Title
Development of a novel Hepatitis B virus vaccine using poliovirus as a vaccine vector

Author
Yim, Taejin,

Publication Date
1995

Peer reviewed|Thesis/dissertation

Development of a Novel Hepatitis B Virus Vaccine Using Poliovirus as a Vaccine Vector

by

Taejin Yim

THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

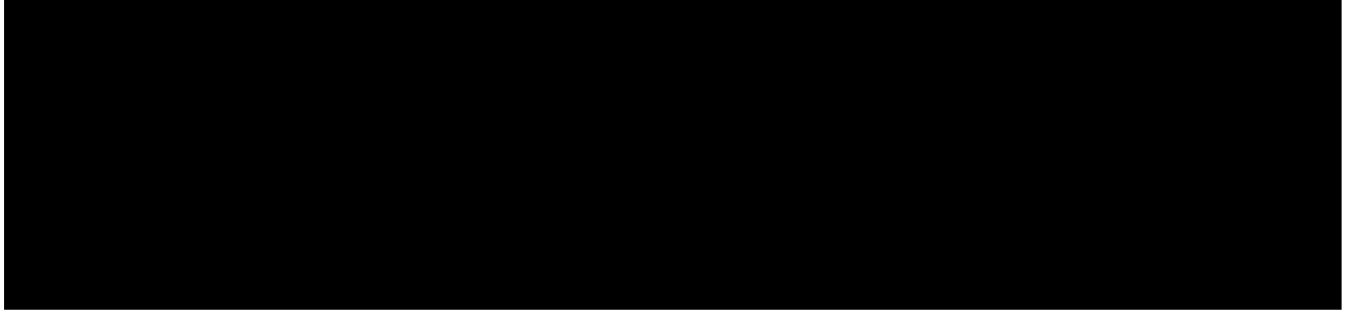


TABLE OF CONTENTS

INTRODUCTION

VACCINE: HISTORY AND SIGNIFICANCE

Rudimentary practice of vaccination is traceable back to eleventh century China, where wisemen inoculated children with the extracts of the smallpox pustules, in an attempt to reduce the detrimental effects of smallpox infection. It was not until 1798, however, when the first recognized successful vaccine was developed. English doctor Edward Jenner, made the observation that milkmaids caught a mild form of "the pox" from cows which protected them from the more harmful effects of smallpox infection. This simple, yet, crucial realization led to the development of a vaccination procedure that used pustules from cows infected with cowpox virus. Recipients of this vaccine (the term derives from the Latin *vacca* meaning cow) suffered only mild symptoms and were spared from the devastating effects of the smallpox disease (15). Since then, the development of vaccines has become increasingly sophisticated and pursued with vigor for its medical, social, and economical importance (8, 11).

The ultimate goal of any vaccine is to protect individuals from identified pathogens which cause injury, disability, and death. Hence, a successful vaccine should be safe, effective, and prevent or limit initial pathogen replication. In addition, the vaccine should be easy to store, transport, and administer in a cost effective manner. The latter requirements could have the greatest impact in the developing world, where most of the viral epidemics occur and vaccines could have the greatest impact in public health. The beneficial attributes of Sabin live, attenuated poliovirus vaccine has greatly facilitated in controlling the widespread paralytic poliomyelitis epidemic. The live poliovirus vaccine is easily administered by the oral route, has a low cost for distribution, induces both serum antibodies and intestinal mucosal resistance, and confers long lasting immunity (8).

Given the favorable characteristics of the Sabin poliovirus vaccine, number of investigators have attempted to adapt poliovirus as a vector to express antigens from other pathogens. This report presents the results of the construction and characterization of replication-competent recombinant poliovirus that stably express diverse HBV antigens through a novel approach. We hope that this novel method of engineering recombinant poliovirus that carry and express HBV antigen will yield an improved novel HBV vaccine.

HEPATITIS B VIRUS

There are more than 250 million people today infected with HBV worldwide. The majority of these individuals live in "third world" countries; for example, in Southeast Asia, China, and sub-Saharan Africa, prevalence of HBV infection reaches as high as 5-20% of the total population. In the U.S., where the prevalence rate is among the lowest (0.1-1%) in the world, approximately 200,000 cases of new HBV infections occur each year (10, 13). Despite the existence of two effective vaccines (plasma derived and HBV surface-antigen obtained by DNA recombinant techniques), HBV continues to be a major cause of acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (10). The lack of success in controlling HBV infection in highly endemic areas, is attributed to several major factors: lack of an inexpensive (a crucial criterion since most of the HBV carriers and infections occur in the developing and therefore poorer nations), and effective vaccines; lack of an appropriately tested strategy for the use of current vaccines; the complicated current vaccination regimen; and the need for revaccination (13). These factors represent a significant handicap for the current HBV vaccines, as evidenced by the United Nations' Children's Vaccine Initiative's recognition of HBV vaccine as one of the existing vaccines that needs to be improved (14). We hope that

some of the above criteria for an effective vaccine could be addressed by the HBV recombinant-poliovirus approach reported here.

MUCOSAL IMMUNE SYSTEM

The major mode of horizontal transmission for HBV is through sexual contact. Although the biology of sexual transmission of HBV is poorly understood, it is clear that the first essential step in this process is the deposition of infectious virus in the mucosal surfaces of the vagina or the rectum of the recipient. For a vaccine to be effective in blocking the horizontal transmission of HBV, it must be able to stop the virus or significantly reduce the viral burden at the portal of entry. Therefore, likely way of interrupting HBV transmitted by sexual routes would be to have an immune barrier extending the mucosal surfaces. Hence, the process of induction and the concept of mucosal immunity is of particular interest (24, 28). The mucosal membrane surface area in humans covers over 400 square meters (largely, in the gut) and serves as the portal of entry for many pathogens. The mucosal immune system (MIS) serves to protect this vulnerable border against both local and systemic disease (22, 37). The MIS produces 70% of the body's antibodies, and has been shown to prevent passage of pathogens through the mucosal surface, as well as triggering the systemic immune response (35). The MIS is anatomically and functionally divided into inductive and effective sites. The major inductive sites lie in the gastrointestinal (GI) tract. These gut-associated lymphoreticular tissues (GALT) consist of Peyer's patches (PP), appendiceal, colonic and cecal patches, and isolated lymphoid aggregates. Along with mesenteric lymph nodes, and the lymphoid elements of the gut epithelium, and lamina propria, GALT account for most of body's lymphoid mass (18). These groups of lymphoid tissue are responsible for inducing the cell-mediated and the humoral immune responses. Among the GALT, the PP-organized lymphoid tissues of the small intestine-play a significant role in the

function of the MIS at the inductive sites. The epithelium of the PP contains specialized cells known as microfold cells which transport intact antigens gathered at the apical membrane (the surface exposed to the lumen of the GI tract) across the cell to the basolateral membrane, where the antigens may be passed on to antigen-presenting cells (APCs). APCs further process these antigens, and present them to other cells of the immune system (such as T and B lymphocytes), via the regional and the systemic lymph nodes and via the blood circulation. These ultimately mount a concerted immune response against the foreign antigen at the effector sites in the genital mucosa, salivary glands, respiratory tracts, pharynx, and mammary glands. The most prevalent and important immunoglobulin of the MIS is the secretory IgA. Released by B lymphocytes along the mucosal surface, this antigen-specific antibody is present in external secretions (*e.g.* , saliva, tears, *etc.*), where it binds to foreign antigens to form immune complexes which serve to inhibit the interaction of the bound antigen with the mucosal surface, thus attenuating microorganisms' ability to enter the host (40). This protective mechanism of the MIS may be the key in achieving the needed protection against pathogens that are introduced through sexual activity. Hence, the theoretical and the practical hope in harnessing the MIS, in regards to vaccine development, is to stop the HBV before it enters the human host at the mucosal membrane, the portal of entry, by producing specific antibodies at the remote effective sites and in the circulation by stimulating the inductive sites, thereby propagating the immune response throughout the host. Due to the compartmentalization of the secretory and the systemic immune systems, parentally administered vaccines do not consistently stimulate mucosal immunity. Relevant example is provided by the comparison between the Salk inactivated whole poliovirus vaccine and the Sabin attenuated live vaccine, both of which are highly effective in protecting individuals against paralytic poliomyelitis. The parenterally administered Salk vaccine induces a strong systemic antibody response that blocks viral dissemination to the blood stream and the central nervous system; however, it does not elicit significant

secretory IgA production, which blocks initial viral replication in the gut and compromises infected individuals' ability to transmit virulent poliovirus. On the contrary, by the virtue of its ability to replicate in the intestine, the Sabin live, attenuated poliovirus vaccine induces long-lasting mucosal immunity that greatly limits poliovirus replication following exposure to the virus. Sabin attenuated poliovirus vaccine replicates extensively in the regional and the systemic lymph nodes like their wild-type parents. Furthermore, it also induces levels of polio-reactive serum IgG and IgA antibodies equivalent to those seen following intramuscular administration of inactivated Salk vaccine (35, 37, 40, 41).

POLIOVIRUS

The picornaviridae family comprises a variety of humans and plants pathogens. Within this family, poliovirus is classified as an enterovirus as it enters the host through and propagates within the alimentary tract, resulting in various sequelae ranging from severe, permanent paralysis to minor or fatal unidentified febrile illnesses (1). Given its medical, social, and economical consequences, it is not surprising that the development and the study of modern virology and vaccinology have focused greatly on the picornaviridae family (20, 33).

Poliovirus is presumed to be an ancient human pathogen, but poliomyelitis as a paralytic disease did not emerge as a clinical problem until the late 19th century, when epidemic cases began to appear in northern Europe (31). The epidemic form of poliomyelitis, as opposed to the endemic form, in which paralysis seldom occurs, most likely due to viral exposure early in life, resulted directly from increased economic and social development which resulted in improved community and personal hygiene. This reduced the opportunities for infections among young children and infants, and accordingly, increased the number of people who encountered poliovirus in later

childhood or in adult life, when infections are more likely to cause paralytic disease (20). With the introduction of poliovirus vaccine however, the disease is under control in many countries and where it is not, it is well on its way. It is notable that the present success of polio vaccination has been achieved without understanding the molecular basis of the virus (4).

The poliovirus genome is a single strand positive sense RNA molecule of approximately 7500 nucleotides. It is polyadenylated at the 3' end and carries a small viral protein VPg (virion protein, genome) linked to its 5' end, which is implicated in initiation of viral RNA synthesis and viral RNA packaging (33). The coding region of the genome is flanked by nontranslated regions (ntr). The 5' ntr is involved in both initiation of viral RNA translation and in viral RNA replication (12, 33). The viral genome contains a single reading frame, which is translated by a cap independent mechanism to produce a large single polyprotein precursor. This polyprotein must be proteolytically processed by two viral proteinases ($2A^{pro}$ and $3C^{pro}$) to generate mature poliovirus proteins. The viral proteins then serve to duplicate and encapsidate the viral RNA through a cascade of biochemical reactions, resulting in production of infectious viruses (Fig. 1A). $3C^{pro}$, the major viral protease, recognizes and cleaves at specific amino acid sequences (AXXQG) exposed within flexible polyprotein domains.

POLIOVIRUS AS A VACCINE VECTOR

Notable advantages of the existing oral poliovirus vaccine (OPV) are its documented efficacy (data on which has been compiled for 40 years of world wide usage), safety, affordability, and induction of mucosal immunity. Because of these characteristics, OPV has been genetically manipulated by several investigators for its use as a vaccine vector. In a pioneering effort, Almond *et al* and Girard *et al* created several poliovirus chimeras by substituting the nine amino acid residues of the linear B-C loop of

VP1 with antigenic sites of other pathogens, including HIV-1. VP1 is one of four structural viral proteins (VP1-VP4) that form the poliovirus capsid and present certain flexibility at the B-C loop. Their strategy was to create a replicating poliovirus recombinant from the live attenuated Sabin 1 strain which expresses epitopes of other foreign pathogens buried within the viral capsid, and thereby elicit immune responses against them (6, 9). Using the same approach, Girard *et al* synthesized a recombinant poliovirus that was able to elicit an immune response against inserted HIV-1 epitopes (7). This approach is limited, however, by the small size of tolerated insert sequences, the destruction of important antigenic epitopes of the poliovirus vaccine, and abrogation of viral replication by the introduced sequences. Wimmer's group has also constructed replication competent poliovirus by introducing a second internal ribosomal entry site (IRES), which converts poliovirus mRNA into a bicystronic messenger. In this manner, the foreign polypeptide can be expressed from the second IRES. The limitation to this approach is the genetic instability of the inserted sequences (second IRES and foreign sequences), which are deleted after few rounds of replication in tissue culture (1). Finally, Morrow *et al* used poliovirus minireplicon genomes in which VP2 and VP3 were replaced by foreign sequences, and obtained poliovirus chimera that expressed foreign proteins (HIV-1 *gag* and *pol*). However, in this case, it is necessary to provide *in-trans* poliovirus capsid protein from a helper virus in order to obtain encapsidated poliovirus minireplicons, and theoretically, they will undergo only one round of replication *in vivo* (27).

NOVEL APPROACH FOR THE GENERATION OF A HEPATITIS B VIRUS VACCINE

Our replication competent recombinant poliovirus vaccine vector is designed to serve as a vehicle that is able to propagate from cell to cell without the need of a helper

virus and that will stably carry and express genetic sequences of other pathogenic agents (2). This approach utilizes basic aspects of the viral life cycle. Through the genetic manipulation of poliovirus molecular clones, we have inserted additional amino acid sequences at the amino terminus of the poliovirus polyprotein precursor and placed an artificial recognition sequences for the poliovirus 3C protease. In this way, a larger than normal precursor is initially made, but is appropriately cleaved into the usual array of constituent proteins by the 3C protease. The 3C protease accurately recognizes and cleaves the inserted synthetic proteolytic site, freeing the exogenous protein sequences from the Vp0 protein. Thus, all of the poliovirus proteins are correctly produced and normal viral replication proceeds (Fig. 1A). The inserted amino acid sequences are produced in significant amounts in infected cells, but are not included in the poliovirus particle (2, 26, 33).

Using the above vector, we have constructed the following poliovirus chimeras: HBV.S1 (poliovirus expressing preS1 region of HBV), HBV.S2 (poliovirus expressing preS2 region of HBV), and HBc.155 (poliovirus expressing core region of HBV and derivatives of HBV core). Inserts were introduced into the poliovirus genome in frame with the poliovirus polyprotein, allowing these chimeric poliovirus to replicate normally. The extended fusion polyproteins were produced and proteolytically processed to give rise to all the encoded viral proteins, plus the exogenous protein, which was not incorporated into the mature chimeric virions. The exogenous sequences were stably maintained in culture and *in vivo* through successive rounds of replication. Infection of poliovirus-infection susceptible mice with the recombinants elicited immune response to both foreign and poliovirus proteins.

This type of HBV recombinant poliovirus vaccine may present the aforementioned advantages of OPV. In particular, this new vaccine may induce mucosal immunity, and therefore, protect against sexually transmitted HBV. In addition, this type of recombinant HBV-poliovirus vaccine would provide simultaneous defense against

both poliovirus and HBV. This advantage may allow for a more efficient and cost effective way to deliver the vaccine to potential recipients.

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 from Boehringer Mannheim Biochemicals, Indianapolis, Indiana; T4 DNA ligase was from New England BioLabs, Inc., Beverly, Massachusetts; and shrimp alkaline phosphatase was from Unites States Biochemical Corp., Cleveland, Ohio. 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 (MoV-1.5) (29). The Mahoney vector cDNA has been modified to include an in-frame 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 ($3C^{pro}$) at the 3' border of the polylinker (2, 23, 25, 38); and is preceded by a polyglycine tract that increases flexibility at the artificial cleavage site.

Exogenous DNA sequences were amplified by PCR (3) 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 preS1 region of HBV was amplified with primers #1 and #2; preS2 region of HBV was amplified with primers #3 and #4 ; and HBV core region was amplified with primers #5 and #6. All three sequences were amplified using plasmid containing HBVadw2 sequences as a template (kind gift from D. Ganem laboratory). All 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 (19).

Replication competent polioviruses were recovered by transfection of RNA transcribed *in vitro* using the recombinant cDNA clones as templates into HeLa cells (17).

CELLS AND VIRUS STOCKS

HeLa cells were grown in suspension in Joklik modified minimal essential medium (MEM; Sigma Chemical Company, St. Louis Missouri.) supplemented with 10% horse serum (GIBCO Diagnostics, Madison, Wisconsin), 1% penicillin-streptomycin, and 1% L-glutamine (both from Mediatech, Inc. Herndon, Virginia) (5).

HeLa cell monolayer on 100mm dishes were grown in DMEM/F12 (GIBCO) and transfected with 1-10 μg of recombinant viral RNA by DEAE-dextran procedure (17). Single plaques were isolated and expanded for each poliovirus chimeras by standard procedures to generate stocks that were used for further characterization (17).

VIRUS INFECTION

One-step growth curve was performed in 6-well multiwell plates (Becton Dickinson Labware, Lincoln Park, New Jersey) containing $1.5x10^5$ of HeLa cells . In all other experiments, 100mm dishes containing approximately $3x10^6$ cells were used. The dishes were washed once with phosphate-buffered saline (PBS) and appropriate multiplicity of infection (MOI) or dilutions of virus in 100 μl of PBS were added. The virus were uniformly distributed and cells were incubated at room temperature for 30 minutes to allow virus to adsorb to the cells. After the adsorption, 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) were added and the dishes were placed in 37°C incubator as

required by each specific experiments (5). At the time of collection, plates were washed once with PBS and cells were resuspended in 1 ml of PBS. Cells were pelleted by centrifugation at low speed and stored at -20°C until analysis.

ONE-STEP GROWTH CURVE

HeLa cell monolayers in 100mm dishes were infected as described above with each recombinants with an MOI of 10. Viral yield (plaque-forming units per milliliter[pfu/ml]) at each time point was determined by plaque asssay (39).

RT-PCR

Total RNA from cells infected with recombinant virus was prepared by phenol-chloroform extraction after 9 hours of infection and was precipitated in ethanol overnight in -20°C. Reverse transcription (RT) was performed with AMV RTase at 42°C for 60 minutes with primer immediately 3' to the inserted sequences and the enzyme was inactivated by incubating in 100°C for 3 minutes. Polymerase chain reaction (PCR) (3) amplification was performed with primers #7 and #8, to amplify the region of the poliovirus genome that contain the inserted sequences. PCR was performed for 25 cycles at 94°C for 1 minute, 50°C for 1 minute , 72°C for 1 minute with Taq polymerase.

IMMUNOBLOTTING

HeLa cells infected with wild-type and HBV recombinant polioviruses (MOI of 10) were incubated for 4, 6, and 8 hours at 37°C. Cells were harvested and lysed in buffer H (buffer H: 10 mM Hepes [pH=7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1% Triton X-100, and 0.1 mM phenymethylsulfonyl fluoride [PMSF]) and the nuclei

were removed by centrifugation (39). 4 μg of total lysates were subjected to electrophoresis through a 12% SDS-polyacrylamide gel, and analyzed by immunoblotting. Antisera against poliovirus capsid was prepared in rabbit; antisera to HBV preS1 region was a kind gift from D. Ganem laboratory; antisera to HBV preS2 region was a kind gift from Patricia Reilly (American Cyanamid) and antisera to HBc is from DAKO, Carpinteria, California. Secondary antibodies (both anti-mouse and anti-rabbit) were from Amersham, Arlington Heights, Illinois. Immunoblotting procedure has been described by the manufacturer of ECL chemiluminescense detection kit (Amersham).

MEASUREMENT OF VIRAL RNA SYNTHESIS

HeLa cells were infected as previously described with an MOI of 10 with each recombinants and wild type poliovirus. After 30 minutes of adsorption of virus at 37°C, 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, Massachusetts, was added at 1 hour post-infection. 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 (5).

EXPRESSION OF RECOMBINANT POLIOVIRUS PROTEIN IN *E. COLI*

We used the pGEX expression system developed by Smith and Johnson (36) to produce various proteins used in our ELISA experiments. Plasmids were constructed using pGEX -4T-1 Glutathione S-transferase (GST) Gene Fusion Vector System (Pharmacia Biotech Inc., Piscataway, New Jersey). Inserts were amplified by PCR using

primers #9 and #10 for pGEX-4T-1-S1A; primers #11 and #12 for pGEX-4T-1-S2B; and primers #13 and #14 for pGEX-4T-1-HBc. PCR fragments were digested with Bam HI and Not I restriction enzymes and ligated to the pGEX -4T-1 that was also digested with the same enzymes (19). The proteins were expressed and purified according to the protocol recommended by the manufacturer (Pharmacia).

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Mice (TgPVR1-17 strain) (30), generously provided by American Cyanamid, were infected intraperitoneally with 100 µl of $1x10^7$ pfu/ml of wild type and recombinant poliovirus HBV.S2. As control, mice were mock infected by intraperitoneal injection of 100 µl of PBS. Mice were inoculated and bled at 0, 14, 28, 49, 70, and 91 days. Sera obtained from infected mice were analyzed by ELISA. Plates were coated with 10 µg/ml of following antigens: purified polio 3CD (expressed in T7 *E. Coli* expression system), polio capsid (purified by CsCl), and purified GST preS2 (expressed in pGEX). 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. Background controls included assay plates coated with 2% milk, antibodies against 3CD, capsid, and preS2, and pre-immune sera. Rest of the ELISA procedure was conducted as described by Ausbel *et al* (3).

SUCROSE GRADIENT

Lysates from HeLa cells infected with recombinant HBc.155 or wild-type virus were analyzed by sucrose gradient (10-60%) as described by Zhou and Standring (42).

Fourteen fractions (100 µl each) were collected and HBV or poliovirus specific proteins were detected by immunoblotting as previously described.

LIST OF OLIGONUCLEOTIDE PRIMERS USED

#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 GGA GGT TGG TCA TCA

#10: GTC ACG ATG CGG CCG CTC GGC CTG AGG ATG ACT

#11 TTC CCG GGT CGA CTC CAG TGG AAT TCC ACT

#12: GTC ACG ATG CGG CCG CTC GTT CGT CAC AGG GTC

#13: GTT CCG CGT GGA TCC CTT GGG TGG CTT TGG

#14: GTC ACG ATG CGG CCG CTC AAC AAC AGT AGT TTC

RESULTS

CONSTRUCTION OF CHIMERIC POLIOVIRUS AND THEIR REPLICATIVE CHARACTERISTICS

Poliovirus vector (MoV-1.4) was constructed by genetic manipulation of biologically active cDNA clones corresponding to wild-type Mahoney type 1 strain, and has been recently described (29). In brief, an in-frame synthetic polylinker, containing several restriction enzyme recognition sites (Eco RI, Not I, Bss HII, and Xho I), was inserted at the amino terminus of the poliovirus open reading frame (Fig. 1). The insert includes sequences encoding the recognition site for the poliovirus protease, $3C^{pro}$, and a polyglycine tract that provides greater flexibility to the poliovirus polyprotein at the artificial proteolytic cleavage site (MoV-1.5, later vector version, carries a longer polyglycine tract).

Six different chimeric poliovirus were prepared by inserting nucleotide sequences encoding for HBV antigenic proteins into poliovirus vectors MoV-1.4 or MoV-1.5 (refer to Materials and Methods section for details). HBV DNA sequences were amplified by PCR from a plasmid encoding the entire HBV genome. Appropriate restriction sites were included in the PCR primers to facilitate the insertion of the exogenous sequences into the poliovirus vector plasmid. Inserted HBV sequences are summarized in figure 1B. They encode for: preS1 and preS2 (118 and 54 amino acids, respectively) corresponding to the amino termini of the HBV pre-surface antigens; portion belonging to the amino end of the HBV core protein (155 amino acids); and hybrid HBV core proteins containing immunogenic epitope of preS1 and preS2 proteins (185 and 169 amino acids, respectively). It has been shown that immunogenicity of the inserted amino acid peptides are greatly enhanced when they are expressed on the surface of the HBV core particle (34). Hence, to ensure expression of the antigenic peptides on the surface of the HBV core particle, antigenic portions of the HBV preS proteins were inserted in a region of the

core sequence that has been previously shown to correspond to the surface of the core particle (34).

All chimeric poliovirus-cDNAs yielded replication-competent virus after transfection in HeLa cells with *in vitro* synthesized RNA. At 32°C and at 37°C, recombinants HBV.S1, HBV.S2, and HBc.155 replicate to produce plaques with quasi-wild-type phenotype. However, at 39°C, recombinants produced plaques that are much smaller than the wild-type (Fig. 2A). One-step growth curve of poliovirus wild-type and recombinants confirm the temperature sensitive nature of the chimeric poliovirus (Fig. 2B). At 39°C and at 37°C, both wild-type and recombinants achieved maximal titers at six hours post-infection. However, recombinant viruses replicated slower than the parental wild-type and yielded approximately 1% of wild-type titers. At 32°C, both wild-type and recombinants exhibit delayed replication but, in contrast to the growth curve at 37°C and 39°C, at 12 hours post-infection, chimeric viruses yielded 50-70% of wild-type titers. The genetic structure of recombinant HBV.S2 proved to be stable after six rounds of replication in HeLa cells as confirmed by RT-PCR. However, recombinant HBV.S1 retained the insert sequences only up to four rounds of replication in tissue culture as seen by the emergence of wild-type fragment (Fig. 2C).

EXPRESSION OF FOREIGN PROTEINS BY POLIOVIRUS RECOMBINANTS

To determine whether recombinant viruses correctly expressed and processed both viral and foreign polypeptides, we infected HeLa cells with wild-type and chimeric polioviruses and cytoplasmic extracts were obtained at different time points post-infection and analyzed by immunoblotting. Capsid proteins were produced and processed by recombinant viruses in a wild-type manner. However, HBV.S1 and HBV.S2 recombinants' capsid production was delayed when compared to the wild-type strain (Fig. 3A, lanes 1-10). All recombinants also expressed the proper foreign proteins

in the infected cells as detected by specific 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 free foreign polypeptide accumulates in infected cells. Recombinant HBV.S2 produced two distinct bands that were recognized by anti-preS2 antibodies (Fig. 3A, lanes 17 and 18). The molecular weight of the larger band suggests that it is a fusion between preS2 and Vp0. Extracts from cells infected with recombinants HBc.pS1 and HBc.pS2 displayed only single immunoreactive polypeptide band corresponding to the HBV core protein containing antigenic peptide inserts derived from preS1 and preS2 proteins (Fig. 3B, lanes 10-13 and 14-17, respectively). In contrast, extracts from cells infected with poliovirus recombinant` HBc.155 showed three polypeptides that reacted with anti-HBcore antibodies corresponding to fusions between HBV core and capsid precursors P1 or Vp0-Vp3, and free HBV core protein (Fig. 3B, lanes 6-9).

MEASUREMENT OF RECOMBINANT POLIOVIRUS VIRAL RNA SYNTHESIS

Replication characteristics of poliovirus recombinants were further analyzed by measuring their ability to synthesize viral RNA. All recombinants' RNA synthesis rates were remarkably similar to the wild-type (Fig. 4), suggesting that subtle reduction in recombinant polioviruses' replication rate, as observed by one step growth curve, is not due to the impairment of RNA synthetic capability. This may be the result of a different step in viral replication; perhaps capsid assembly or packaging.

HBV CORE PARTICLE FORMATION

As mentioned before, peptides fused to HBV core are more immunogenic than free peptides. This enhanced immunogenicity appears to depend on the assembly of 100s HBV core protein particle. To determine whether core proteins expressed by the poliovirus recombinant assembled into a particle, HeLa cells were infected with the recombinant HBc.155 for 7 hours, after which cytoplasmic extracts were analyzed by sucrose gradient followed by immunoblotting. Fractions 8 to 12, corresponding approximately to 100s, contained polypeptides that were detected with anti-HBV core antibodies (Fig. 5). The molecular weights of these polypeptides suggest that these particles are free HBV core protein, and fusions of HBV core protein and Vp0 or Vp0-Vp3.

IMMUNE RESPONSES TO THE INSERTED SEQUENCES

The immunogenicity of the recombinant poliovirus was examined in transgenic mice that express the human poliovirus receptor and therefore are susceptible to poliovirus infection. Mice were inoculated intraperitoneally on four occasions with recombinant HBV.S2. Serum antibodies recognizing poliovirus capsid proteins were detected by ELISA after 2 weeks post-inoculation. And antibodies recognizing other proteins, poliovirus 3CD and HBV preS2 polypeptides, were detected after 4 weeks post-inoculation (Fig. 6). Interestingly, the titers of antibodies elicited against HBV preS2 were similar to the ones elicited against poliovirus proteins (capsid and 3CD).

CONCLUSION AND DISCUSSION

In this work, I report the construction of several replication-competent poliovirus recombinants carrying and expressing preS1, preS2, and core proteins of HBV (Fig. 1B). Recombinants underwent normal poliovirus replication and infection cycle. Single plaques of poliovirus recombinants were isolated after transfection with the *in vitro* synthesized RNA, giving rise to stocks of infectious viruses. Infection of cells with the recombinant viruses produced extended fusion polyprotein that was proteolytically processed into complete series of viral proteins, along with high level production of inserted foreign peptide. However, the foreign protein was not incorporated into the mature chimeric virions as they proceeded through viral replication. The foreign sequences inserted were retained after several rounds of serial passages in tissue culture. Inoculation of mice with the recombinants induced high titers of antibodies against both poliovirus and foreign proteins.

RNA viruses present a great deal of genetic variations due to its biological nature, mainly due to the absence of proof-reading mechanism of the RNA-dependent RNA-polymerase. These variations manifest themselves during viral replication in the form of point mutations, insertions, deletions, *etc.* In general, sequences that do not confer beneficial attributes to viral replication and propagation, are either mutated or actively removed by the virus from its genome.

Our poliovirus chimeras, particularly the inserted HBV sequences, does not contain any known beneficial attributes to the poliovirus replication. Nonetheless, engineered poliovirus not only tolerates, but stably carries these inserted foreign sequences in its genome through several rounds of replication in tissue culture. Presumably the inserted HBV sequences, although not beneficial to the poliovirus, is certainly not harmful, and therefore retained in the genome as seen in RT-PCR. HBV.S2 recombinant proved to be extremely stable and inserted preS2 sequences were retained

for more than six passages in HeLa cells. In contrast, HBV.S1 recombinant is not as stable as HBV.S2 since deletions of the inserted sequences were detected after four to five passages in tissue culture. In light of this result, one may assert that size of the insert may play a major role in this biological phenomenon of deletion/reversion (HBV.S1 sequence is double the size of HBV.S2, Fig. 1B). However, this is not necessarily the case, in previously reported work, where poliovirus recombinants were constructed by the same approach, it was shown that the size is not the limiting factor, but the nature of the inserted sequences played a decisive role in poliovirus tolerating the foreign insert (2). Furthermore, comparison was made of the kinetics of viral RNA synthesis, and it was shown that inserted sequences did not have a deleterious effect in RNA synthesis. Indeed at 37°C, all poliovirus recombinants exhibited RNA synthesis rates that were strikingly similar to the wild-type. Then, why does poliovirus recombinants display reduced rate of viral replication as observed by one step growth curve? One possibility is that the artificial proteolytic cleavage site is not efficiently utilized, leaving exogenous proteins fused to the poliovirus polyprotein, and therefore, altering the normal function of the viral protein and reducing recombinant viruses' ability to replicate efficiently. However, western blot analysis indicates that the exogenous proteins (preS1 in particular) are proteolytically processed away from the rest of the poliovirus polyprotein in a very efficient manner (Fig. 3A). One possible explanation, although it has not been fully investigated, is that HBV proteins somehow produce a specific detrimental effect in poliovirus replication. Additionally, because HBV is an enveloped virus, some of its proteins are membrane associated. In contrast, poliovirus is non-enveloped virus, and its entire life-cycle takes place in the cytoplasm, therefore, all poliovirus proteins are localized in the cytoplasm. HBV proteins, produced by the poliovirus recombinants, are not in its natural environment, but are forced to be in the cytoplasm. Furthermore, the truncated versions of the HBV proteins (HBV preS1 and HBV preS2 proteins) are expressed without the endogenously present HBV surface-protein (HBV-S). Thus,

recombinant polioviruses expressing HBV proteins may experience difficulties in carrying out wild-type-like viral replication due to inappropriate folding of the truncated HBV proteins in the cytoplasm that causes inductive defect in the folding of poliovirus proteins which may target poliovirus' essential proteins for rapid degradation.

Poliovirus recombinant expressing HBV core (HBc.155) virus was created based on two main attractive characteristics. First, particulate HBV core protein, although it is an internal component of HBV, has been shown to exhibit high immunogenicity after HBV infection. Second, hybrid of the core created by fusing foreign epitopes to the core greatly enhances the immunogenicity of the foreign epitope. Interestingly, the inserted sequences do not reduce immunogenicity of the HBV core protein itself . For example, HB core/preS1 hybrid particles carrying preS1 epitopes elicited higher immune response in mice against preS1 protein than when they were inoculated with the entire preS1 protein. Hence, we want to harness this immunogenic characteristic of the HBV core particulate by utilizing it as an internal adjuvant for the recombinant poliovirus. In fact, HBV core antigen has been shown to efficiently elicit responses of both T cells and B cells (in a T-cell-independent manner). In this way, HBV core and its hybrid particulate can elicit stronger immune response with the cooperation of T-helper cells to produce antibodies against both the core and the inserted sequences of the hybrid (34). Essentially, we have created a new vaccine vector utilizing the immunogenic benefits of both HBV core hybrids and the poliovirus that may harness several clusters of the immune system.

HBV core particles expressed by poliovirus recombinant proved to be stable and the self-assembling nature of the core protein was maintained as indicated by sucrose gradient analysis. Free HBV core particles and fusions of HBV core protein and Vp0 or Vp0-Vp3 were detected. It is remarkable that the HBV core protein was able to form a particle even with large portion of poliovirus capsid protein still fused to it and remain stable as a particle (Fig. 5). There are two viable possibilities to the existence of the

fusion core protein: the polio protein is sequestered inside the core particle; or the polio protein is on the outer surface of the core particulate. In either case, poliovirus protease $3C^{pro}$ must not be able to gain access to its cleavage site since the fusion proteins are very stable once formed into a particle. In contrast, fractions containing free non-particulate proteins (Fig. 5, lanes 1-5) do not display HBV core fusion proteins.

Immunogenic effects of recombinant polioviruses were evaluated in transgenic mice that express human poliovirus receptor, and, therefore, are susceptible to poliovirus infection. Mice were inoculated intraperitoneally with poliovirus recombinant HBV.S2. Immunoglobulin G (IgG) serum antibodies recognizing poliovirus capsid protein was detected 14 days after first inoculation in all the mice. However, IgG against poliovirus 3CD and preS2 proteins were detected only after 28 days of the first inoculation. This delay perhaps is due to the need for the virus to go through several rounds of replication before producing enough proteins to effectively stimulate the mouse immune system. Interestingly, mice #280 did not sero-convert against preS2 protein, yet it sero-converted against poliovirus proteins (capsid and 3CD). Possible explanation for this observation is that a poliovirus reverent has been selected in this particular mouse, and induced anti-poliovirus immune response that consequently reduced the ability of recombinant poliovirus to replicate in subsequent inoculations (32). Noteworthy observation in this evaluation is the comparable IgG titer levels of anti-capsid and anti-preS2 in mice #226 and #231. Considering the fact that poliovirus capsid is known to be the most immunogenic protein among all the known poliovirus proteins, similar levels of immune response against preS2 and capsid proteins suggests that preS2 protein expressed in our poliovirus vaccine vector is highly immunogenic.

The possible benefits of this novel HBV vaccine and this particular poliovirus vaccine vector are innumerable. Furthermore, the work presented here provides additional evidence of the possibility of creating a multivalent vaccines that can infer immunity to several pathogens presumably with all the benefits of OPV. This is very

promising since poliovirus recombinants may allow simultaneous vaccination against several pathogens through the use of a "cocktail" of several different types of recombinant polioviruses (2). Considering the fact that there are several pathogens urgently requiring vaccines (*e.g.* HIV, Hepatitis A and C, Dengue, other bacterial pathogens, *etc.*), I hope this work may provide at least an iota of contribution to the current efforts in developing the "ideal vaccine".

I would like to close this work with the statement made by Dr. Barry Bloom, who eloquently expressed the urgency and the responsibility of the scientific community in developing and utilizing vaccines.

"This is the third world, in which 75% of the planet's population lives, where 86% of all children are born and 98% of all infant and child deaths occur, and where 10 kids die of vaccine-preventable illness every minute"

FIGURE LEGENDS

Fig. 1. (A) Schematic diagram of a recombinant poliovirus vector and strategy for expression of foreign 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 shadowed box. Sequences surrounding the point of insertion of the exogenous sequence are indicated: the start codon, additional amino acids flanking the exogenous sequence that provide 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 length (in nucleotides and amino acids) is indicated.

Fig 2. (A) Plaque assay phenotype of two recombinant polioviruses (HBV.S1 and HBV.S2). Plaque assay was performed in HeLa cells at different temperatures (32°C, 37°C, and 39°C) for 48 hr. (B) One-step growth curve of four recombinant polioviruses. HeLa cell monolayers were infected (MOI=10) with wild-type poliovirus, recombinant HBV.S1 (carrying 118 amino acids of HBV preS1), HBV.S2 (54 amino acids of HBV preS2), HBc.155 (155 amino acids of HBV core). Total virus production (pfu/ml) was determined at each time point by plaque assay. (C) Analysis of the structure of recombinant poliovirus genomes. HeLa cells were infected with recombinant (HBV.S1 or HBV.S2) stocks obtained after 2, 3, 4, 5, or 6 successive passages in HeLa cells. RT-PCR reaction was performed using total cytoplasmic RNA as a template for reverse

transcription and primers that specifically amplify the region of poliovirus genome where the foreign sequences have been inserted.

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 (a-capsid, (A) lanes 1-10), preS1 (a-S1, (A) lanes 11-14), preS2 (a-S2, (A) lanes 15-18), HBV core (a-HBV-core, (B) lanes 1-17). Mock, mock infected; wild-type, poliovirus wild-type; HBV.S1, HBV preS1-recombinant; HBV.S2, HBV preS2-recombinant; HBc.155, HBV core-recombinant; HBV.pS1, HBV core carrying preS1 peptide-recombinant; and HBV.pS2, HBV core carrying preS2 peptide-recombinant. Poliovirus capsid proteins are indicated by shaded triangles. Molecular weight markers indicate relative mobility. Based on molecular weight, the bands detected by specific antibodies directed against the inserted sequences are: (S1) free preS1; (S2) preS2-Vp0 fusion; (S2') free preS2; core.S1 (HBV core containing preS1 26 amino acid peptide), core.S2 (HBV core containing preS2 11 amino acid peptide)

Fig. 4. Analysis of the HBV core intermediate particles produced in recombinant poliovirus infected cells. Extracts from HeLa cells infected with 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 immunoblot using antibodies directed against HBV core. Molecular weight markers indicate relative mobility. Bands corresponding to HBV core, HBV core-Vp0-

Vp3 and HBV core-Vp0 fusions are indicated by shadow arrows. Fractions 8-12 contained HBV pseudo-particles.

Fig. 5. Viral RNA synthesis of wild-type and recombinant poliovirus. HeLa cells were infected with an MOI=10 and incubated at 37°C 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.

Fig. 6. Immunization of transgenic mice with HBV.S2-recombinant poliovirus elicited antibodies against the HBV preS2 protein. Several dilutions of sera from three mice (#226, #231, and #280) infected with HBV preS2-recombinant virus (HBV.S2) were analyzed by ELISA (see Materials and Methods). Recombinant poliovirus stocks were produced in HeLa cells in 100 mm petri dishes by infection with a MOI of 5 to 10. Infected cells were incubated overnight at 37°C, and cells were lysed by freeze-thawing in 1.5 ml of DMEM. Mice were infected by intraperitoneal injection with HBV.S2-recombinant poliovirus (100 μl of $1x10^8$ pfu per ml stock) or mock infected with PBS alone. PVR-transgenic mice received four identical injections separated by a period of 2 or 3 weeks. Antibodies reacting with the HBV preS2 and poliovirus proteins were visualized with anti-mouse IgG-specific second stage antibodies conjugated to peroxidase.

REFERENCES

1. Alexander L, Lu HH, Wimmer E (1994). Polioviruses containing picornavirus type 1 and/or type 2 internal ribosomal entry site elements: genetic hybrids and the expression of a foreign gene. Proc Natl Acad Sci USA 91:1406-1410.

2. Andino R, Silvera D, Suggett SD, Achacoso PL, Miller CJ, Baltimore D, Feinberg MB (1994). Engineering poliovirus as a vaccine vector for the expression of diverse antigens. Science 265:1448-1451.

3. Ausbel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K, eds. Current Protocols in Molecular Biology. New York: John Wiley & Sons, 1994.

4. Baltimore D (1984). Molecular genetics of poliovirus. Rev Infect Dis 6[Suppl 2]:484-486.

5. Bernstein HD, Sonenberg N, Baltimore D (1985). Poliovirus mutant that does not selectively inhibit host cell protein synthesis. Mol Cell Biol 5:2913-2923.

6. Burke KL, Almond JW, Evans DJ (1991). Antigen chimeras of poliovirus. Prog Med Virol 38:56-68.

7. Dedieu J-F, Ronco J, Van Der Werf S, Hogle JM, Henin Y, Girard M (1992). Poliovirus chimeras expressing sequences from the principal neutralization domain of human immunodeficiency virus type 1. J Virol 66(5):3161-3167.

8. Dowdel WR, Orenstein WA (1994). Quest for life-long protection by vaccination. Proc Natl Acad Sci USA 91:2464-2468.

9. Evans DJ, McKeating J, Meredith JM, Burke KL, Katrak K, John A, Ferguson M, Minor PD, Weiss RA, Almond JW (1989). An engineered poliovirus chimera elicits broadly reactive HIV-1 neutralizing antibodies. Science 339:385-388.

10. Hollinger FB, "Hepatitis B virus" In Virology (2nd ed.), eds. BN fields and DM Knipe. 2171-2236. New York: Raven Press, 1990.

11. Horaud F (1993). Albert B. Sabin and the development of oral polio vaccine. Biologicals 21:311-316.

12. Kirkegard K (1992). Genetic analysis of picornaviruses. Curr Opin Gen and Devel 2:64-70.

13. Krugman S, "Hepatitis B vaccine" In Vaccines, eds. SA Plotkin and EA Mortimer Jr. 458-473. Philadelphia: Saunders, 1988.

14. La Montagne JR, Rabinovich R, "Challenges and opportunities in vaccine research" In Genetically engineered vaccines, eds. JE Ciardi, JR McGhee, and JM Keith. 293-299. New York: Plenum Press, 1992.

15. Levy JA, Fraenkel-Conrat H, Owens RA, eds. Virology (3rd ed.). 1-11. Englewood Cliffs, N.J.: Prentice Hall, 1994.

16. Lewin B (1990). Potential for change. Scand J Infect Dis [Suppl] 69:199-205.

17. Luthman H, Magnuson (1983). High efficiency polyoma DNA transfection of chloroquin treated cells. Nucleic Acids Res 11:1295-1308.

18. MacDonald TT, Spencer J, "Gut-associated lymphoid tissue" In Handbook of Mucosal Immunity, eds. PL Ogra, ME Lamm, JR McGhee, J Mestecky, W Strober, and J Bienenstock. 415-424. San Diego, CA: Academic Press, Inc., 1994.

19. Maniatis T, Fritsch EF, Sambrook J, eds. Molecular Cloning: a laboratory manual (2nd ed.). Cold-Spring Harbor, NY: Cold-Spring Harbor Laboratory, 1989.

20. Melnick JL, "Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and new enteroviruses" In Virology (2nd ed.), eds. BN fields and DM Knipe. 549-605. New York: Raven Press, 1990.

21. Melnick JL, "Live attenuated poliovaccines" In Vaccines, eds. SA Plotkin and EA Mortimer Jr. 115-157. Philadelphia: Saunders, 1988.

22. Mestecky J, Abraham R, Ogra PL, "Common mucosal immune system and strategies for the development of vaccines effective at the mucosal surfaces" In Handbook of Mucosal Immunity, eds. PL Ogra, ME Lamm, JR McGhee, J Mestecky, W Strober, and J Bienenstock. 357-372. San Diego, CA: Academic Press, Inc., 1994.

23. Namoto A, Omata T, Toyoda H, Kuge H, Horie H, Kataoka Y, Nakano Y, Imura N (1982). Complete nucleotide sequence of the attenuated poliovirus Sabin 1 strain genome. Proc Natl Acad Sci USA 79:5793-5797.

24. Norkrans G (1990). Epidemiology of Hepatitis B Virus (HBV) infections with particular regard to current routes of transmission and development of cirrhosis and malignancy. Scand J Infect Dis 69:43-47.

25. Palmenberg AC (1990). Proteolytic Processing of picornaviral polyprotein. Annu Rev Microbio 44:602-623.

26. Porter A (1993). Picornavirus nonstructural proteins: Emerging roles in virus replication and inhibition of host cell functions. J Virol 67(12):6917-6921.

27. Porter DC, Ansardi DC, Choi WS, Morrow CD (1993). Encapsidation of genetically engineered poliovirus minireplicons which express human immunodeficiency virus type 1 gag and pol proteins upon infection. J Virol 67(7):3712-3719.

28. Purcell RH (1994). Hepatitis viruses: Changing patterns of human disease. Proc Natl Acad Sci USA 91:2401-2406.

29. Racaniello VR, Baltimore D (1981). Cloned poliovirus complementary DNA is infectious in mammalian cells. Science 214:916-919.

30. Ren RB, Costantini F, Gorgacz EJ, Lee JJ, Racaniello VR (1990). Transgenic mice expressing a human poliovirus receptor: a new model for poliomyelitis. Cell 63:353-362.

31. Robbins FC, "Polio-Historical" In Vaccines, eds. SA Plotkin and EA Mortimer Jr. 98-114. Philadelphia: Saunders, 1988.

32. Robinson WS, "Hepadnaviridae and their replication" In Virology (2nd ed.), eds. BN fields and DM Knipe. 2137-2169. New York: Raven Press, 1990.

33. Rueckert RR, "Picornaviridae and their replication" In Virology (2nd ed.), eds. BN fields and DM Knipe. 507-548. New York: Raven Press, 1990.

34. Schödel F, Moriarty AM, Peterson DL, Zheng J, Hughes JL, Will H, Leturcq DJ, McGee JS, Milich DR (1992). The position of heterologous epitopes inserted in hepatitis B virus core particle determines their immunogenicity. J Virol 66:106-114.

35. Service RF (1994). Triggering the first line of defense. Science 265:1522-1524.

36. Smith DB, Johnson KS (1988). Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene 67:34-40.

37. Staats HF, Jackson RJ, Marinaro M, Takahashi I, Kiyono H, McGhee JR (1994). Mucosal immunity to infection with implication for vaccine development. Curr Opin Immunol 6:572-583.

38. Toyoda H, Nicklin MJH, Murray MG, Anderson CW, Dunn JJ, Studier FW, Wimmer E (1986). A second virus-encoded proteinase involved in proteolytic processing of poliovirus polyprotein. Cell 45:761-770.

39. Trono D, Andino R, 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.

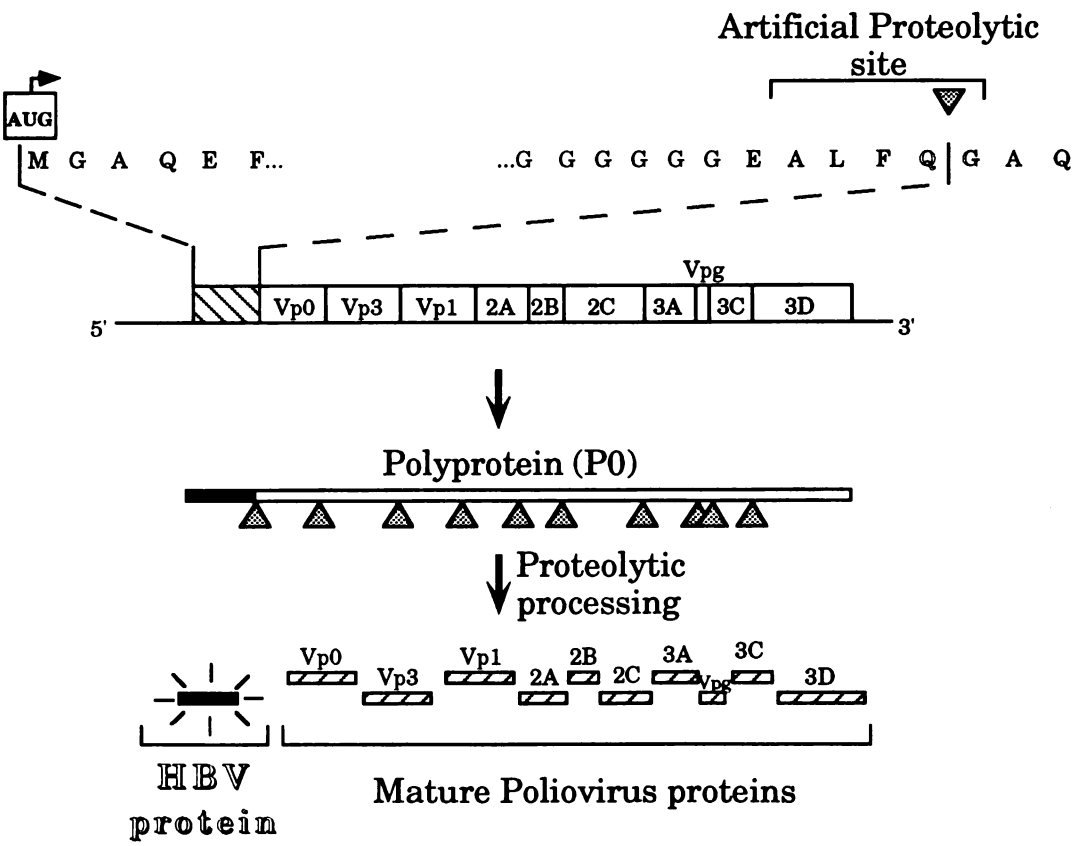
40. Underdown BJ, Mestecky J, "Mucosal immunoglobulin" In Handbook of Mucosal Immunity, eds. PL Ogra, ME Lamm, JR McGhee, J Mestecky, W Strober, and J Bienenstock. 79-97. San Diego, CA: Academic Press, Inc., 1994.

41. Wigzell H (1990). Vaccines against STDs: Specific considerations?. Scand J Infect Dis [Suppl] 69:207-211.

42. Zhou S, Standering DN (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.

Fig. 1

(A)



(B)

rec-POLIO	INSERT	LENGTH	
HBV.S1	pre S1Ag	354 nt	118 aa
HBV.S2	pre S1Ag	162 nt	54 aa
HBc.155	core	465 nt	155 aa
HBc.XS	core/linker	483 nt	161 aa
HBc.pS1	core/preS1	555 nt	185 aa
HBc.pS2	core/preS2	507 nt	169 aa

Fig. 2

(A)

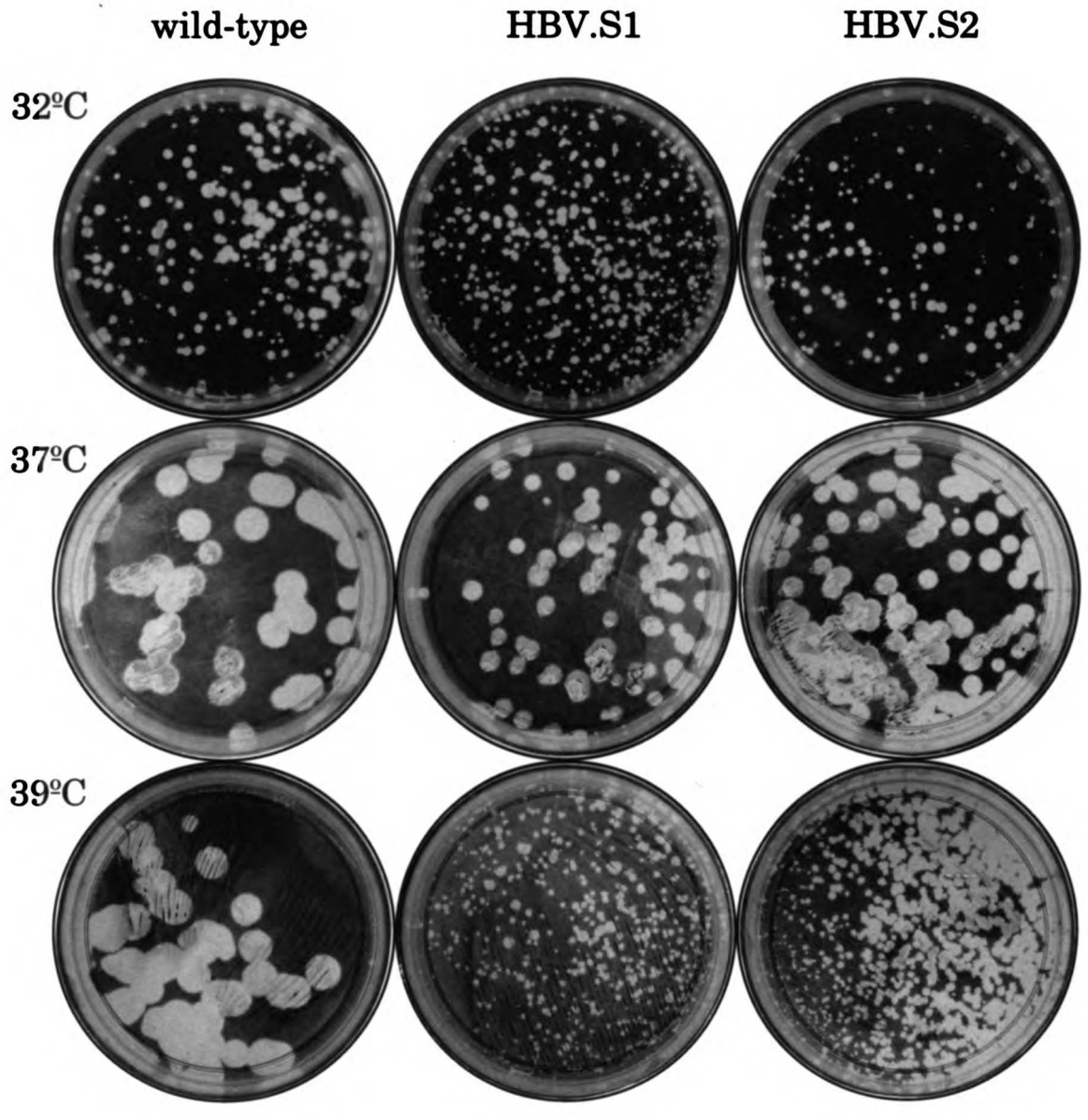


Fig. 2

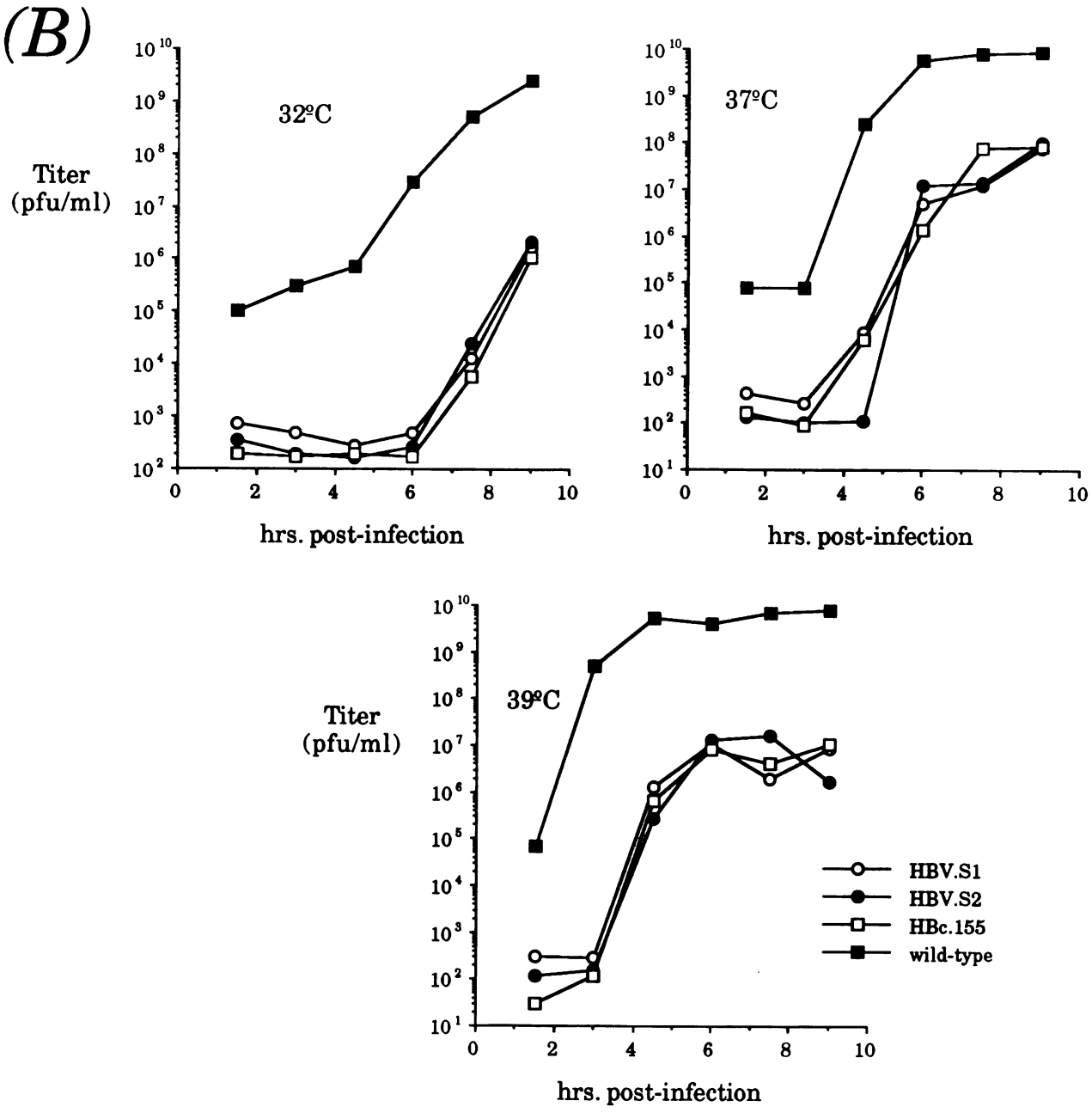

(B)
Titer
(pfu/ml)
32ºC
37ºC
39ºC
hrs. post-infection
hrs. post-infection
hrs. post-infection
HBV.S1
HBV.S2
HBc.155
wild-type

Fig. 2

(C)

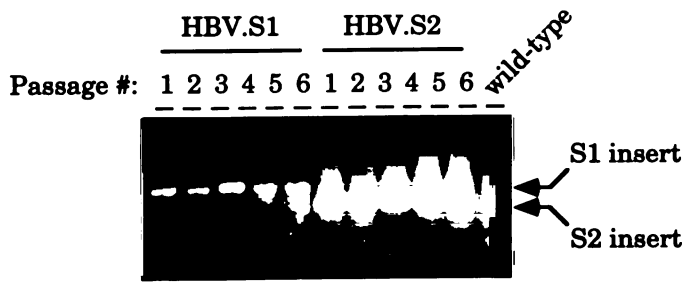

HBV.S1
HBV.S2
Passage #: 1 2 3 4 5 6 1 2 3 4 5 6
wild-type
S1 insert
S2 insert

Fig. 3

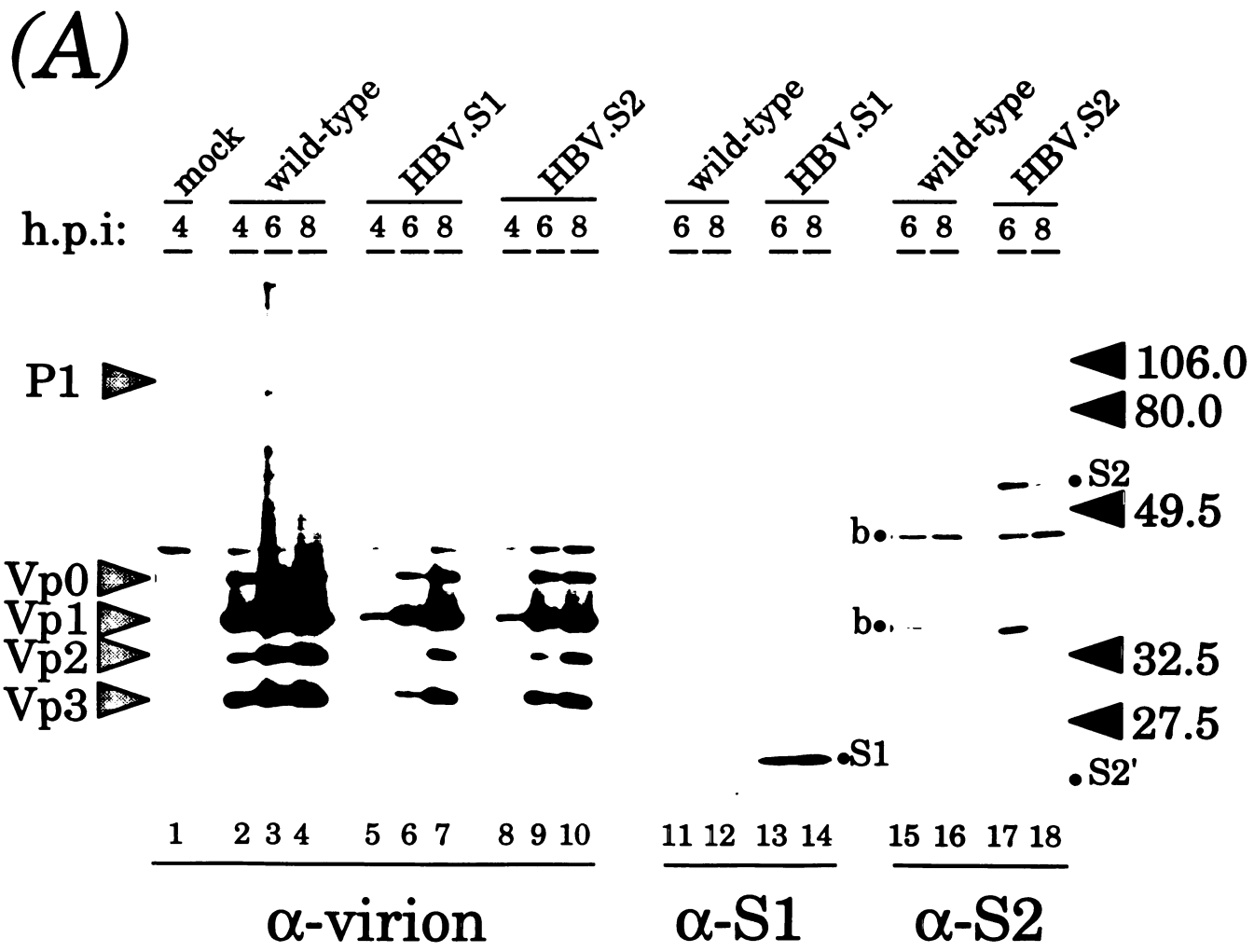

(A)
mock
wild-type
HBV.S1
HBV.S2
wild-type
HBV.S1
wild-type
HBV.S2
h.p.i:
4
4 6 8
4 6 8
4 6 8
6 8
6 8
6 8
6 8
P1
106.0
80.0
•S2
49.5
b•
b•
Vp0
Vp1
Vp2
Vp3
32.5
27.5
•S1
•S2'
1 2 3 4 5 6 7 8 9 10
11 12 13 14
15 16 17 18
α-virion
α-S1
α-S2

Fig. 3

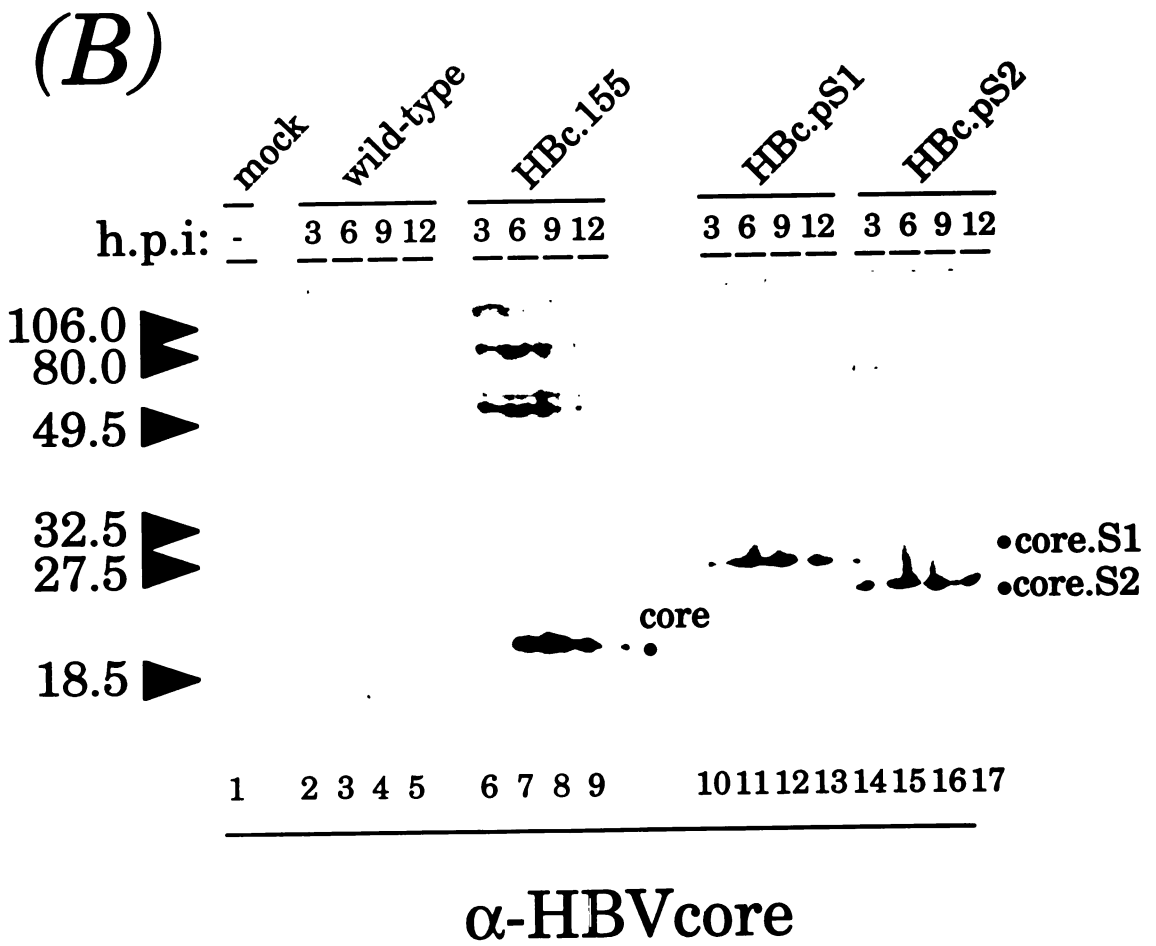

(B)
mock
wild-type
HBc.155
HBc.pS1
HBc.pS2
h.p.i: - 3 6 9 12 3 6 9 12 3 6 9 12 3 6 9 12
106.0
80.0
49.5
32.5
27.5
18.5
•core.S1
•core.S2
core
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
α-HBVcore

Fig. 4

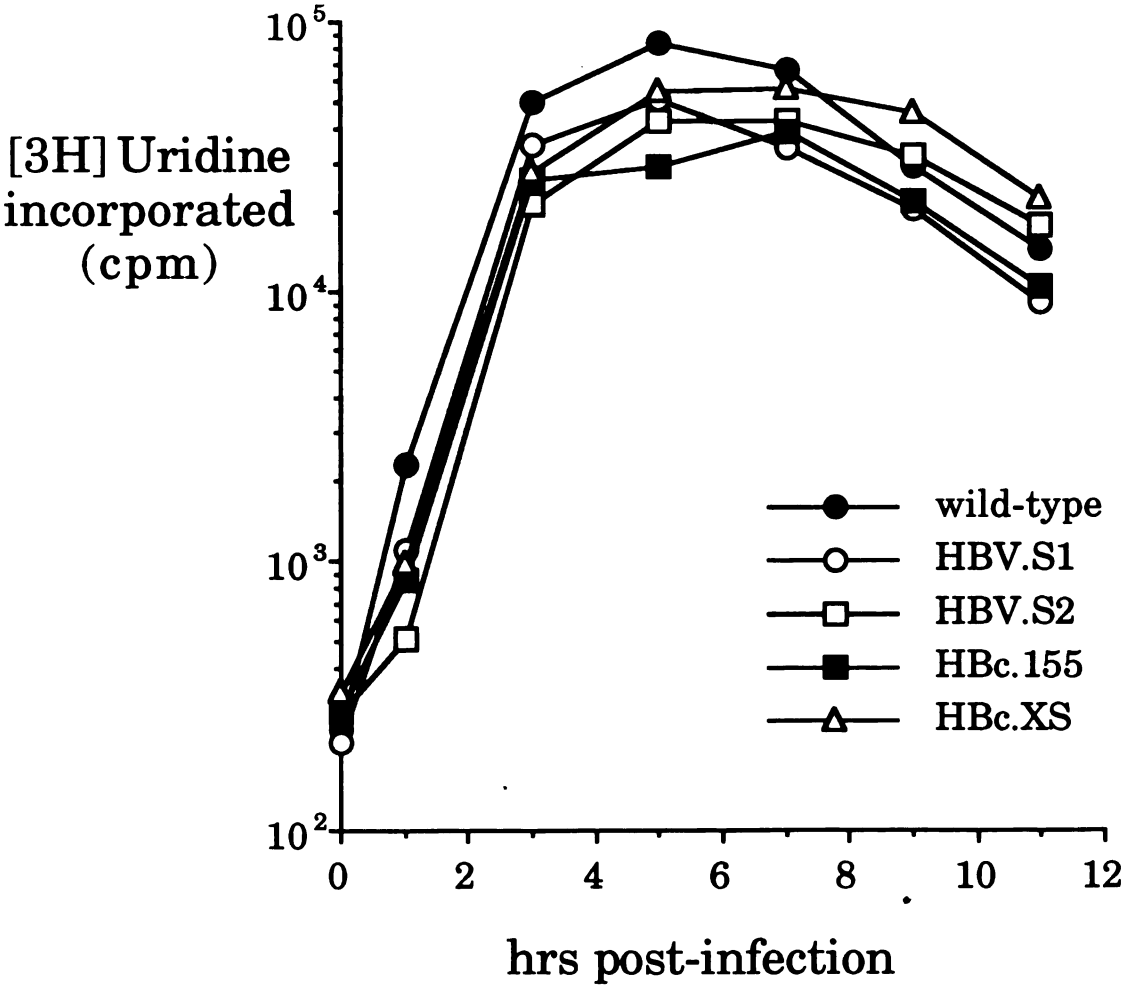

[3H] Uridine incorporated (cpm)
10^5
10^4
10^3
10^2
0
2
4
6
8
10
12
hrs post-infection
wild-type
HBV.S1
HBV.S2
HBc.155
HBc.XS

Fig. 5

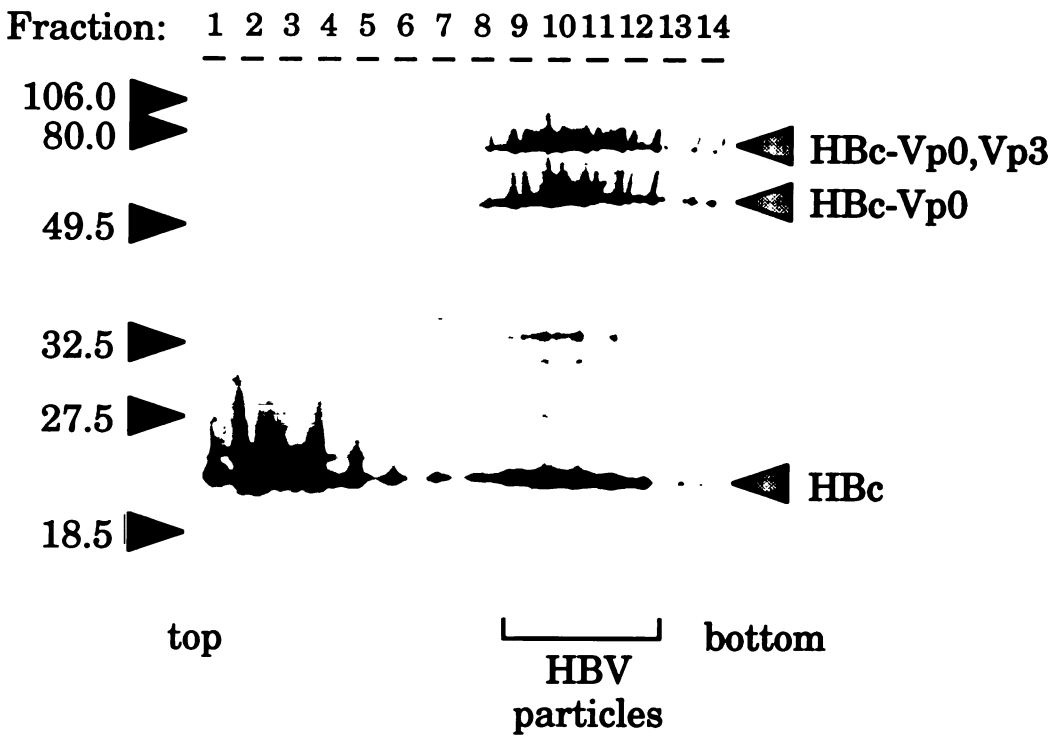
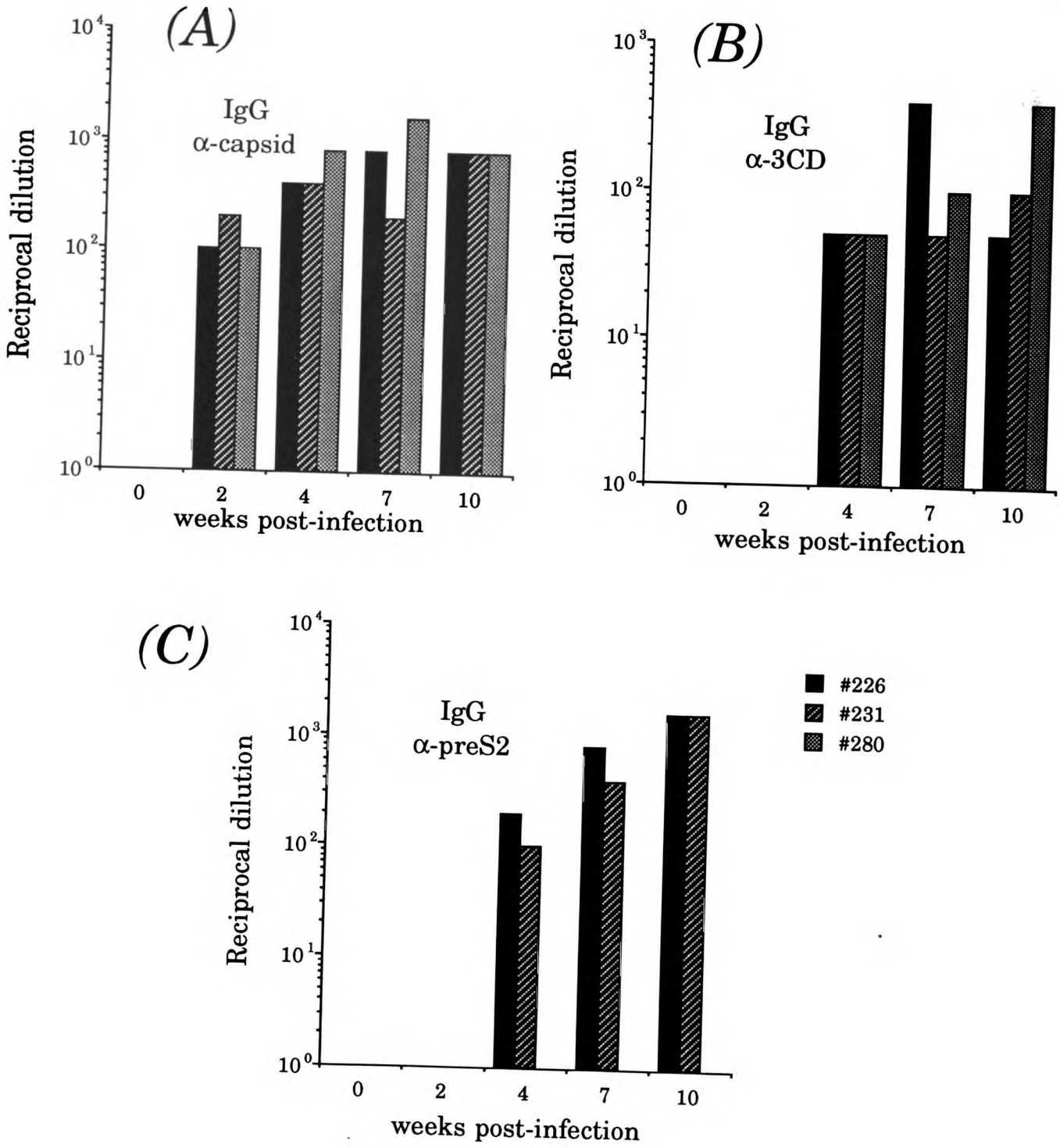
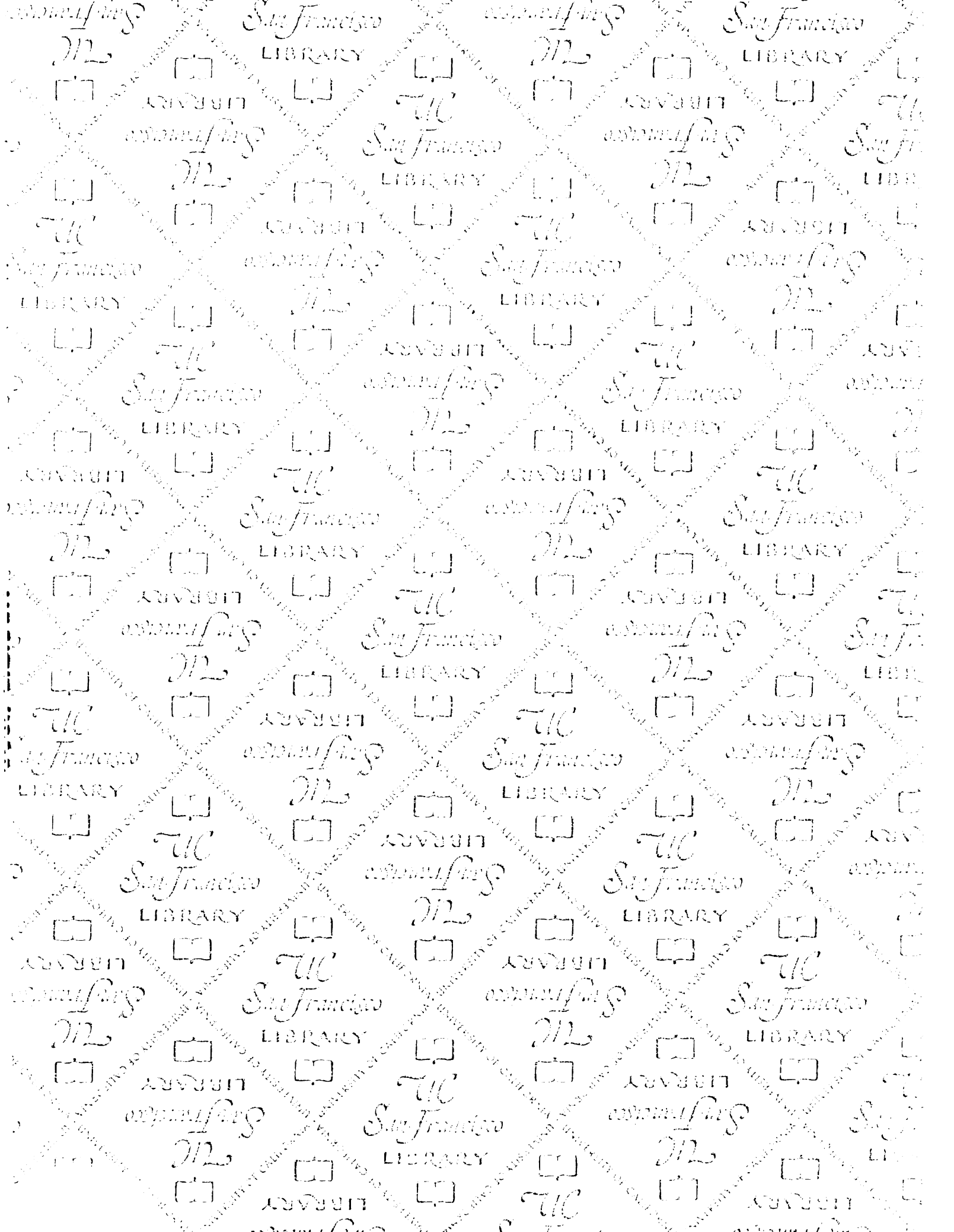

Fraction: 1 2 3 4 5 6 7 8 9 10 11 12 13 14
106.0
80.0
49.5
32.5
27.5
18.5
HBc-Vp0,Vp3
HBc-Vp0
HBc
top
bottom
HBV particles

Fig. 6


(A)
IgG
α-capsid
Reciprocal dilution
10^4
10^3
10^2
10^1
10^0
0
2
4
7
10
weeks post-infection
(B)
IgG
α-3CD
Reciprocal dilution
10^3
10^2
10^1
10^0
0
2
4
7
10
weeks post-infection
(C)
IgG
α-preS2
Reciprocal dilution
10^4
10^3
10^2
10^1
10^0
0
2
4
7
10
weeks post-infection
#226
#231
#280


San Francisco
LIBRARY