people infected with HBV. The majority of these individuals live in developing countries. In Southeast Asia, China, and sub-Saharan Africa, the prevalence of HBV infection is as high as 5–15% of the total population. In the United States, where the prevalence rate of chronic hepatitis is among the lowest in the world (0.1–1%), approximately 200,000 cases of new HBV infections occur each year (Hollinger, 1990; Krugman, 1988).

The lack of success in controlling global HBV infection in highly endemic areas is due in large part to the lack of an inexpensive vaccine and the complex vaccination regimen which requires several consecutive administrations of the vaccine (Krugman, 1988). Indeed, the United Nations' Children's Vaccine Initiative has identified the HBV vaccine as one existing vaccine that needs to be improved (La Montagne and Rabinovich, 1992).

Given the favorable characteristics of the Sabin poliovirus vaccine, a number of investigators have attempted to adapt poliovirus as a vector to express antigens from other pathogens. The poliovirus genome consists of a single strand, positive sense RNA molecule of approximately 7500 nucleotides. A unique open-reading frame encodes a large polyprotein precursor, which must be proteolytically processed by two viral proteinases ($2A^{pro}$ and $3C^{pro}$) in order to generate mature structural and nonstructural poliovirus proteins. The major viral protease, $3C^{pro}$, recognizes and cleaves at specific amino acid sequences (AXXQG) within exposed polyprotein domains.

Several strategies have been reported in the engineering of poliovirus vectors. Through one method, small antigenic epitopes are inserted into one of the capsid proteins, VP1 (Burke *et al.,* 1988; Evans *et al.,* 1989). Through another method, dicistronic poliovirus RNAs are constructed by duplicating the 5′ noncoding region of the poliovirus genomic RNA (IRES). In this manner, the foreign polypeptide is expressed using one IRES and essential viral proteins are produced using the other (Alexander *et al.,* 1994; Lu *et al.,* 1995). A third method uses poliovirus minireplicon genomes in which poliovirus structural protein genes are replaced by foreign sequences (i.e., HIV-1 *gag, env,* and *pol*), in this case minireplicon genome must be packaged by supplying poliovi-

[1] To whom correspondence and reprint requests should be addressed. Fax: (415) 476-0939; e-mail: andino@cgl.ucsf.edu.

0042-6822/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

rus capsid proteins *in trans* (Choi *et al.*, 1991; Porter *et al.*, 1995; Ansardi *et al.*, 1995). Limitations of these strategies include the small size of the tolerated insert, genetic instability of the inserted sequences, or a requirement for helper virus for viral propagation.

We have pursued a different approach that utilizes basic aspects of the viral life cycle and permits the generation of replication-competent recombinant polioviruses that are able to replicate without the need for a helper virus and that stably carry and express genetic sequences of other pathogens (Andino *et al.*, 1994). Sequences are inserted at the amino terminus of the poliovirus polyprotein precursor, separated by an artificial 3C protease cleavage site. In this way, a larger than normal precursor is initially made, but is appropriately cleaved into the usual array of constituent proteins. The 3C protease accurately recognizes and cleaves the inserted synthetic proteolytic site, freeing the exogenous protein sequences from the rest of the poliovirus polyprotein (Fig. 1A). In this manner, all of the poliovirus proteins are correctly produced and normal viral replication proceeds.

This method has been used to express antigenic determinants derived from a large number of bacterial and viral pathogens (Andino *et al.*, 1994; Mattion *et al.*, 1994; Altmeyer *et al.*, 1994). Importantly, Altmeyer and co-workers have employed a different picornavirus, Mengovirus, to express a cytotoxic T-cell epitope from lymphocytic chorio-meningitis virus (LCMV). The mengovirus recombinant can elicit immune responses that protect vaccinated mice against challenge with pathogenic strain of LCMV (Altmeyer *et al.*, 1995).

As a first step toward the development of a alternative, cheaper, and more effective HBV vaccine we have described in this report the construction and characterization of poliovirus recombinants carrying surface and core HBV antigenic sequences. Foreign HBV amino acid sequences were produced in significant amounts in poliovirus chimera-infected cells, but the foreign peptides were not included in the poliovirus particle. Infection of susceptible mice with the poliovirus recombinants elicited humoral immune responses to both HBV and poliovirus proteins.

MATERIALS AND METHODS

Poliovirus recombinant construction and DNA procedures

Restriction enzymes, T4 DNA polymerase, Taq polymerase, T7 RNA polymerase, and avian myeloblastosis virus reverse transcriptase (AMV RTase) were obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); T4 DNA ligase was obtained from New England BioLabs, Inc. (Beverly, MA); and shrimp alkaline phosphatase came from the United States Biochemical Corp. (Cleveland, OH). All enzymes and compounds were used as recommended by the manufacturers.

Poliovirus chimeras were constructed by individually cloning three specific antigenic HBV proteins into a molecular clone of Mahoney Type 1 wild-type poliovirus vaccine vector (pMoV-1.4) (Andino *et al.*, 1994). The Mahoney vector cDNA has been modified to include an inframe synthetic polylinker containing *Eco*RI, *Not*I, *Bss*HII, and *Xho*I sites that facilitate the insertion of foreign sequences; an artificial recognition and cleavage site (AXXQG) for the major poliovirus protease ($3C^{pro}$) was placed at the 3′ border of the polylinker and was preceded by a six polyglycine tract.

Exogenous DNA sequences were amplified by PCR (Ausubel *et al.*, 1994) with primers that included restriction enzyme recognition sites to facilitate the insertion of the PCR amplified exogenous DNA into the vector polylinker. Coding sequences for the preS1 region of HBV was amplified with primers 1 and 2; the preS2 region of HBV was amplified with primers 3 and 4; and a HBV core region was amplified with primers 5 and 6. All three sequences were amplified using plasmid containing HBVadw2 sequences as a template (a gift from the Don Ganem laboratory). PCR fragments used in cloning were digested with *Bss*HII and *Not*I restriction enzymes and ligated to the vector digested with the same enzymes (Ausubel *et al.*, 1994). Restriction sites (*Sal*I and *Xho*I) were introduced at codon 79 of the HBV core insert using overlap PCR extension technique with primers 16 and 14 and 15 and 17. This recombinant, named HBc.SX, was used to construct polioviruses carrying HBV core chimeric particles which display on their surface HBV preS1 and preS2 antigenic peptides. Antigenic peptides coding sequences were PCR amplified and ligated to HBc.SX plasmid. HBV preS1 sequences were amplified using primers 18 and 19, and HBV preS2 was constructed by inserting the double-strand oligonucleotide (20 and 21). Replication-competent chimeric polioviruses were recovered by transfection of HeLa S3 cells with *in vitro* transcribed RNA from recombinant cDNA clones (Luthman and Magnusson, 1983).

Cells and virus stocks

HeLa cells, clone S3, were grown in suspension in Jokliks modified minimal essential medium (MEM; Sigma Chemical Company, St. Louis, MO) supplemented with 10% horse serum (GIBCO Diagnostics, Madison, WI), 1% penicillin–streptomycin, and 1% L-glutamine (both from Mediatech, Inc., Herndon, VA).

HeLa cell monolayers in 100-mm dishes were grown in DMEM/F12 (GIBCO) and transfected with 1–10 μg of recombinant viral RNA by a DEAE–dextran procedure (Luthman and Magnusson, 1983). Single plaques were isolated and expanded for each poliovirus chimera by standard procedures to generate stocks that were used for further characterization.

Virus infections

In all experiments, 100-mm dishes containing approximately 3×10^6 cells were used. The dishes were washed once with phosphate-buffered saline (PBS) and the appropriate amounts of virus were added in order to yield the desired multiplicity of infection (m.o.i.). Plates were incubated at room temperature for 30 min, allowing the virus to bind to the cells, and then 10 ml of Dulbecco's Modified Eagle Medium [Nutrient Mixture F-12 (DMEM/F12) 1:1 Mixture (Mediatech)] supplemented with 10% newborn calf serum (GIBCO), 1% penicillin–streptomycin, and 1% L-glutamine (Mediatech) was added to the dishes and placed at appropriate temperature, as required for each specific experiment. At the time of collection, plates were washed once with PBS, and cells were resuspended in 1 ml of PBS. Cells were collected by centrifugation at low speed and stored at −20° until analysis.

One-step growth curve

HeLa cell monolayers in 100-mm dishes were infected with each recombinant, as described above, with an m.o.i. of 10 and incubated at different temperatures. At each time point, infected cells were collected and lysed by freeze-thaw. Viral yield (plaque-forming units per milliliter[pfu/ml]) at each time point was determined by plaque assay (Trono *et al.,* 1988).

RT–PCR

Each passage on tissue culture was performed by infecting HeLa cells with a m.o.i. of 10, and incubating the infected cells at 37° for 24 hr. Total RNA from cells infected with a recombinant virus was prepared by phenol–chloroform extraction 9 hr postinfection and precipitated with ethanol. Reverse transcription (RT) was performed with AMV RTase at 42° for 60 min using oligo 13 as a primer, complementary to the viral RNA sequences located approximately 60 bases downstream from the inserted sequences. After the reaction was completed, the enzyme was inactivated by incubating it at 100° for 3 min. Polymerase chain reaction (PCR) amplification was performed with primers 7 and 8 to amplify the region of the poliovirus genome that contained the inserted sequences. Note that these primers amplify both wild-type and recombinant sequences, and the method is semiquantitative. PCR was performed for 25 cycles at 94° for 1 min, 50° for 1 min, and 72° for 1 min using Taq polymerase.

Western blot analysis

HeLa cells infected with wild-type and HBV recombinant polioviruses (m.o.i. of 10) were incubated for 4, 6, and 8 hr at 37°. Cells were harvested and lysed in buffer H [buffer H: 10 m*M* Hepes (pH 7.9), 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* DTT, 1% Triton X-100, and 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF)] and the nuclei were removed by centrifugation (Andino *et al.,* 1993). Four micrograms of proteins of total lysates was subjected to electrophoresis through a 12% SDS–polyacrylamide gel and analyzed by immunoblotting. Antisera against the poliovirus capsid proteins was prepared in a rabbit. A monoclonal antibody that reacts with HBV preS1 region was a gift from the Mena Ostapchuck's laboratory (Suny–Stony Brook), antisera to the HBV preS2 region was a gift from Patricia Reilly (American Cyanamid), and antisera to the HBc was obtained from DAKO (Carpinteria, CA). Antisera directed against poliovirus capsid proteins were obtained by inoculating rabbits with purified poliovirus. Secondary antibodies (both anti-mouse and anti-rabbit) were obtained from Amersham (Arlington Heights, IL). The immunoblotting procedure was provided by the manufacturer of the ECL chemiluminescense detection kit (Amersham).

Measurement of viral RNA synthesis

HeLa cells were infected as previously described with an m.o.i. of 10 for each of the recombinant and wild-type polioviruses. After 30 min of adsorption of the virus at 37°, the cells were washed with PBS, and DMEM/F12 containing Actinomycin D (5 μg/ml) was added. [^{3}H]-Uridine (20 μCi/ml) (purchased from Du Pont NEN Research Products, Boston, MA) was added at 1 hr postinfection. Cells were collected as previously described at various time points. Acid insoluble materials were collected onto Millipore glass microfiber filters, and radioactivity was determined by scintillation counting (Bernstein *et al.,* 1985).

Cesium chloride gradient analysis

Viruses were metabolically labeled by infecting Hela cells with wild-type or recombinant poliovirus for 8 hr at 37° in the presence of 25 μCi/ml of ^{35}[S]methionine. Cells were lysated in buffer H and cytoplasmic extracts were clarified by centrifugation and loaded in a CsCl solution (1.4 g/ml). Gradients were submitted to centrifugation for 20 hr at 35000 rpm; then they were fractionated in aliquots of 0.5 ml, the radioactivity in each aliquot determined by scintillation counting.

Sucrose gradient analysis

Lysates from HeLa cells infected with a recombinant, HBc.155, or a wild-type virus were analyzed by sucrose gradient (10–60%) as described by Zhou and Standring (1991). Fourteen fractions (100 μl each) were collected and HBV or poliovirus-specific proteins were detected by immunoblotting as previously described.

Expression of HBV proteins in *E. coli*

The pGEX expression system (Smith and Johnson, 1988) was used to produce HBV proteins used in ELISA

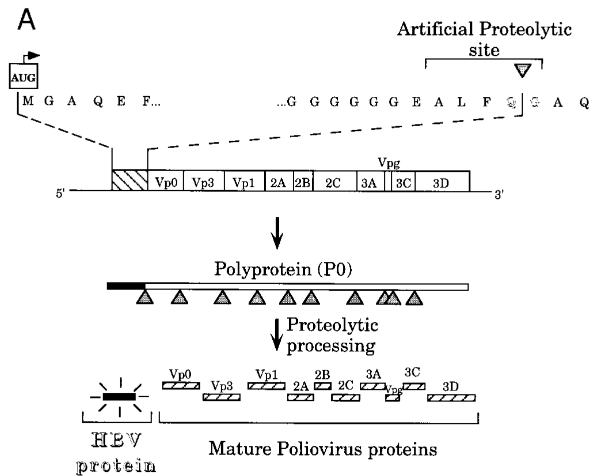
determinations. Plasmids were constructed using the pGEX -4T-1 glutathione *S*-transferase (GST) Gene Fusion Vector System (Pharmacia Biotech, Inc., Piscataway, NJ). Inserts were amplified by PCR using primers 9 and 10 for pGEX-4T-1-S2B and primers 11 and 12 for pGEX-4T-1-HBc. PCR fragments were digested with *Bam*HI and *Not*I restriction enzymes and ligated to the pGEX -4T-1 which was also digested with the same enzymes. The proteins were expressed and purified according to the protocol recommended by the manufacturer (Pharmacia).

Inoculation of transgenic mice with recombinant poliovirus and determination of antibody titers by enzyme-linked immunosorbent assay (ELISA)

Mice (TgPVR1-17 strain) (Ren *et al.,* 1990), generously provided by American Cyanamid, were infected intraperitoneally with 100 μl of 1×10^8 PFU/ml stocks of either wild-type or recombinant polioviruses (HBV.S2 or HBc-155). Also as a negative control, mice were mock infected by intraperitoneal injection of 100 μl PBS. Mice were inoculated at 0, 2, 5, and 8 weeks. Sera obtained from infected mice at 0, 2, 5, 8, and 11 weeks after the first inoculation were analyzed by ELISA. ELISA procedure was basically conducted as described (Ausubel *et al.,* 1994). Plates coated with 5 μg/ml of purified GST preS2 fusion protein or GST–HBV core fusion protein (expressed in pGEX) were incubated with doubling dilution of test samples. Bound antibody was detected by incubation with antibodies to mouse Ig antibodies conjugated to horseradish peroxidase (Amersham). Enzymatic activity was determined with ABTS tablets (Boehringer). The absorbance was measured at 405 nm. Results are expressed as the reciprocal of the lowest dilution that gave an absorbance of 0.1–0.15 units above the background. Background controls included assay plates coated with 5 μg/ml of GST as well as evaluation of preimmune samples. The reproducibility of the ELISA after three repeated assays of the same sample of serum was within one dilution.

Oligonucleotides

1: GAA TTC GGA GCG GCC GCT GGA GGT TGG TCA TCA AAA
2: TGA CTC GAG GCG CGC GGC CTG AGG ATG ACT GTC
3: GAA TTC GGA GCG GCC GCT CAG TGG AAT TCC ACT GCC
4: TGA CTC GAG GCG CGC GTT CGT CAC AGG GTC CCC
5: GGC TGC TCA GGA ATT CCT TGG GTG GCT TTG GGG C
6: CAA CCC CGA GGC GCG CAA CAA CAG TAG TTT CCG G
7: AGT TAT TTC AAT CAG ACA AT
8: TGA GTT TTC ATG TGC GCC CAC
9: TTC CCG GGT CGA CTC CAG TGG AAT TCC ACT
10: GTC ACG ATG CGG CCG CTC GTT CGT CAC AGG GTC
11: GTT CCG CGT GGA TCC CTT GGG TGG CTT TGG
12: GTC ACG ATG CGG CCG CTC AAC AAC AGT AGT TTC
13: GCC CAC TTT CTG TGA
14: GCT GCT CAG GAA TTC CTT GGG TGG CTT TGG G
15: CCA ACC CCG AGG CGC GCA ACA ACA GTA GTT TCC GG
16: AGC CTC GCC TCC ACC TCC GCC AAC CCC GAG GCG CGC AAC AAC AGT AGT TTC
17: CGG AGG TGG AGG CGA GGC TTT GTT TCA AGG TGC TCA GGT TTC ATC
18: TAC CTG GGT GGA GCT CCA TCA GTT GGA CCC T
19: ACG ACT GGC CAG CAC TCG AGA ATA ATT T



B

rec-POLIO	INSERT	LENGTH	
HBV.S1	pre S1Ag	354 nt	118 aa
HBV.S2	pre S2Ag	162 nt	54 aa
HBc.155	core	465 nt	155 aa
HBc.pS1	core/preS1	555 nt	185 aa
HBc.pS2	core/preS2	507 nt	169 aa

FIG. 1. (A) Schematic diagram of a recombinant poliovirus vector and strategy for expression of HBV proteins. The bar represents recombinant poliovirus genomic RNA. Viral genes are indicated within corresponding boxes, and the exogenous sequence at the 5′-end of the open-reading frame is indicated by a striped box. Sequences surrounding the insertion point of the exogenous sequence are detailed: the start codon, additional amino acids flanking the exogenous sequence providing a restriction enzyme polylinker, a poly-glycine tract, the $3C^{pro}$ artificial cleavage site, and the amino terminus of the viral polyprotein. Following translation of the viral RNA, a larger than wild-type polyprotein is produced, but proteolytic processing of the natural and artificial cleavage sites (indicated by triangles) results in the release of the foreign peptide and the generation of mature and functional viral proteins. (B) Denomination of each HBV-recombinant poliovirus constructed. The inserted HBV proteins and their lengths (in nucleotides and amino acids) are indicated.

20: GAG CTC CCC AGA GTC AGG GGT CTG TAT CTT CCT GCT GGT CTC GAG
21: CTC GAG ACC AGC AGG AAG ATA CAG ACC CCT GAC TCT GGG GAG CTC

RESULTS

Replicative characteristics of HBV-recombinant poliovirus

The poliovirus vector (pMoV-1.4) was constructed from biologically active cDNA clones of wild-type Mahoney type 1 strain, as recently described (Andino *et al.,* 1994). Mahoney rather than Sabin type 1 was chosen because the available strain of susceptible mice does not well support Sabin type 1 replication (Andino, 1994). Five chimeric polioviruses were prepared by inserting nucleotide sequences encoding for HBV antigens into MoV-1.4 (Fig. 1B). The antigens include the

amino-termini of preS1 and preS2 HBV presurface antigens (118 and 54 amino acids, respectively); 155 amino acids from the amino terminus of the HBV core protein; and HBV core hybrids that display immunogenic epitopes derived from preS1 (amino acids 27 to 53: HQLDPAFGANSNNPDWDFNPVKDDWPA) and preS2 (amino acids 133 to 143: PRVRGLYLPAG) on their surface (inserted between amino acid 79 and 80). It has been shown that peptide immunogenicity can be greatly enhanced by presentation on the surface of HBV core particles (Schodel *et al.*, 1993); thus, antigenic portions of the HBV preS proteins were inserted in a region of the core sequence shown to be expressed on the surface of the core particle (Crowther *et al.*, 1994).

All chimeric poliovirus-cDNAs yielded replication-competent viruses after transfection of HeLa cells with *in vitro* synthesized RNA. At 32° and at 37°, recombinants HBV.S1, HBV.S2, and HBc.155 produced plaques similar in size to wild-type plaques. However, at 39°, recombinants produced plaques that were smaller than those corresponding to wild-type virus (Fig. 2A). A one-step growth curve of poliovirus wild-type and recombinants confirmed the temperature-sensitive nature of the chimeric poliovirus (Fig. 2B). At 39° and at 37°, both wild-type and recombinants achieved maximal titers at 6 hr postinfection. However, recombinant viruses replicated more slowly than the parental wild-type, and yielded approximately 1% of wild-type titers. At 32°, both wild-type and recombinants exhibit delayed replication, but by 12 hr postinfection, chimeric viruses achieved 50–70% of wild-type titers. Replication of recombinants HBc.pS1 and HBc.pS2 in HeLa cells was indistinguishable from HBc.155 (data not shown).

To determine the genetic stability of recombinant polioviruses, the regions of the genome containing the inserted sequences were amplified by RT–PCR (Fig. 2C). Primers were chosen to amplify either wild-type or recombinant sequences around the site of insertion. Because the PCR assay is semiquantitative, the ratio between bands corresponding to wild-type and recombinant sequences should represent ratio between recombinant and revertants present in a given viral stock. The genetic structure of recombinant HBV.S2, HBc.155, HBc.pS1, and HBc.pS2 were stable after six rounds of replication in HeLa cells. Recombinant HBV.S1 appears to be less stable; the inserted sequences were fully retained for only four rounds of replication, and smaller bands corresponding to wild-type sequences emerged after the fifth and sixth passages. However, even in this recombinant, a substantial proportion of the virus population still retained the foreign insert after the sixth passage. Based on these results, all further experiments presented in this report were performed using viral stocks obtained after three passages in HeLa cells.

Expression of the foreign proteins by poliovirus chimeras

To determine whether recombinant viruses expressed the foreign polypeptides, HeLa cells were infected with wild-type and chimeric polioviruses, and cytoplasmic extracts were obtained at different times following infection and analyzed by immunoblotting. Poliovirus capsid proteins were produced and correctly processed in recombinants HBV.S1 and HBV.S2; however, poliovirus proteins accumulated at lower levels in recombinant infected cells than in wild-type infected cells (Fig. 3A, lanes 1–10). All recombinants also expressed the expected foreign proteins in the infected cells as detected by antibodies directed against the foreign proteins (Fig 3A, lanes 13, 14, 17, and 18; Fig. 3B, lanes 6–17). In cells infected with recombinant HBV.S1, anti-preS1 antibodies detected a single polypeptide (Fig. 3A, lanes 13 and 14). The molecular weight of this polypeptide suggests that the detected band corresponds to the preS1 free polypeptide. Recombinant HBV.S2 produced two distinct bands that were recognized by anti-preS2 antibodies (Fig. 3A, lanes 17 and 18). Based on molecular weight (see Fig. 3 legend), the larger band probably corresponds to a fusion between preS2 and Vp0 + Vp3, and the smaller band to the free preS2 protein. Extracts from cells infected with recombinants HBc.pS1 and HBc.pS2 displayed only a single immunoreactive polypeptide band of expected molecular weight corresponding to the HBV core protein fused to the antigenic peptide derived from preS1 and preS2 proteins (Fig. 3B, lanes 10–13 and 14–17, respectively). Extracts from cells infected with poliovirus recombinant HBc.155 showed three polypeptides that reacted with anti-HBV core antibodies. Based on molecular weight, these bands probably correspond to fusions between the HBV core and capsid precursors Vp0 or Vp0-Vp3 and the free HBV core protein (Fig. 3B, lanes 6–9).

RNA synthesis of poliovirus recombinants

Replication characteristics of poliovirus recombinants were further analyzed by measuring their ability to synthesize viral RNA. HeLa cells were infected with wild-type or recombinant viruses, and infected cells were incubated at 37° in the presence of [^{3}H]uridine and Actinomycin D to inhibit cellular RNA synthesis. Incorporation of [^{3}H]uridine into viral RNA was determined by acid precipitation at several time points. Rates of synthesis for all recombinant polioviruses were very similar to that of the wild-type virus (Fig. 4A), suggesting that reduction in replication indicated by the one-step growth curve does not result from impaired RNA synthesis (see Discussion).

Cesium chloride gradient analysis

The physical properties of the recombinant poliovirions were examined using cesium chloride (CsCl) gra-

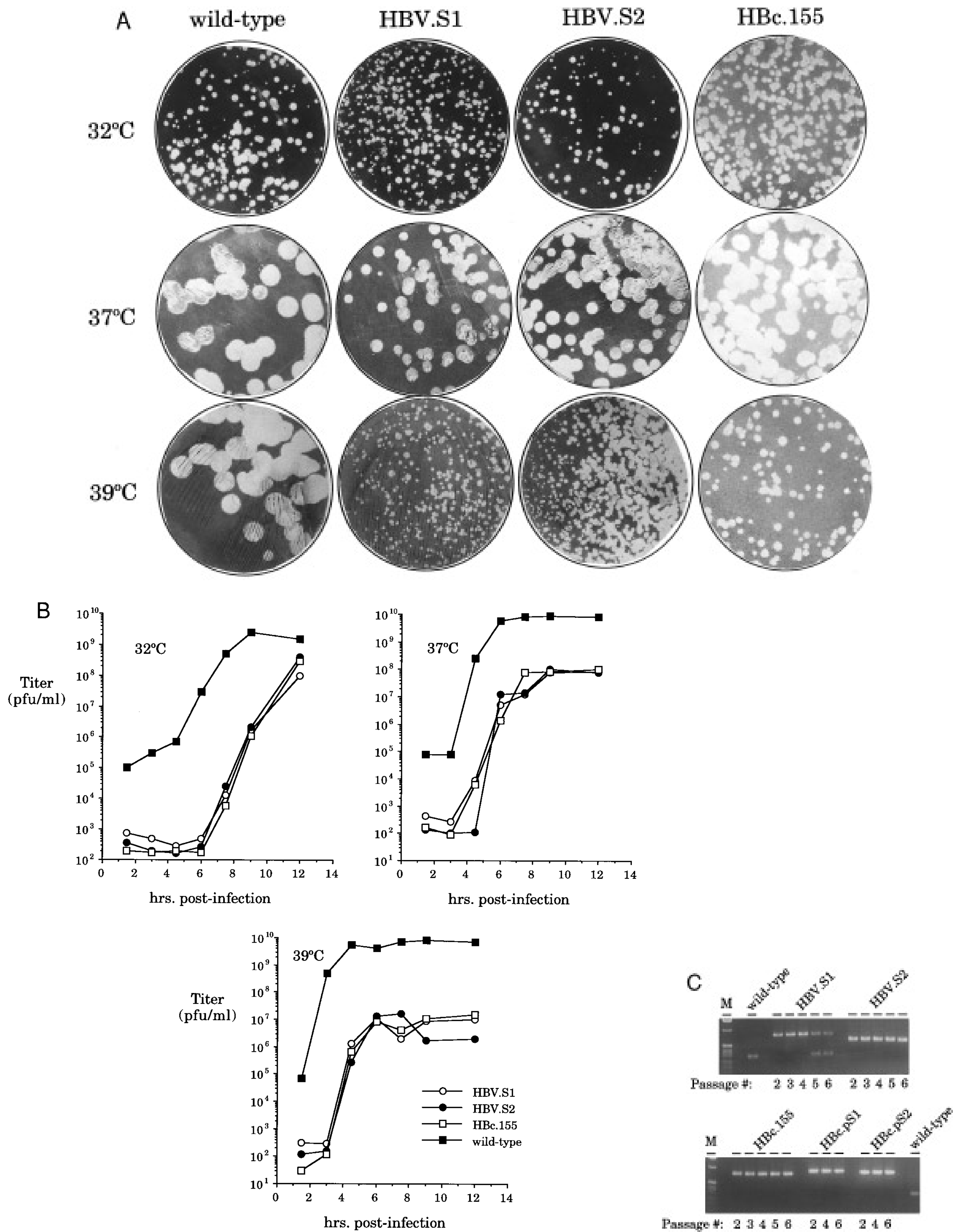


FIG. 2. Growth characteristics of poliovirus recombinants. (A) Plaque assays of wild-type and recombinant HBV.S1 (carrying 118 amino acids of HBV preS1), HBV.S2 (54 amino acids of HBV preS2), and HBc.155 (155 amino acids of HBV core) were performed in HeLa cells at different temperatures (32°, 37°, and 39°) for 48 hr. (B) One-step growth curves for three recombinant polioviruses. HeLa cell monolayers were infected (m.o.i.

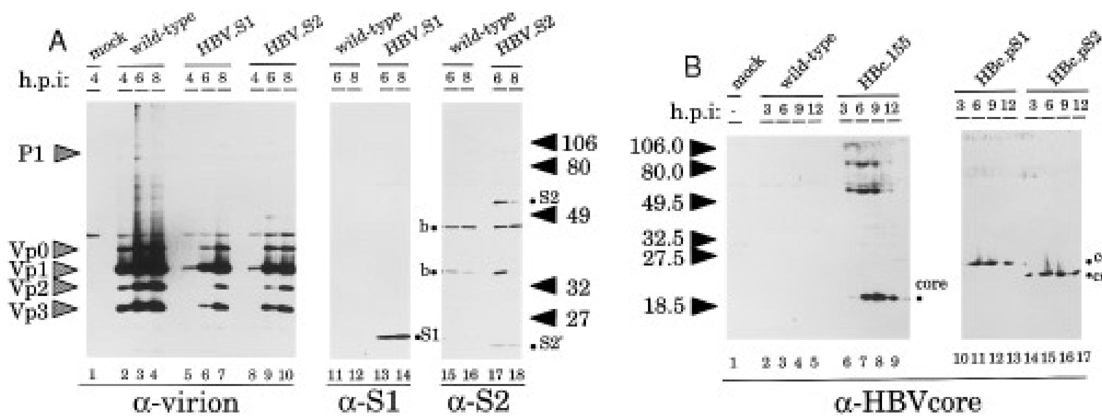


FIG. 3. Expression and processing of the exogenous proteins in cells infected with recombinant polioviruses. Cytoplasmic lysates from HeLa cells infected with wild-type poliovirus or various recombinant viruses were analyzed by Western blot with antibodies directed against poliovirus virions [α-capsid, (A) lanes 1–10], preS1[α-S1, (A) lanes 11–14], preS2 [α-S2, (A) lanes 15–18], HBV core [α-HBV-core, (B) lanes 1–17]. Mock: mock-infected HeLa cells; wild-type: poliovirus wild-type; HBV.S1: HBV preS1-recombinant; HBV.S2: HBV preS2-recombinant; HBc.155: HBV core-recombinant; HBV.pS1: HBV core fused to preS1 peptide-recombinant; and HBV.pS2: HBV core fused to preS2 peptide-recombinant. Poliovirus capsid proteins are indicated by shaded triangles. Molecular weight markers indicate relative mobility. Bands detected by specific antibodies directed against the inserted sequences are indicated by dots: (S1) free preS1 (16.5 kDa); (S2) preS2-Vp0 + Vp3 fusion (64 kDa); (S2′) free preS2 (9.7 kDa); (core) free HBV core (19.5 kDa), (core.S1) HBV core containing preS1 26 amino acid peptide (23 kDa), and (core.S2) HBV core containing preS2 11 amino acid peptide (21 kDa). Background bands recognized by anti-S2 antibodies are represented by a "b."

dients. Viruses were metabolically labeled by infecting HeLa cells with wild-type or recombinant polioviruses in the presence of [^{35}S]methionine. Eight hours postinfection, viruses were extracted from infected cells in buffer H, then loaded onto a 1.4 g/ml CsCl solution. Following centrifugation, radioactivity present in each fraction was determined by scintillation counting (Fig 4B). The buoyant densities determined for wild-type and recombinant viruses were almost identical (1.35 g/ml), suggesting that the recombinant particle is very similar to the wild-type particle. It is interesting that samples from HBV.S2- and HBc.155-infected cells showed more radiolabeled material at the top of the gradient, perhaps reflecting instability of the recombinant virus particles under the gradient conditions. It is somewhat surprising that the larger size of the genome of recombinant viruses (102 to 106% of the wild-type size) did not result in a higher density particle; perhaps the difference in density (1.38 g/ml vs 1.35 g/ml) is too small to be detected under our experimental conditions, or the volume of the recombinant viral particle is changed by the larger genome of recombinant viruses.

Because the HBV peptides are expressed as fusions to the poliovirus capsid proteins, we asked whether some foreign antigens may remain fused and become incorporated into recombinant particles. CsCl-purifed recombinant viruses were analyzed by Western blot using antibodies against the foreign proteins. None contained detectable foreign proteins (data not shown), suggesting that exogenous proteins are excluded from the mature recombinant virus particle.

HBV core particle formation

The HBV core particle is a strong immunological adjuvant. Since peptides fused to the HBV core protein elicit longer-lasting and much stronger antibody responses against the inserted peptides than do the free peptides, it has been proposed that HBcAg may act as an efficient T-cell carrier moiety (Schodel *et al.*, 1993; Milich *et al.*, 1987). In fact, the HBV core antigen has been shown to efficiently elicit responses from both T cells and B cells (in a T-cell-independent manner). This enhanced immunogenicity appears to depend in part on the ability of HBV core hybrids to assemble into particles that display on their surface the inserted antigenic peptide (Schodel *et al.*, 1993; Clarke *et al.*, 1988; Milich *et al.*, 1987).

To determine whether HBV core, expressed by the poliovirus recombinants, indeed assembled into particles, HeLa cells were infected with the recombinant HBc.155 for 7 hr, after which cytoplasmic extracts were analyzed by sucrose gradient sedimentation followed by immunoblotting. Fractions 8 to 12 (∼100s) contained polypeptides that were detected with anti-HBV core antibodies (Fig. 5). The molecular weights of these polypeptides suggest that HBV particles formed after poliovirus

= 10) with wild-type poliovirus, recombinant HBV.S1, HBV.S2, and HBc.155. Virus production (PFU/ml) was determined at each time point. (C) Analysis of the stability of recombinant poliovirus genomes. HeLa cells were infected with recombinant viruses obtained after 2, 3, 4, 5, or 6 successive passages in HeLa cells. The presence of the foreign sequence insertion was analyzed by RT-PCR, using total cytoplasmic RNA of infected cells as a template for reverse transcription.

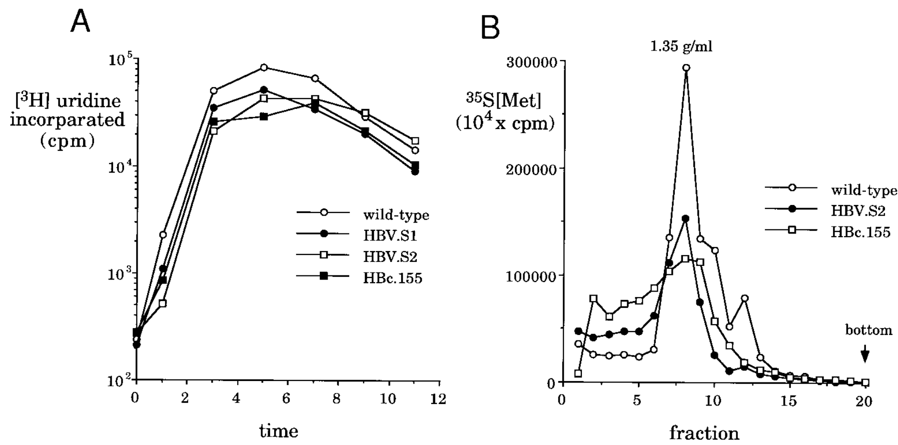


FIG. 4. (A) Viral RNA synthesis of wild-type and recombinant polioviruses. HeLa cells were infected with an m.o.i. = 10 and incubated at 37° in DMEM containing 5 μg/ml of actinomycin D and 20 μCi/ml of [^{3}H]uridine. Cytoplasmic extracts were obtained at each time and [^{3}H]uridine incorporated to an acid-insoluble fraction was measured by scintillation counting. (B) Cesium chloride gradient of wild-type and recombinant polioviruses. Metabolically labeled viruses were submitted to centrifugation in CsCl gradient. Aliquots of 0.5 ml were collected and radioactivity determined. The buoyant density determined by weighing each fraction was 1.35 g/ml for both wild-type and recombinant particles.

infection consist of free HBV core protein and fusions of the HBV core protein with poliovirus Vp0 or Vp0–Vp3. In addition, antibodies directed against poliovirus capsid proteins immunoprecipitated HBV free core polypeptide from HBc.155 infected-cell extracts, suggesting that the HBV core particle is constituted by free and fused HBV core protein. It is remarkable that the HBV particle could still be formed by the HBV core protein fused to a large portion of poliovirus capsid proteins.

Immune responses to the HBV sequences

The immunogenicity of the recombinant poliovirus was examined in transgenic mice that express the human poliovirus receptor and are susceptible to poliovirus infection (Ren *et al.,* 1990). Mice were inoculated intraperitoneally on four occasions (0, 2, 5, and 8 weeks), either with HBV.S2 or HBV.155, or controls (wild-type poliovirus or saline). Serum samples were obtained at the day of the first inoculation and at 2, 5, 8, and 11 weeks after. The induction of specific antibodies directed against HBV proteins was analyzed by ELISA. Serum antibodies recognizing HBV preS2 and core polypeptides were readily detected in all poliovirus recombinant-infected animals within 2 weeks of the first inoculation (Fig. 6). Interestingly, antibody response elicited against the HBV proteins achieved similar titers to those obtained against poliovirus capsid proteins (~10^3 reciprocal dilution; data not shown). In addition, wild-type and recombinant poliovirus elicited similar antibody response against poliovirus capsid proteins. Finally, though all recombinants used are based on wild-type type 1 Mahoney strain none of the mice were paralyzed, suggesting that the insertion of foreign sequences at the polyprotein amino terminus somehow attenuates poliovirus virulance.

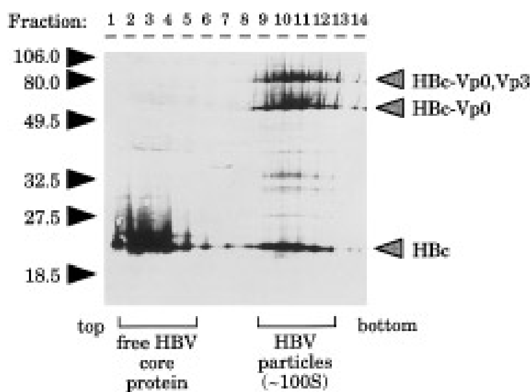


FIG. 5. HBV core particles produced in recombinant poliovirus-infected cells. Extracts from HeLa cells infected with the HBc.155-recombinant poliovirus (carrying 155 amino acids of HBV core) were submitted to sucrose density gradient analysis (10 to 60%). Aliquots from each fraction were analyzed by 10% SDS–PAGE and by immunoblots using antibodies directed against HBV core. Molecular weight markers indicate relative mobility. Bands corresponding to the HBV core (19.5 kDa), HBV core-Vp0-Vp3 (83 kDa), and HBV core-Vp0 (57 kDa) fusions are indicated by shaded arrows.

DISCUSSION

In this study, we have shown that replication-competent poliovirus can be generated when HBV antigens are fused to the poliovirus polyprotein. In general, recombinants replicated more slowly than the wild-type virus, and the difference was greater at 39° than at 32°. Perhaps faster replication rates at higher temperatures accentuate slight defects in one or more replicative steps.

Because RNA viruses in general lack efficient proofreading mechanisms, one concern about using poliovirus as a vaccine vector is that inserted sequences may be genetically unstable. We found that the recombinants described here stably carry foreign sequences for at

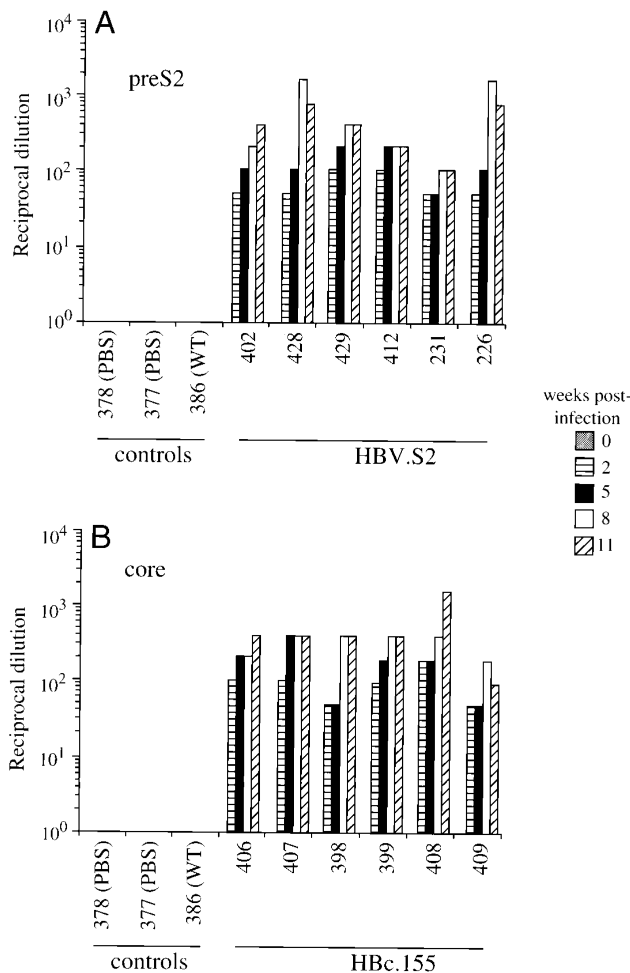


FIG. 6. Immunization of poliovirus receptor (PVR)-transgenic mice with the HBV.S2- or HBC.155-recombinant poliovirus elicited a humoral response against the HBV inserted proteins. Six PVR-transgenic mice (Nos. 402, 428, 429, 412, 226, and 231) were infected with the HBV preS2-recombinant virus (HBV.S2), and six mice (Nos. 406, 407, 398, 399, 408, 409) with HBV core-recombinant poliovirus (HBc.155). As a control mice were inoculated with either poliovirus wild-type (WT) or saline (PBS). Animals were infected by intraperitoneal injection with 100 μl of 1×10^8 PFU/ml of viral stock; they received four identical inoculations separated by a period of 2 or 3 weeks. At 0, 2, 5, 8, and 11 weeks after the first inoculation serum samples were obtained and analyzed by ELISA, using multiwell plates coated with preS2 (A) or HBV core (B) GST-fusion proteins. Antibodies reacting with the HBV proteins were visualized with anti-mouse total Ig-specific second-stage antibodies conjugated to horseradish peroxidase.

least six rounds of replication in tissue culture (Fig. 2C). However, one of them (HBV.S1) appears to be somewhat less stable since deletions were detected after four to five passages in tissue culture. We currently do not know the reason for this phoneme. However, because HBV.S1 carries a smaller insert than some of the other chimeras (i.e., HBc.155, HBc.pS1, HBc.pS2) it seems that the nature of the inserted sequences, rather than their size, plays an important role in determining the genetic stability of the recombinant.

We measured the kinetics of viral RNA synthesis in wild-type and recombinant polioviruses and found no deleterious effect of the insert. Because most poliovirus polypeptides, except the structural proteins, participate in RNA synthesis, the growth defect observed for poliovirus recombinants is more likely to relate to capsid formation, RNA packaging, or particle release.

Expression of the HBV core protein by HBc.155 results in production of HBV hybrid particles composed of free HBV core protein and fusions of HBV core to Vp0 or Vp0–Vp3. It appears that the poliovirus protease inefficiently cleaves at the artificial site once the HBV hybrid particle has been formed, since fusions of HBV core and poliovirus capsid proteins are readily detected in fractions containing HBV particle (Fig. 5, lanes 9–13). In contrast, fractions corresponding to free nonparticulate proteins (Fig. 5, lanes 1–5) contained no detectable fusion proteins.

All mice inoculated with poliovirus recombinants (HBV.S2 and HBc.155) developed an antibody response against the HBV proteins, whereas control mice (inoculated with nonrecombinant poliovirus or saline) showed no immunoreactivity (Fig. 6). Antibody titers elicited against preS2 and HBV core were similar to those produced against poliovirus capsid proteins (data not shown). Because the poliovirus capsid proteins are very immunogenic, it appears that preS2 and core expressed by poliovirus vectors are also highly immunogenic. However, it will be important to compare antibody titers obtained after exposure to recombinant poliovirus with those obtained by direct inoculation of HBV proteins as used in the current vaccine formulation. We did not attempt to address this question in the transgenic mouse model because it would be difficult to extrapolate any result to the human situation. A more relevant system (such as chimpanzees or humans) would be necessary to further investigate the potential of poliovirus recombinants as a human HBV vaccine.

In addition to the beneficial attributes of Sabin live attenuated vaccine, HBV-recombinant polioviruses would be expected to simultaneously elicit immunity against both poliovirus and HBV; indeed, is possible to imagine a broad range vaccination against multiple pathogens by using a "cocktail" of recombinant polioviruses, each expressing a different antigen.

ACKNOWLEDGMENTS

We are grateful to Drs. Don Ganem for plasmid encoding for HBV genome, Mena Ostapchuck for anti-preS1 antiserum, Patricia Reidly for anti-preS2 antiserum, and Deborah Silvera, Mark B. Feinberg, Alan Frankel, and Don Ganem for their useful comments on the manuscript. This work was supported by funds provided by the Department of Microbiology and Immunology, University of California, San Francisco, and the Gladstone Institute of Virology and Immunology to R.A, and a graduate student fellowship from Genentech Foundation to T.J.Y.

REFERENCES

Alexander, L., Lu, H. H., and Wimmer, E. (1994). Polioviruses containing picornavirus type 1 and/or type 2 internal ribosomal entry site ele-

ments: Genetic hybrids and the expression of a foreign gene. *Proc. Natl. Acad. Sci. USA* **91,** 1406–1410.

Altmeyer, R., Escriou, N., Girard, M., Palmenberg, A., and van der Werf, S. (1994). Attenuated Mengo virus as a vector for immunogenic human immunodeficiency virus type 1 glycoprotein 120. *Proc. Natl. Acad. Sci. USA* **91,** 9775–9779.

Altmeyer, R., Girard, M., Van Der Werf, S., Mimic, V., Seigneur, L., and Saron, M. (1995). Attenuated Mengo virus: A new vector for live Recombinant vaccine. *J. Virol.* **69,** 3193–3196.

Andino, R. (1994). Unpublished results.

Andino, R., Rieckhof, G. E., Achacoso, P. D., and Baltimore, D. (1993). Poliovirus RNA synthesis utilizes an RNP complex formed around the 5′-end of viral RNA. *Embo. J.* **12,** 3587–3598.

Andino, R., Silvera, D., Suggett, S. D., Achacoso, P. L., Miller, C. J., Baltimore, D., and Feinberg, M. B. (1994). Engineering poliovirus as a vaccine vector for the expression of diverse antigens. *Science* **265,** 1448–1451.

Ansardi, D. C., Pal Ghosh, R., Porter, D., and Morrow, C. D. (1995). Encapsidation and serial passage of a poliovirus replicon which expresses an inactive 2A proteinase. *J. Virol.* **69,** 1359–1366.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994). *Curr. Protocols Mol. Biol.* **1,** 3.0.1–3.19.8.

Bernstein, H. D., Sonenberg, N., and Baltimore, D. (1985). Poliovirus mutant that does not selectively inhibit host cell protein synthesis. *Mol. Cell. Biol.* **5,** 2913–2923.

Burke, K. L., Almond, J. W., and Evans, D. J. (1991). Antigen chimeras of poliovirus. *Prog. Med. Virol.* **38,** 56–68.

Burke, K. L., Dunn, G., Ferguson, M., Minor, P. D., and Almond, J. W. (1988). Antigen chimaeras of poliovirus as potential new vaccines. *Nature* **332,** 81–82.

Choi, W. S., Pal-Ghosh, R., and Morrow, C. D. (1991). Expression of human immunodeficiency virus type 1 (HIV-1) gag, pol, and env proteins from chimeric HIV-1-poliovirus minireplicons. *J. Virol.* **65,** 2875–2883.

Clarke, B. E., Newton, S. E., Carroll, A. R., Francis, M. J., Appleyard, G., Syred, A. D., Highfield, P. E., Rowlands, D. J., and Brown, F. (1988). Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Br. Med. J. (Clin. Res. Ed.)* **296,** 381–384.

Crowther, R. A., Kiselev, N. A., Bottcher, B., Berriman, J. A., Borisova, G. P., Ose, V., and Pumpens, P. (1994). Three-dimensional structure of hepatitis B virus core particles determined by electron cryomicroscopy. *Cell* **77,** 943–950.

Dowdle, W. R., and Orenstein, W. A. (1994). Quest for life-long protection by vaccination. *Proc. Natl. Acad. Sci. USA* **91,** 2464–2468.

Evans, D. J., McKeating, J., Meredith, J. M., Burke, K. L., Katrak, K., John, A., Ferguson, M., Minor, P. D., Weiss, R. A., and Almond, J. W. (1989). An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralizing antibodies. *Nature* **339,** 385–388.

Hollinger, F. B. (1990). Hepatitis B virus. *In* "Virology" (B. N. Fields and D. M. Knipe, *et al.*), pp. 2171–2236. Raven Press, New York.

Krugman, S. (1988). Hepatitis B vaccine. *In* Vaccines (S. A. Plotkin and E. A. Mortimer, Jr., Eds.), pp. 458–473. Saunders, Philadelphia.

La Montagne, J. R., and Rabinovich, R. (1992). Challenges and opportunities in vaccine research. *In* "Genetically Engineered Vaccines" (J. E. Ciardi, J. R. McGhee, and J. M. Keith, Eds.), pp. 293–299. Plenum Press, New York.

Lu, H., Alexander, L., and Wimmer, E. (1995). Construction and genetic analysis od dicistronic polioviruses containing open reading frame for epitopes of human Immunodeficiency virus type 1 gp120. *J. Virol.* **69,** 4797–4806.

Luthman, H., and Magnusson, G. (1983). High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res.* **11,** 1295–1308.

Mattion, N. M., Reilly, P. A., DiMichele, S. J., Crowley, J. C., and Weeks-Levy, C. (1994). Attenuated poliovirus strain as a live vector: expression of regions of rotavirus outer capsid protein VP7 by using recombinant Sabin 3 viruses. *J. Virol.* **68,** 3925–3933.

Milich, D. R., McLachlan, A., Moriarty, A., and Thornton, G. B. (1987). Immune response to hepatitis B virus core antigen (HBcAg): Localization of T cell recognition sites within HBcAg/HBeAg. *Nature* **329,** 547–549.

Porter, D. C., Ansardi, D. C., and Morrow, C. D. (1995). Encapsidation of poliovirus replicons encoding the complete human immunodeficiency virus type 1 gag gene by using a complementation system which provides the P1 capsid protein in trans. *J. Virol.* **69,** 1548–1555.

Ren, R. B., Costantini, F., Gorgacz, E. J., Lee, J. J., and Racaniello, V. R. (1990). Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. *Cell* **63,** 353–362.

Schodel, F., Moriarty, A. M., Peterson, D. L., Zheng, J. A., Hughes, J. L., Will, H., Leturcq, D. J., McGee, J. S., and Milich, D. R. (1993). The position of heterologous epitopes inserted in hepatitis B virus core particles determines their immunogenicity. *J. Virol.* **66,** 106–114.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione *S*-transferase. *Gene* **67,** 31–40.

Trono, D., Andino, R., and Baltimore, D. (1988). An RNA sequence of hundreds of nucleotides at the 5′ end of poliovirus RNA is involved in allowing viral protein synthesis. *J. Virol.* **62,** 2291–2299.

Zhou, S. L., and Standring, D. N. (1991). Production of hepatitis B virus nucleocapsidlike core particles in Xenopus oocytes: Assembly occurs mainly in the cytoplasm and does not require the nucleus. *J. Virol.* **65,** 5457–5464.