MOLECULAR COMPONENTS OF HEPATITIS B VIRUS

DEVELOPMENTS IN MOLECULAR VIROLOGY

Yechiel Becker, Series Editor
Julia Hadar, Managing Editor

Other books in the series:
Becker, Y. (ed.) *Herpesvirus DNA* (1981)
Becker, Y. (ed.) *Replication of Viral and Cellular Genomes* (1984)
Kohn, A. and Fuchs, P. (eds.) *Mechanisms of Viral Pathogenesis: from Gene to Pathogen* (1984)
Becker, Y. (ed.) *Antiviral Drugs and Interferon* (1984)
Becker, Y. (ed.) *Recombinant DNA Research and Viruses* (1985)

MOLECULAR COMPONENTS OF HEPATITIS B VIRUS

Mark Feitelson
Fox Chase Cancer Center
Institute for Cancer Research
Philadelphia, PA 19111

Martinus Nijhoff Publishing
a member of the Kluwer Academic Publishers Group
Boston/Dordrecht/Lancaster

Distributors for North America:
Kluwer Academic Publishers
190 Old Derby Street
Hingham, MA 02043

Distributors Outside North America:
Kluwer Academic Publishers Group
Distribution Centre
P.O. Box 322
3300 AH Dordrecht
The Netherlands

The figure on the cover is from Robinson, W.S. in Comprehensive Virology, *14:471–526, Plenum Press, 1979. Reprinted with permission. This figure is also reproduced on page 3 of this book.*

Library of Congress Cataloging in Publication Data

Feitelson, Mark.
Molecular components of hepatitis B virus.

(Developments in molecular virology)
Bibliography: p.
1. Hepatitis B Virus. 2. Host-virus relationships.
3. Molecular biology. I. Title. II. Series.
[DNLM: 1. Hepatitis B Virus—immunology. 2. Hepatitis B Antigens—analysis. W1 DE998DG v.6 / QW 170 F311m]
QR201.H46F45 1985 616.3'623071 84-22619

ISBN-13: 978-1-4612-9615-7 e-ISBN-13: 978-1-4613-2573-4
DOI: 10.1007/978-1-4613-2573-4

CONTENTS

This work was supported by USPHS grants CA-06551, RR-05539 and CA-06927 from the National Institutes of Health and by an appropriation from the Commonwealth of Pennsylvania.

PREFACE

Since the discovery of Australia antigen and its association with type B hepatitis, molecular characterization of the components making up hepatitis B virus (HBV) have been pursued with worldwide interest. Over the past two decades, such characterization has led to the development of sensitive assays to screen and exclude contaminated units from blood banks and has recently resulted in the licensing of several HBV vaccines. That more than 200 million people worldwide are chronically infected with HBV, and that they are at a high risk for the development of chronic hepatitis and hepatocellular carcinoma, still represent formidable problems in our understanding of host-virus relationships on the molecular level. In the absence of a suitable tissue culture system, and with a very limited host range of infection, characterization of HBV on the molecular level has made remarkable progress recently with the advent of genome cloning, sequencing and expression of individual virus genes by recombinant DNA technology. The presence of hepatitis B-like viruses in an expanding number of animal hosts, and the possibility of virus replication in cells other than hepatocytes, provide great promise that future work will elucidate the molecular mechanisms operative in the various outcomes of HBV infection. In an effort to help develop this background upon which such future work depends, this monograph was written to present a detailed description of each HBV gene and gene product, to discuss how the various gene products might interact during the virus life cycle and in disease, to review various models, and to pose unanswered questions for future research. The monograph discusses the molecular characterization of HBsAg, HBcAg, HBeAg, HBsAg associated receptor for polymerized albumin, HBV DNA, HBV DNA polymerase activity, core associated protein kinase activity, the relationship between HBV and hepatocellular carcinoma, HBV gene expression in disease and by recombinant DNA techniques, the 5' covalently bound protein of HBV DNA, and the replication scheme for HBV and related viruses. Although the approach is comprehensive, and information from earlier portions of the monograph are integrated into later sections for clarity, the monograph is constructed in the hope that it will be useful to all who have an interest in the field.

MOLECULAR COMPONENTS OF HEPATITIS B VIRUS

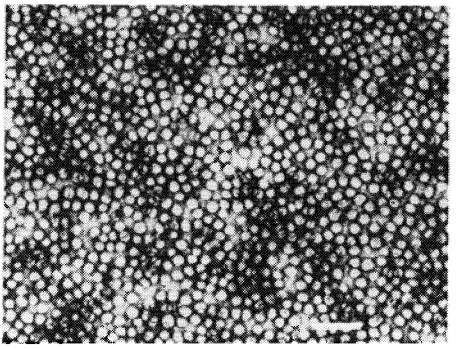
THE MOLECULAR COMPONENTS OF HEPATITIS B VIRUS

MARK FEITELSON
Division of Clinical Research, Fox Chase Cancer Center, Philadelphia, PA

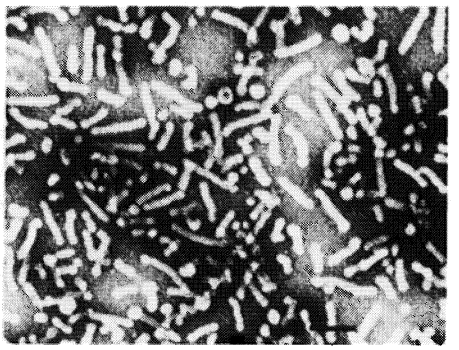
INTRODUCTION

The search for new antigenic polymorphisms among different human populations, undertaken by B. S. Blumberg and colleagues in the early 1960's, resulted in the discovery of a substance in the sera of an Australian aborigine which reacted in immunodiffusion analysis with a component in the serum of a hemophilia patient in the United States (1-3). The finding of this "Australia antigen" (Au) in the sera of leukemia prone Down's syndrome patients, and the seroconversion of one patient from Au^- to Au^+ associated with raised serum transaminase levels, suggested liver involvement, and first established a link between Au and acute viral hepatitis (4). Further work confirmed these findings (5-8) and revealed that Au is, or is part of, a transmissible agent capable of establishing evidence of infection (serum Au or anti-Au) in drug addicts (9, 10) and in patients undergoing hemodialysis (11, 12) or otherwise exposed parenterally to blood or blood fractions (5, 13-16). The subsequent screening for Au^+, or hepatitis B virus surface antigen (HBsAg) positive sera, dramatically reduced the incidence of post-transfusion hepatitis (3, 17) and paved the way for both isolation and characterization of HBsAg as well as future vaccine development.

The antibody directed against surface antigen, anti-HBs, detects three forms of HBsAg in the sera of individuals with acute or chronic hepatitis B virus infection. The predominant form observed by immune electron microscopy consists of 18-22 nm diameter spherical particles, while smaller amounts of variably long filamentous particles of the same diameter were also present (18). In much lower concentrations, many HBsAg positive sera also possess larger spherical or Dane particles (19), which are 42 nm in diameter (Figure 1) and contain an outer

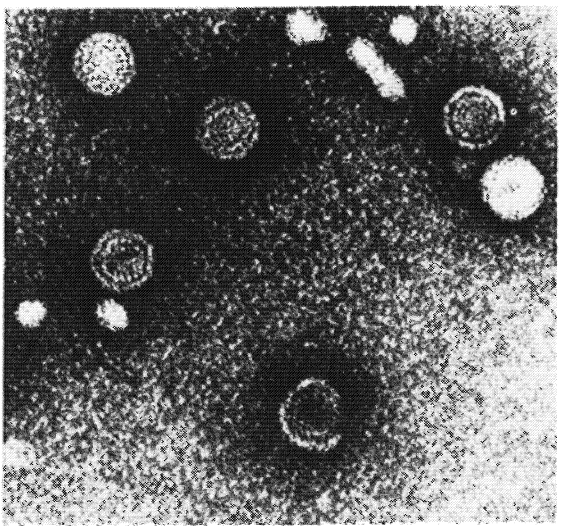


1A



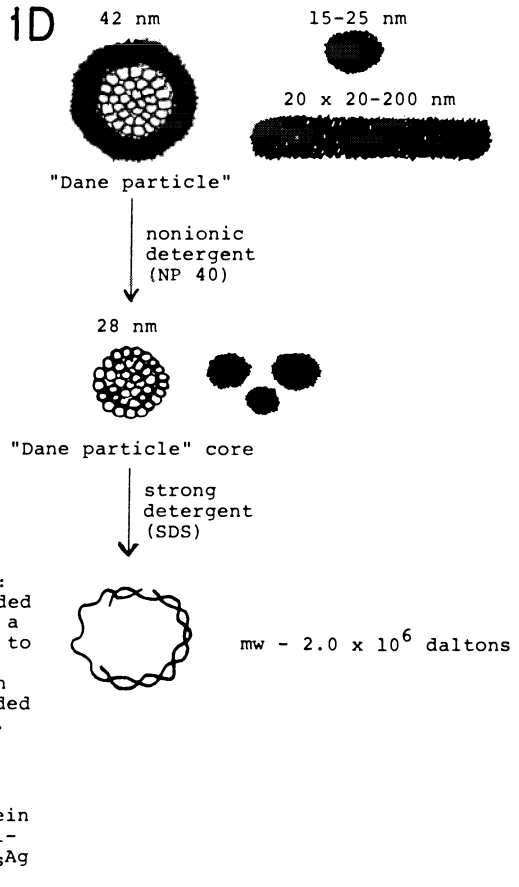
1B

envelope (having HBsAg reactivity) and an inner core, or nucleocapsid (having HBcAg reactivity) which are antigenically distinct (20). The HBsAg, present on all of these particles, consists of a group specific antigen "a" (present in all HBsAg preparations (21-23)) and at least two subtype determinant systems, "d/y" and "w/r," which behave in a quasiallelic manner (only one or the other subtype found in HBsAg from a single source) and may represent the expression of distinct HBV genotypes (22, 24-28). HBsAg also contains host lipid and possibly host serum or hepatocyte cell surface proteins and/or glycoproteins (3, 29-40). In addition to HBsAg, Dane particles alone possess an electron dense inner core (HBcAg) which is approximately 28 nm in diameter, have several protein components of distinct molecular weight (41-45, 56),



1C

have a small circular, partially double stranded DNA molecule which is 1.6 x 10^6 daltons in size (42, 46, 47), and have associated DNA polymerase activity (48-50, 557) (Figure 1). Further, both Dane and liver derived core particles possess a serologically defined e antigen (HBeAg) activity, which is cryptic in nature (51-53), as well as associated protein kinase and apparent protease activities (54, 55). Additional information that core particles have been found in the nuclei of infected hepatocytes (57-60), that Dane particles display virus-like morphology, and epidemiological evidence linking DNA polymerase containing Dane particles with the development of anti-HBc (61-63) and clinical disease (64, 65) strongly support the Dane particle as the infectious agent of hepatitis B. Since the 22 nm spherical and filamentous forms do not contain core components (HBcAg, HBeAg), DNA, or any of the associated enzyme activities, it is widely held that these forms represent incomplete viruses produced in large excess after infection. A subset of patients with acute hepatitis and some HBsAg chronic carriers, however, have been found to contain yet another serological specificity, delta antigen, cryptically associated with HBsAg in the absence of other HBcAg associated antigenic or enzymatic markers (66-69). Although transiently found in serum, delta antigen, like core antigen, is found in the nuclei of infected hepatocytes.



1. HB_sAg:
Particles with HB_sAg (a, d/y, w/r) on their surfaces.

2. HB_cAg:
Dane particle core with HB_cAg on its surface and DNA polymerase activity.

3. DNA and DNA Polymerase:
Circular, double stranded Dane particle DNA with a single stranded gap up to 1/3 of circle length. DNA polymerase reaction makes the single stranded region double stranded.

HB_eAg:
$\sim 3 \times 10^5$ dalton protein antigenically and physically distinct from HB_sAg and HB_cAg.

Figure 1. The major serum forms associated with HBV infection. (A) Electron micrograph of 22 nm small spherical HBsAg particles; (B) Electron micrograph of variably long filamentous HBsAg particles. Bars indicate 100 nm [Copyright 1980 by Alan R. Liss, Inc. (ref. 244)]; (C) Electron micrograph of intact Dane particles [Reprinted with permission from Robinson, W.S. in Comprehensive Virology 14:471-526 (ref. 974). Copyright 1979 by Plenum Press]; (D) Diagrammatic representation of serum derived particles from infected patients (from ref. 974).

Electron microscopic evidence shows that when the human liver is infected with hepatitis B virus (HBV), dilation and disruption of the rough endoplasmic reticulum with the detachment of ribosomes and concomitant decrease in hepatogenous protein synthesis occurs (70), eventually proceeding to necrosis of single and small groups of hepatocytes and their replacement by inflammatory lymphocytes and histiocytes (71). In hepatocytes, HBsAg has been found in association with the nuclear envelope, along the rough surfaced endoplasmic reticulum (ER) and in the cisternae of the ER. Particulate HBsAg was observed only in the ER cisternae, suggesting that polypeptide synthesis occurred at the rough ER followed by secretion into the cisternae (512, 772-776). Further, these results suggest that the membranes of the rough ER actively participating in HBsAg synthesis, processing and secretion are those areas which become dilated and disrupted. Damage to the hepatocyte plasma membrane also occurs and results in the release of aminotransaminases as well as other enzymes during acute hepatitis. The release of hepatocellular antigens from tissue becoming progressively necrotic may stimulate autoimmune or autoaggressive immune responses and thereby produce, at least in part, the chronic, self-perpetuating form of viral hepatitis (71, 72). While the production of HBsAg/anti-HBs immune complexes in persistent HBV infection might also contribute to the stimulation of autoaggressive immune responses (85), their presence is associated with low frequencies of extrahepatic syndromes such as polyarteritis nodosa (73-77), membranous glomerulonephritis (73, 74, 78-80), and infantile papular acrodermatitis. The latter condition has been characterized by mixed cryoglobulinemia and cryoprecipitates containing HBsAg (81-83) in most but not all cases (84). In addition, lymphocytes sensitized to viral (85) or altered self antigens (86) on infected hepatocyte cell membranes may cause immunologically mediated hepatocyte destruction (71, 87). Although a direct cytopathic effect (CPE) is not completely ruled out by the available evidence, the long incubation periods during which evidence of viral replication is observed in the absence of illness or tissue injury argue against direct CPE as being central to the pathogenesis of HBV infection (88). The most important component of this massive liver inflammation is fibrosis which often leads to postnecrotic cirrhosis (71), usually of the macronodular type. Many epidemiological studies

have demonstrated an association between macronodular cirrhosis and primary hepatocellular carcinoma (PHC), which suggests a possible etiological relationship of HBV to PHC (89-98). As the HBV associated antigens were discovered and their detection became possible by sensitive radioimmunoassays, many studies reported a relatively high frequency of HBsAg among patients with PHC throughout the world (93, 97, 99-102). Further investigation showed a relationship between anti-HBc and PHC (97, 103, 104). Furthermore, reports that HBsAg, cirrhosis and hepatoma occur more commonly in males than females and that they often cluster if families further suggest that all three may be causally interconnected (97). The hypothesis put forth that HBV infection leads to the sequence of acute hepatitis → chronic hepatitis → cirrhosis → hepatoma, then, is not only supported by the results already discussed, but is also substantiated by the fact that neoplastic cells make their first appearance in areas of long-term cirrhosis (71), and that viral DNA sequences have been found in association with high molecular weight DNA in chronically infected human livers and in PHC tissue (105-112). The establishment of the human hepatoma cell line, PLC/PRF/5, which has viral sequences associated with high molecular weight DNA, possesses at least some viral specific transcripts (114-116), secretes 22 nm spherical HBsAg particles into the culture medium (113), and forms tumors in nude mice (117-119), also supports the close association between hepatitis B virus and primary hepatocellular carcinoma. The recent finding of a hepatitis B-like virus in association with hepatocellular carcinoma in woodchucks (120-123) further demonstrates this type of association.

There are more than 200 million carriers of HBV throughout the world (as indicated by the presence of serum HBsAg, anti-HBs or anti-HBc). Since acute and chronic hepatitis are often debilitating diseases, associated primary hepatocellular carcinoma often fatal, and adequate treatments still lacking, infection with hepatitis B virus is still a major public health problem. An adequate understanding of the molecular biology, biochemistry, replication, immunology and molecular pathogenesis of disease with HBV have been hampered by the lack of a suitable tissue culture system (124-131) and restricted host range of infection. The PLC/PRF/5 (113) as well as other human hepatoma cell lines that secrete HBsAg into the culture media (141-146) have thus far

shown little evidence of virus replication (147). Further, the consistent finding of HBV replication in hepatocytes (59, 149) and less suggestive data delineating an association between HBV and the pancreas (150, 151) point to a narrow tissue and cell tropism. Host range is also very narrow, consisting of man and several lower primates (134, 139, 140) including chimpanzees (132-136, 140), gibbons (133, 137) and African green monkeys (132, 138). Despite the difficulties present in being able to study various molecular parameters of the virus and host-virus interactions with only infected biopsy or autopsy material and HBV containing blood bank units from infected patients, the discovery of related viruses in woodchucks (120), ground squirrels (152) and Pekin ducks (151) have provided more suitable models for experimental manipulation (153, 154). The molecular cloning and restriction endonuclease mapping of the mammalian hepatitis virus DNA's (47, 161-166), and the recent nucleotide sequencing of HBV (155-157), woodchuck hepatitis virus (WHV (158-160)), ground squirrel hepatitis virus (GSHV (913)) and duck hepatitis B virus (DHBV (912)) DNA clones, have resulted in considerable information concerning their relatedness and genetic organization. Recombinant DNA technology has also permitted assessment of the state of viral DNA in acute and chronic hepatitis as well as in hepatoma tissue (105-112). The detection of HBsAg and HBcAg in bacterial or mammalian cells transformed by a variety of recombinant phage vectors or plasmids, respectively, is being used to study expression of the various HBV genes, used for vaccine production, and used to further characterize HBV associated polypeptides now available only in minute quantities (167-179). The recent characterization of some hepatitis virus associated polypeptides by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and tryptic peptide mapping has further established the relationships of the various animal viruses to each other and to HBV as well as suggested the possible genetic organization on these viral genomes leading to expression of the characterized polypeptides (55, 56, 180, 181). The deduction of sequential determinants in polypeptides from the nucleic acid sequence of HBV, their synthesis in vitro, and testing of their abilities to produce protective and neutralizing antibodies (182-187) represent the hope that a future synthetic vaccine against HBV can be made. The recent

suggestion that the hepatitis B-like virus from duck liver may use reverse transcription in part of its replication cycle (188, 189) would not only be unique among DNA viruses, but will permit study of the enzymology of replication and a clearer understanding of the mode of replication which may be common to all hepadnaviruses. In this monograph an attempt will be made to describe the molecular characteristics of the various HBV associated components in order to better understand HBV itself and virus-host interactions on the molecular level.

HEPATITIS B SURFACE ANTIGEN (HBsAg)

Nature of the "a" determinant.

The observations (a) that antibody directed against surface antigen (anti-HBs) becomes most prevalent following recovery from acute type B hepatitis; (b) that it is often present in asymptomatic carriers without detectable HBsAg and (c) that anti-HBs may persist for many years after initial exposure to HBV suggest that anti-HBs containing serum contains protective antibodies (3, 353, 354). Further, some high risk human populations could be protected from infection with HBV by immunization with highly purified HBsAg particles (3, 274-279) and by injection of immunoglobulin preparations containing a high titer of anti-HBs (280, 281). In contrast, the persistence of serum HBsAg following acute infection has often signaled procession to chronic hepatitis, and similar persistence of HBsAg in chronic hepatitis (with or without an acute disease phase) has been associated with continued chronic liver disease, indicating that persistent HBsAg expression can be associated with progressive disease (3). Therefore, studies directed toward the isolation and physiochemical characterization of HBsAg particles (mostly the spherical, 22 nm forms) for the purposes of eventual vaccine production were undertaken in many laboratories.

The association of serum components with 22 nm spherical HBsAg particles, and repeated attempts to purify HBsAg away from these components, has resulted in only partial agreement as to the physical and chemical properties of these HBV associated forms (29, 557). Some of these properties are listed in Table 1. Small spherical HBsAg particles have been isolated in part by gel filtration through Sepharose 4B (224), Sephadex G-200 (197, 231) or Biogel A5-M (180, 181), by rate

Table 1. Some physical and chemical properties of spherical 22 nm HBsAg particles.

Parameter measured/treatment	ref(s)	result
1. particle diameter	212	16-25 nm
surface subunit diameter	18	3 nm
particle diameter and ether	214	12-21 nm
2. particle molecular weight (daltons)	196	3.7×10^6 (ad)
	196	4.6×10^6 (ay)
	220	2.4×10^6
	224	2.5×10^6
	221	2.74×10^6
- by Sepharose 4B	45	3.6×10^6 (ad)
- after 50%, 1, 1', 3, 3'-tetramethylurea	260	3×10^6
3. particle density (gm/ml), CsCl	419	1.18
CsCl	216	1.20
- in e antigen positive sera	873	1.213
- in anti-HBe positive sera	873	1.202
sucrose	199	1.17
potassium tartarate	216	1.15
amido trizoate	217	1.19
- after 50%, 1, 1', 3, 3'-tetramethylurea; CsCl	260	1.27
- in immune complexes; CsCl	190	1.25
4. particle sedimentation coefficient	190	54S
	218	34.1S
	19	30.8S
	220	40.2S
	221	40.5S
	222	33S (ad)
	222	40.1S (ay)
- 1% SDS, 65mMDTT, 100°C, 2 min	261	25S
- 2% Triton X-100, 37°C overnight	210	3.9S
5. particle isoelectric point(s) (pH)	419	3.6-4.0
	196	3.9,4,4.2,4.5, > 6 (ad)
	196	3.9,4.1,4.2, 4.4,4.9,5.3, > 6 (ay)
	197	4.7 (ad)
	197	4.9 (ay)
- 15-19 nm diameter	198	3.65
- 20-22 nm diameter	198	3.95
	265	4.8-5.0
- 1% Tween 80	33	4.7-5.2, 5.3-5.7, 4.2-4.6

Table 1. (Cont.)

Parameter measured/treatment	ref(s)	result
6. half life; *in vivo*, humans	215	3.3d
in vivo, rabbits	376	10-12 hrs
- in rabbits with prior neuraminidase	376	< 10 min
7. electrophoretic mobility	220	α_2-βglobulins
	197	α_2 globulin
8. extinction coefficient (280 nm)	221	29.9
	223	25
	192	37.26
9. concentration in blood; mg/kg	220	2.1
(ave) particles/ml	220	3.35×10^{13}
(ave) µg/ml	220	194
10. diffusion constant; cm^2/sec	220	2.278×10^{-7}
11. alpha helical content (ORD/CD)	242	70-80%
12. total protein content	29	40-70%
	769	70%
13. total carbohydrate content	195	3.6-6.5%
	364	7.5%
	375	7.6%
- fucose (µg/mg HBsAg)	375	1.1
- glucose (µg/mg HBsAg)	375	trace
- mannose (µg/mg HBsAg)	375	12.1
(µg/mg p26-29)	298	8.41
- galactose (µg/mg HBsAg)	375	8.3
(µg/mg p26-29)	298	12.06
- N-acetylglucosamine (µg/mg HBsAg)	375	45.8
(µg/mg p26-29)	298	28.35
- sialic acid (µg/mg HBsAg)	376	0.19
(µg/mg HBsAg)	375	8.5
% of carbohydrate	295	1.64%
% of carbohydrate	375	0.87%
- total hexose (µg/mg HBsAg)	375	25
- total neutral sugar (% of carbohydrate)	295	2.4%
(% of carbohydrate)	375	2.15%
14. total lipids	364	22.5%
- neutral lipids (% of total lipid)	221	19.3%
- cholesterol (% of neutral lipid)	221	36%
- acid phospholipids (% of total lipid)	221	78%

Table 1. (Cont.)

Parameter measured/treatment	ref(s)	result
- phosphatidyl choline (% of phospholipid)	221	82%
(% of total lipid)	31	65%
- sphingomyelin (% of total lipid)	31	30%
- lysophosphatidyl choline (% of total lipid)	31	5%

zonal centrifugation on sucrose gradients (220), by density equilibrium ultracentrifugation (180, 181, 216), by DEAE-cellulose chromatography (218, 232, 233), by polyethylene glycol precipitation (180, 234, 237), isoelectric focusing (196, 235), and by affinity chromatography (236-238, Table 1). This heterogeneity or physical polymorphism was examined more closely under controlled conditions where alterations in the physical properties of HBsAg particles were assessed following exposure to various ranges of pH or ionic strength, to various peptidases and nucleases, and to a variety of organic denaturing agents (213) (Table 2). The repeating cross striations observed by electron microscopy in filamentous HBsAg particles, and the progressive uncoiling of these particles by exposure to low pH (sodium acetate, pH 5) or to increasing ionic strength suggest that HBsAg contains helical protein structures which may partially unwind under these conditions. The disappearance of filamentous forms and stability of the small spherical forms at low pH suggest a dissociation of filamentous forms to small spherical HBsAg particles. The finding that these variably long filamentous forms, but not Dane or small spherical HBsAg particles, bind to the SDS exposed hydrophobic interior of 22 nm spherical particles further suggests this relationship (244). The nearly identical chemical and antigenic composition of the filamentous forms and small spherical HBsAg particles also suggests a close relationship. Other properties are also shared among spherical and filamentous HBsAg (Tables 1 and 3). The enlargement of small spherical HBsAg particles following treatment with 0.1% chymotrypsin (213), complement (245), or exposure to high ionic strength solutions suggests that HBsAg particles could be uncoiled by these

Table 2. Effects of various treatments upon the integrity of 22 nm spherical HBsAg particles.

Treatment	morphology	antigenicity
1. heating; 6 hrs, 37°C		+ (30)
1 hr, 56°C		+ (246)
6 hrs, 56°C		+ (30)
18 hrs, 56°C		+ (216)
85°C	+ (246)	- (246)
85°C		- (283)
100°C	+ (246)	- (246)
- remaining activity,		
5 min, 100°C		50% (283)
15 min, 100°C		- (248)
- remaining activity,		
20 min, 100°C		25% (283)
- remaining activity,		
40 min, 100°C		10% (283)
autoclave		- (248)
2. freezing/thawing (40 cycles)		+ (30)
3. putrefaction (rm temp, 4 wks)		+ (30)
4. enzymes/detergents; pepsin		+ (30)
pepsin, 3 hrs, 37°C		± (283)
1% SDS, then pepsin		± (30)
pepsin, then RNase + DNase		+ (30)
pepsin, then trypsin		+ (30)
trypsin	± (213)*	+ (30)
- RIP buffer, 0.1% trypsin,		
4 hrs, 37°C		- (32)
1 mg/ml trypsin		+ (204)
trypsin		+ (283)
1% SDS, then trypsin		± (30)
chymotrypsin	± (213)*	+ (30)
2% SDS, then 1 mg/ml		
chymotrypsin		+ (204)
1 mg/ml chymotrypsin		+ (204)
1% SDS, then chymotrypsin		± (30)
subtilisin		± (30)
1% SDS, then subtilisin		± (30)
subtilisin		± (204)
subtilopeptidase A		± (30)
1% SDS, then subtilo-		
peptidase A		± (30)
0.2% bromelin	- (213)	

Table 2. (Cont.)

Treatment	morphology	antigenicity
- RIP buffer, 0.1% bromelin		
4 hrs, 37°C		+ (32)
pronase	± (213)*	+ (30)
ethanol:HoAc (3:1 v/v),		
then pronase		- (246)
pronase, 16 hrs, 37°C		+ (283)
1% SDS, then pronase		± (30)
pronase		± (204)
lysozyme		+ (30)
1% SDS, then lysozyme		± (30)
pancreatic lipase		+ (30)
1% SDS, then pancreatic lipase		± (30)
RNase and DNase	+ (213)	+ (30)
RNase		+ (283)
DNase		+ (283)
alpha-amylase		+ (246)
wheat germ lipase		+ (246)
neuraminidase		+ (246)
glycosidases/neuraminidase, 24 hrs, 37°C		+ (283)
phospholipase C		+ (283)
5. acids; pH 2-5, 3 hrs, 37°C		+ (30)
sodium acetate, pH 5, 0.1M	± (213)	
sodium acetate, pH 5, 0.25M	- (213)	
pH 2.7		+ (216)
periodate		- (252)
periodate, pH 5.7		± (283)
pH 1.8, 2 wks		+ (248)
6. reduction/alkylation; DTT, then alkylation	+ (242)	- (242)
0.1M DTT, then iodoacetamide		± (241)***
0.1M DTT, 8M urea, then iodoacetamide	- (233)	- (233)
0.2M 2-ME, then 0.2M iodoacetamide		- (239)
immunogenicity, humoral	- (239)	
0.05M DTT, then 0.2M iodoacetamide		- (239)
0.1M DTT, then 0.5M iodoacetamide	+ (240)	- (240)
immunogenicity, humoral	- (240)	
0.1M DTT, 8M urea, then iodoacetamide	- (240)	- (240)
immunogenicity, humoral	± (240)	
1% 2-ME, then iodoacetamide		- (243)
0.1 M DTT, then 0.1M iodoacetamide		- (283)
2-ME, 5%, rm temp, 24 hrs	± (213)*	+ (30)
4% DTT	- (213)	
DTT	+ (242)	- (242)

Table 2. (Cont.)

Treatment	morphology	antigenicity
0.1M DTT, 8M urea		- (233)
0.1M DTT, 8M urea, then reoxidized	+ (233)	+ (233)
0.2M 2-ME		+ (239)
0.05M DTT		+ (239)
reduction, then dialysis		± (283)
0.5M iodoacetamide	+ (240)	+ (240)
immunogenicity, humoral	+ (240)	
0.5M iodoacetamide, 8M urea	+ (240)	+ (240)
immunogenicity, humoral	+ (240)	
0.2M iodoacetamide		+ (239)
7. Detergents; 0.1% SDS, rm temp, 24 hrs		+ (30)
0.5% SDS, rm temp, 24 hrs		+ (30)
1.0% SDS, rm temp, 24 hrs	+ (213)	± (30)
1.0% SDS, rm temp, 24 hrs		- (242)
2% SDS		+ (204)
(aggregated) 2.0% SDS, rm temp, 24 hrs		- (30)
1% SDS, then diethyl ether		± (30)
1% SDS, then 5% chloroform		- (30)
1% SDS		- (246)
1% Tween 80	± (33)	
1% Tween 80	+ (32)	+ (32)
1% Tween 80		+ (30)
1% Tween 80 (2 hrs, 25°C)	± (266)	- (266)
1% sodium deoxycholate	- (213)	
1% sodium deoxycholate		+ (30)
2% Triton X-100, 18 hrs, 37°C, pH 7.3		+ (210)
50% 1,1',3,3' tetramethyl urea	+ (204)	+ (204)
N-cocoyl-L-arginine ethyl ester, DL-pyroglutamic acid salt		- (247)
benzalconium chloride, 5 min		- (248)
8M urea, rm temp, 3 hrs		+ (30)
8M urea	+ (242)	+ (242)
4M urea	± (243)*	
6M urea, 3 hrs, 37°C		+ (239)
delipidation	+ (204)	+ (204)
delipidation with aggregation		- (30)
8. Other treatments; diethyl ether, 2 hrs, 0°C		+ (30)
chloroform:methanol (2:1 v/v)		- (30)
water	+ (213)	
0.1M NaCl	+ (213)	
0.25M-0.5M NaCl	± (213)**	
0.05M magnesium chloride	+ (213)	
0.1M-0.5M magnesium chloride	± (213)**	

Table 2. (Cont.)

Treatment	morphology	antigenicity
phosphate buffered saline, pH 7	± (213)**	
periodate, pH 7.2		+ (283)
chloroform:methanol (2:1)	+ (204)	+ (204)
formalin, undiluted, 1 hr		+ (248)
formalin, 1.5%, 7 days		+ (248)
80% ethanol, 3 hrs		+ (248)
ether, 5 hrs		+ (248)
phenol, 2%, 24 hrs		+ (248)
chloroform, 3 days		+ (248)
ether, chloroform	+ (246)	+ (246)
ether		+ (216)

*native conformation absent, multistrand and ring form of antigen present (multistrand particles are unwound forms showing four strands).

**ring forms, which stain penetrates; and uncoiled forms, which are large particles displaying loose coiling, were observed.

***a determinant resistant to reduction and alkylation is noted as AuRe. (The common "a" and subtype determinants are sensitive under these conditions.)

+ = property retained following treatment.

± = property decreased following treatment.

- = property destroyed following treatment.

Table 3. Some physical properties of variably long filamentous HBsAg

Parameter measured	ref(s)	result
1. particle diameter	212	20 nm
diameter of nonhelical transverse striations	212	3 nm
2. particle length	18,212	50-200 nm
3. particle density (in CsCl)	194	1.20
4. particle isoelectric point(s); (pH)	198	4.1

these various procedures. However, the resistance of small spherical HBsAg particle morphology and antigenicity to a variety of denaturing agents, proteolytic enzymes, and to penetration by phosphotungstic acid following negative staining for electron microscopy (29, 213, Table 2) suggest a highly compact physical structure and remarkably stable antigenic structure for these particles. Most of the morphological forms in which a suggested helical content of the protein was preserved (by electron microscopy) following physical or chemical treatment of HBsAg particles still agglutinated with anti-HBs. The appearance of amorphous conglomerates of protein following treatment of HBsAg with dithiothreitol (DTT), and their failure to agglutinate with anti-HBs under these same conditions (233, 239-241) suggests that a secondary structure or a tertiary structure necessary to "lock in" the configuration of one or more antigenic determinants was required for neutralizing antibodies to bind to the "helical" structures of HBsAg, independent of the different morphological forms of surface antigen. The conformation of small spherical HBsAg particles, studied by optical rotating dispersion (ORD) and circular dichroism (CD) showed that 70-80% of the protein is alpha-helical in nature (242). However, treatment of intact HBsAg with 1% SDS, DTT or by carbamidomethylation rendered the particles nonantigenic while not affecting their ORD or CD spectra. Protein secondary structure, then, may play a role in forming critical antigenic sites, but the tertiary structure may play an equally important role in bringing two or more of these helicies into the correct juxtaposition for antibody binding.

The importance of tertiary protein conformation to both the common and subtype specific determinants of small spherical HBsAg particles has been demonstrated by the consistent finding that reduction and alkylation results in loss of antigenic activity with respect to these determinants (233, 239-241, 343, Table 1) and that reoxidation of the reduced disulfide bonds often results in the re-establishment of at least some of this serological activity. In some studies, however, particle morphology, as studied by electron microscopy, became indistinct following reduction, or reduction and alkylation (213, 233), while other studies showed no alteration in the morphology (240, 242) or sedimentation coefficient (239) of small spherical HBsAg following

identical modification (Table 2). While reduction and alkylation of HBsAg abolished binding to most anti-HBs containing sera, the finding of a delayed type hypersensitivity response in guinea pigs (239) and the precipitation of such chemically modified HBsAg with a subset of antibodies in sera directed against native HBsAg (241) has resulted in the designation of "Re" determinant(s) which are resistant to reduction and alkylation. Further studies using intact HBsAg or reduced and alkylated HBsAg as immunogens, followed by testing homologous or heterologous antisera with each antigen, were used to study the relationship among these determinants in greater detail (243). Reduced and alkylated HBsAg not only failed to bind antibodies directed to the common "a" and subtype specific determinants of native HBsAg, but also failed to elicit antibodies to these determinants. In contrast, reduced but nonalkylated forms of HBsAg bound antibodies to native, but not reduced and alkylated antigen, suggesting that alkylation results in the appearance of new antigenic sites in HBsAg. HBsAg reduced in 8M urea, but not alkylated, generated antibodies reacting with both intact and reduced plus alkylated antigen, suggesting successive appearance of new antigenic determinants after unfolding, reduction, and finally alkylation. If the type of immunological response to HBsAg is dependent upon the proportion of differing morphological forms of antigen appearing early after infection, then the appearance and titer of neutralizing antibody, which would limit the spread of virus in the liver, as well as the kinetics and final levels of virus specific cell mediated immune response, which would be instrumental in eliminating productively infected cells, may be partially regulated in such a fashion.

The high percentage of alpha helical content in HBsAg, the presence of disulfide bonds essential to its antigenicity, and the resistance of the 22 nm spherical forms to a variety of physical and chemical treatments (Table 2) suggest a very compact physical structure for surface antigen and the likelihood that its major immunodominant determinants are largely conformation dependent. The chemical modification of various surface amino acid residues of HBsAg, as well as the fragmentation of surface antigen by physical or chemical means were carried out in an attempt to deduce and/or directly identify conformation dependent

antigenic determinants. Since HBsAg possesses a high extinction coefficient (239), an absorption shoulder at 290 nm in a continuous scan of purified antigen which is characteristic of high tryptophan content (250), and a high percentage of tryptophan by amino acid analysis (29, 192, 199), the role of tryptophan residues in the serological activity of surface antigen was assessed by titration with N-bromosuccinimide (NBS) at pH 4 in 8M urea (249). Under the conditions of chemical modification, the great majority of the tryptophan residues were derivatized. Cleavage at trp residues did not generate major dialyzable peptides, and serological activity remained intact. Recent experiments exposing intact HBsAg particles (in the absence of urea) to N-chlorosuccinimide, which modifies and affects cleavage only at tryptophan residues (250a, 792), however, destroys the antigenicity of 22 nm spherical HBsAg completely. In other studies, exposure of HBsAg to periodate treatment eliminated both common "a" and subtype "d" serological activity, while subtypes "w" and "r" were resistant (251, 252). Although it was suggested that the carbohydrate chain(s) attached to some of the polypeptides of HBsAg (see below) may be responsible for the periodate sensitivity of "a" and "d" activity, the exposure of proteins to periodate can also result in very rapid oxidation and cleavage at tryptophan residues (253-256) and can also result in oxidation of serine, threonine, methionine, cystine, tyrosine as well as other amino acid residues in an intact protein. The reaction of intact HBsAg with 2-hydroxy-5-nitrobenzyl bromide (HNB-Br), a reagent highly selective for externally facing tryptophan residues (257-259), also resulted in the blocking of serological reactivity (792). Since HBsAg particles possess a major polypeptide component 22-25,000 daltons in size (p22-25) and a major glycoprotein 26-29,000 daltons in size (p26-29) which, by all criteria measured thus far, is a glycosylated form of p22-25, it would be predicted that p26-29 should give rise to anti-a and anti-d while p22-25 would not if the attached carbohydrate carries these antigenic specificities. The production of antibodies to both common and subtype specificities by either of these individual polypeptides suggests that the carbohydrate moieties are not directly involved in the expression of these immunogenic specificities. These results suggest that the periodate sensitivity of the "a" and "d" determinants

is not the consequence of carbohydrate sensitivity to this treatment. Recent results have shown, however, that tryptophan residues modified by 2,3-dioxo-5-indolinsulfonic acid did not result in a significant reduction of HBsAg antigenicity (260). It is possible that the different results obtained in several laboratories could be a consequence of using different reagents and experimental conditions for modification and the fact that HBsAg has a relatively large number of tryptophan residues which may be derivatized to various extents in each study. Since the conformation of HBsAg was not studied in parallel to any changes in antigenicity following protein modification, the relative importance of conformational changes and direct blocking of one or more antigenic sites by reagents used is difficult to ascertain.

Treatment of HBsAg with citraconic anhydride (260) or with succinic anhydride (257a), which react with the primary epsilon amino groups of lysine residues in surface antigen, dramatically reduces the antigenicity of HBsAg. Decitraconylation resulted in complete restoration of antigenic activity. Cyanogen bromide (CNBr) cleavage of HBsAg 22 nm spherical particles resulted in fragments reacting with antibodies to native, but not denatured, HBsAg (243). Approximately 10^{-4}% of the antigenic activity in native HBsAg was recovered following CNBr cleavage, while further characterization was not possible due to strong aggregation. The possibility that this very low percentage of residual antigenic activity was a consequence of incomplete cleavage, unfortunately, could not be evaluated. In recent experiments (792), CNBr cleavage of HBsAg particles resulted in total loss of antigenic activity in the commercial assay (Ausria II) for surface antigen. Carboxyl groups modified by carbodiimide-promoted amide formation using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide followed by addition of ethylenediamine resulted in a 19 fold decrease in antigenicity (260). Similarly, cleavage of HBsAg at aspartic acid residues in dilute acid for 6-18 hours resulted in total disappearance of antigenic activity (792). Reaction of HBsAg with 1,2-cyclohexanedione or phenylglyoxal, which specifically modify arginine residues, resulted in no decrease in antigenic activity (260). Reaction of tyrosine residues specifically with tetranitromethane (260) or N-acetylimadazole (792) did not alter antigenicity. Cleavage at reduced cystine residues with 2-nitro-5-thiocyanobenzoic acid resulted in a loss of antigenic activity; a

result consistent with the requirement of disulfide bond integrity for the expression of complete antigenicity. Although most of the reactions employed were chosen to react specifically with rarely occurring amino acid residues in HBsAg, it is not proven whether alteration in antigenicity following treatment is a consequence of conformation changes or blockage within an antigenic site. However, modifications of a single or very small number of residues usually results in antigenic alterations limited to the molecular domain of the determinant where modification has occurred (263). The partial sequence of the major polypeptide components in small spherical HBsAg (p22-25 and p26-29 (264)), combined with the nucleic acid sequence of the HBV genome cloned by recombinant DNA techniques from Dane particles (155-160), has permitted further localization of the tentative common "a" determinant(s) in the deduced sequence of the major surface antigen polypeptide.

The antigenic complexity of HBsAg can be appreciated not only from the serological heterogeneity of the common "a" determinant designated as a_1, a_2 and a_3 specificities on antigen preparations from different patients (25), or the frequent appearance of subtype determinants d, y, r and subspecificities w_1, w_2, w_3 and w_4 (22, 24-28), but from the observation of rare subtype determinants q, g, n, x and t as well as the finding of unusual or mixed subtypes in an individual infected with two or more subtypes of HBsAg (25, 27, 268-270). The most common subtypes of HBsAg usually found are ayw, ayw_2, ayw_3, ayw_4, ayr, adw_2 and adr (269), while examples of unusual subtypes such as awr, adwr, adyw, adyr and adywr may be the result of either phenotypic mixing and/or genetic recombination among two or more viruses in a multiple infection (271-273). The knowledge of this antigenic complexity combined with the conformation sensitivity of the "a" determinant(s) resulted in continued efforts to fragment intact HBsAg particles in an attempt to simplify this complexity and isolate antigenically active fragments. The disruption of intact surface antigen in 8M urea by sonication produced a fragment approximately 6000 daltons in size by Sephadex chromatography which retained antigenic activity in a hemagglutination inhibition assay (200). Although amino acid analysis revealed high tryptophan and no detectable cysteine, cystine or

proline, attempts at further characterization have resulted in the finding of a number of polypeptides in this material which have not been adequately resolved for conclusive identification of the active component (29, 282). In another study, intact HBsAg in 8M urea was reduced with DTT, but not alkylated, and then exposed to 0.3M HCl for 1 hour at room temperature (240). Analysis by rate zonal sedimentation in sucrose and Sephadex G-200 chromatography demonstrated the presence of a small fragment 4000-10,000 daltons in size which retained antigenic activity by radioimmunoassay, but was not characterized further. Treatment of intact HBsAg with chymotrypsin (1 mg/ml) in the presence of 1% SDS resulted in cleavage of the small spherical surface antigen particles into fragments much smaller than the starting material (204). SDS-PAGE of affinity chromatography purified fragments revealed two major low molecular weight peaks at 8500 and 7800 daltons. The results of gel electrophoresis were identical whether or not 2-mercaptoethanol (2-ME) was used in dissociation of the sample, suggesting the presence of serological activity in this case was not dependent upon intact disulfide bands. It should also be noted that prior to affinity chromatography on columns of anti-HBs linked to Sepharose, the chymotryptic fragments of HBsAg particles were labeled with the Bolton-Hunter reagent, which modifies the epsilon amino groups of lysine residues and the N-terminal amino group in each of the fragments (267). If, as other studies suggest, the lysine residues of HBsAg are required for anti-HBs binding, then modification of these residues with this reagent and their binding to affinity columns raises the question of the requirement of free lysine residues for binding to anti-HBs. Of course, if the amino termini of a subpopulation of the HBsAg chymotryptic fragments were labeled in the absence of epsilon amino modification, a free lysine residue would still be available for binding to occur. Unfortunately, parallel analysis of HBsAg chymotryptic fragments not bound to the affinity column was not carried out to see if labeled 8500 and 7800 dalton bands (among others) were observed by SDS-PAGE. When chloramine T iodinated HBsAg particles were subjected to a solution of 1% trypsin in 1% SDS, the particulate nature of the antigen was disrupted and considerable anti-HBs binding activity was observed at the top of rate zonal sedimentation gradients used for analysis (283).

The antigenic activity of the released material was stable to proteolytic enzymes (pepsin and pronase) and boiling (50% activity remaining after 5 minutes), while mixed glycosidases and neuraminidase or periodate (16 hours, 4°C, pH 4.5) treatment resulted in small variable reductions in antibody binding activity. Reduction alone or reduction and alkylation resulted in nearly total loss of serological activity. Sephadex G-200 chromatography of the released material sized it in the range of 5000 to 15,000 daltons. It is interesting that at 10 mg/ml trypsin, HBsAg is sensitive to proteolysis by this enzyme in 1% SDS (283), while replacement of chymotrypsin by trypsin at 1 mg/ml under similar conditions did not result in any detectable cleavage products from HBsAg (204). Further, in many of the serologically active cleavage fragments where substantial characterization has been carried out, carbohydrate was often present. The resistance of intact HBsAg to mixed glycosidases and neuraminidase and the relative susceptibility of cleaved material (283) to these enzymes may point to a role for carbohydrate in the stabilization of one or more antigenic determinants. Notwithstanding the discussion above, the oxidation of carbohydrate chains in HBsAg glycoproteins may contribute to decreased anti-HBs binding for the same reasons.

The appearance of autoaggressive immune responses in patients with chronic HBV infection and the association of many serum (host) components with HBsAg particles has prompted detailed study of the components which are viral in nature and those that originate from the host. Only in this manner can the complex antigenic nature of HBsAg be understood in molecular terms and host components eliminated in future vaccine development. In recent years, the nonionic detergents Triton X-100 and octylglucoside have been used to disrupt the envelope of many animal viruses for the purpose of recovering the envelope proteins or spike glycoproteins in antigenically active form, free from host constituents, for vaccine production (284-289). Disruption of purified 22 nm spherical HBsAg with 2% Triton X-100 and analysis of the products by sucrose rate zonal sedimentation resulted in material having a sedimentation coefficient of 3.9 S (210). Since similar disruption of Semliki Forest virus resulted in the appearance of 4.5 S complexes consisting of a single spike protein bound to 75 molecules of Triton

X-100 (284), it is likely that the slowly sedimenting peak of complexes resulting from Triton X-100 treatment of HBsAg particles generates similar complexes. Further characterization of these Triton X-100 solubilized complexes by anti-HBs affinity chromatography and SDS-PAGE showed that the major polypeptide components, p22-25 and p26-29, were the major constituents in which serological activity resided. When purified 22 nm HBsAg particles were treated with 1% SDS for 2 hours at 37°C in the absence of any reducing agent and the treated material analyzed by gel filtration on Sephadex G-200, two peaks of HBsAg activity were observed (290). The leading peak was characterized by roughly spherical HBsAg particles with an irregular surface, as seen by electron microscopy, and possessing an average diameter slightly less than untreated particles. When the second peak, which contained approximately 10% of the HBsAg serological activity in the column, was subjected to SDS-PAGE in the absence of reducing conditions, a major component at 49,000 daltons in size, which retained antigenic titers of common "a," subtype "d" and "r," and reduction plus alkylation resistant "Re" comparable to those in intact HBsAg, were observed. Other polypeptide components seen by gel electrophoresis of this second chromatographic peak possessed little or no serological activity to any of the same determinants present in the native antigen preparation. Further analysis of p49 by gel electrophoresis under reducing conditions yielded the two major components of HBsAg regularly seen under reducing conditions, p22-25 and p26-29. p49, then, is likely to be at least one of the major polypeptide complexes possessing all of the serological specificities of intact antigen found on the surface of HBsAg particles. Further, when guinea pigs were immunized with intact HBsAg, p49, p22-25, or p26-29, it was shown that p49 induced the same titers of humoral antibody response as intact antigen, while p22-25 or p26-29 induced only about 10% of the titers seen with intact antigen or p49. The isolation of the p49 complex, either by treatment of HBsAg with SDS or Triton X-100, then, may yield polypeptide complexes free of both host components and viral nucleic acid suitable for polypeptide vaccines.

The absence of a suitable tissue culture system for HBV has prompted further research into the development of a semisynthetic or

completely synthetic HBV vaccine by further characterization of the antigenicity, immunogenicity and structural relationships among the various proteins and glycoproteins making up surface antigen. Although many studies have been directed toward enumerating the polypeptides associated with HBsAg, and individual investigations have reported anywhere from 2 (192, 206) to 11 (201, 208) bands, there has only been consistent agreement to the presence of the major polypeptide pair, p22-25 and p26-29 (180, 190-196, 199, 201-211, 261, Table 4). Several reports have shown differences in the polypeptide profiles of ad and ay subtypes (195, 196, 202, 203); yet others have not shown any consistent differences (193, 209, 211). SDS-polyacrylamide gel analysis of surface antigen polypeptides associated with Dane particles, variably long filaments, and small spherical HBsAg particles have shown small but reproducible differences in one study (198) and no differences in another (33), but work has not been carried out to confirm or extend these observations. The relatedness among independent isolates of HBsAg and some of the component polypeptides was further studied by amino acid composition data. When various isolates of small spherical HBsAg particles were hydrolyzed and quantitative amino acid analysis carried out, each of the isolates showed similar composition (29, 192, 203, 264, 291, 292, 298, Table 5). The relatively high percentage of proline residues, whose presence in a protein sequence signals interruptions in alpha and beta helical secondary structure, suggests that helical protein domains separated from each other by a series of proline (or prolyl-glycyl) residues may exist (293, 294). The most common amino acid in HBsAg particles is leucine, which has a strong tendency in helical structures to be associated with alpha helicies. The presence of a larger than average percentage of tryptophan in HBsAg and its tendency in helical structures to be associated with alpha helix formation, also suggests a high percentage of this type of secondary structure. The very high percentage of serine residues in HBsAg, which is interesting because serine tends to disrupt beta secondary structure, is also consistent with the above results, suggesting high alpha helical content in intact surface antigen particles and with the ORD and CD spectra discussed above, which also indicate high alpha helical content (242). The existence of 14 amino

Table 4. Apparent molecular weight of polypeptides associated with 22 nm diameter HBsAg particles.

(190)[a]	(191)	(192)	(193, 194)		(195, 196)		(197)[g,h,i]	(198)[g,i]	(199)
			ad	ay	ad	ay			
					120	120		120	
						105		105	
			95	95					
							90		
							82		
			75	75				69	
					69	69			
				65[d]				55	
			55	55		55			
40[b]			40	40	40	40			
				36[d]				40	
	34								39
32[c]		32	32	32	32[e]	32[e]			
							30	35.5	
	28								32
					27[e]	27[e]			
26			26	26					
		25						27	27
	24								
					22[e]	22[e]		24	
						19			
					18				22
								19	
								16	16
									10

Table 4. (Cont.)

(201, 208)[g]	(202)[g] (209, 211)[g,h,i]		(203)[g]		(204)[j]	(205)[g]	(206)[g]
	ad	ay	ad	ay			
			120	120			
				105	85		
97[k,l]		97[k]					
72[f,k,l]	72[k]	72[k]			68		
68[k,l]				69			
	53.5[k]	53.5[k]	55	55			
52[e,k,l]							
45[k,l]						50	
	41.5[k]	41.5[k]					
40[k,l]			40[k]	40[k]	45		
	36[k]	36[k]				38	
35[k,l]			35[e,k]	35[e,k]			
31[e,k,l]							
	29.5[k]	29.5[k]					
27[e,f,k,l]			27[e]	27[e]		29	
25[e,k,l]							
			24[e,k]	24[e,k]	27		27
	23[k]	23[k]					
22[k,l]					24	24	
			19[k]	19			23.5

Table 4. (Cont.)

(207)	(210)g	(180)g	(261)g,i	(292)g	(295)g,n	(296)g
			96			
			79			> 95
		72		80		
	64[e]		60–65	68		68
		63			64	
		56				55
		54			49.5[n]	
			52		48[n]	
		49		44		44
			45			
40						40
35						
	32[e]					35
		31	31			
	30[e]					
		29			32	
	28[e]		23–30			31
27					30	
		25			28[n]	
24				27[e]		27
	23		17–24			25
19					23	
			12–14[m]	22		22

Table 4. (Cont.)

(33)[g]	(145)[o]	(875)[r]	(144)[j]	(143)[q]
108-110				
101-105				
89-96				
68-72		68		
54-59		53		
				49
			47	
40-44		44		
	43	41		
				38
35-36	36p	36		
			34	
				33
30-32	31p	33		
			28	28
26-27		27		
23-24	23p	24	23	
				22
	20			
16-19				

Table 4. (Cont.)

---

[a]Only components reproducibly present in the hands of each author are presented.
[b]Molecular weights are reported x 10^{-3} daltons.
[c]Underscoring indicates a major HBsAg associated polypeptide component.
[d]In study (194), p65 was absent and p36 present.
[e]PAS positive bands.
[f]Polypeptide bands present in (208) in addition to others listed in (201).
[g]Polypeptide gel profile deduced from Coomassie blue staining.
[h]Polypeptide gel profile deduced from reductively methylated HBsAg using ^{3}H and ^{14}C formaldehyde (262).
[i]Polypeptide gel profile deduced from ^{125}I labeled components using chloramine T or lactoperoxidase procedures.
[j]Polypeptide gel profile deduced from ^{125}I labeled components radioacylated with the Bolton-Hunter reagent (267).
[k]Immunogenicity (anti-HBs) to HBsAg demonstrated from animals injected with indicated polypeptide.
[l]Immunogenicity (cellular response) to HBsAg demonstrated from animals injected with indicated polypeptide.
[m]Major component released from HBsAg treated with 1% SDS and 65mM DTT.
[n]Glycoproteins detected by virtue of carbohydrate(s) labeled with tritiated sodium borohydride.
[o]Polypeptides labeled by ^{35}S-methionine and immunoprecipitated prior to SDS-PAGE.
[p]Glycoproteins detected by virtue of tritiated sugars incorporated into metabolically labeled HBsAg.
[q]Polypeptides labeled metabolically using tritiated amino acids.
[r]Polypeptide gel profile deduced from ^{125}I-labeled components using iodogen.

acid residues of cysteine in p25 (Table 5), the failure to resolve the major surface antigen polypeptides on SDS polyacrylamide gels in the absence of a reducing agent (292), and the resistance of HBsAg particles, even in the presence of 6M urea or 1% SDS, to undergo carboxymethylation, suggest that most if not all of the cysteine residues present in p25 are involved in disulfide bonding. That ORD and CD spectra of reduced and alkylated HBsAg are unaltered even though these particles are nonantigenic suggests spatial separation of otherwise intact protein domains which make up one or more recognizable antigenic determinants. Additionally, reduced and alkylated HBsAg retained its morphology while losing both antigenicity and the ability to elucidate anti-HBs (240). Only by further treatment with 8M urea was morphology lost. Since the overall amino acid composition of HBsAg suggests that at least two thirds of the residues are hydrophobic or neutral in character (Table 5), reduction and alkylation alone may cause alterations in the antigenically important alpha helical domains of HBsAg without affecting the strongly hydrophobic core, while further treatment with 6-8M urea would be needed to denature the sequestered hydrophobic regions. The progressive unfolding of HBsAg described here is consistent with the changing immunological specificities noted when intact HBsAg was reduced or reduced and alkylated in the presence or absence of urea, and these treated forms used to raise antibodies (243).

Another approach utilized to dissect the antigenic complexity of HBsAg so that immunodominant, protective determinants could better be defined on the molecular level involves the isolation of individual surface antigen associated polypeptides and their subsequent characterization by structural, antigenic and immunogenic means. A number of studies have indicated that the amino acid composition of p22-25 is very similar to that of p26-29 (264, 291, 292, 298). Limited N-terminal and C-terminal polypeptide sequencing of p22-25 and p26-29 demonstrated identical sequences in the amino terminal 30 and carboxy terminal 3 amino acid residues (264, 292). The presence of carbohydrate associated with p26-29 and absent from p22-25 (201, 208, 210, 292, 295) suggests that the major and perhaps only difference between the two major HBsAg polypeptides is glycosylation. Tryptic peptide mapping in

Table 5. Amino acid composition of HBsAg and some constituent polypeptides.

Amino acid residue	(203) HBsAg	(264) HBsAg	p25	p29	(192) HBsAg	(291) HBsAg DRAA p25	p29	HBsAg 630198 p68	p25	p29	p68
lys	1.7[a]	2.2	2.2	2.0	1.9	2.4	1.7	6.9	1.9	2.1	4.6
his	0.6	0.6	0.7	0.6	0.7	0.5	0.4	2.8	0.8	1.0	1.3
arg	3.1	2.4	2.2	2.1	2.6	2.4	2.1	3.1	2.2	2.3	3.1
asp	5.3	5.0	5.6	5.7	5.5	7.2	7.0	9.2	5.6	5.3	8.1
thr	7.8	7.8	8.4	8.6	8.2	8.1	8.1	5.6	9.6	9.7	7.3
ser	8.3	11.7	11.9	13.0	11.1	13.0	12.6	8.4	12.5	12.5	11.7
glu	5.6	5.0	5.8	5.7	5.5	8.5	5.7	11.6	5.0	4.3	8.7
pro	11.6	12.8	12.5	10.6	12.3	10.7	10.4	8.8	11.7	11.4	7.5
gly	7.7	7.2	6.1	6.6	7.5	8.8	9.1	6.3	9.9	9.1	10.5
ala	4.0	3.4	3.5	3.3	3.6	4.6	4.1	9.0	4.0	3.9	7.3
cys-SH	4.8[b]	6.8[b]	7.2[b]	7.0[b]	6.5[b]	5.7[b]	9.8[b]	4.3[b]	5.8[b]	6.4[b]	4.2[b]
val	5.9	4.7	4.1	3.9	5.1	4.4	4.8	5.6	4.7	4.8	5.6
met	1.4	3.2	3.0	3.1	2.7	2.0	2.0	1.3	2.9	2.3	1.3
ile	6.2	5.6	5.7	5.1	5.5	4.6	4.9	1.7	5.2	5.8	3.3
leu	16.7	11.9	11.2	10.9	12.5	12.2	11.7	10.3	12.0	12.0	9.7
tyr	1.1	2.2	2.4	2.3	2.3	0.9	0.6	ND[c]	1.1	1.5	1.3
phe	8.0	5.1	4.9	5.1	5.9	4.3	5.3	4.8	5.2	5.5	4.3
trp											
gln											
asn											

Table 5. (Cont.)

Amino acid residue	(874) p31	(874) p35	p31 gene	(29) HBsAg	(292) p25	(292) p30	(155) p25 gene[e]	(177) p25 gene[e]
lys	1.1 (3)	1.5 (4)	1.1 (3)	3.4	1.6	1.6	0.88 (2)	1.33 (3)
his	0.75 (2)	1.1 (3)	1.1 (3)	1.1	0.5	0.5	0.44 (1)	0.44 (1)
arg	3 (8)	3.3 (9)	2.8 (8)	3.6	2.3	2.3	2.7 (6)	2.2 (5)
asp	5.2 (14)	5.2 (14)	5.5 (15)	6.1	4.8	4.7	3.5 (8)	4.0 (9)
thr	7.8 (21)	7.5 (20)	7.8 (22)	7.7	8.3	8.3	8.4 (19)	7.5 (17)
ser	11.5 (31)	11.5 (31)	11.4 (32)	10.8	12.8	12.5	11.1 (25)	10.6 (24)
glu	4.8 (31)	4.8 (13)	4.3 (12)	5.3	4.8	4.6	4.0 (9)	4.0 (9)
pro	11 (30)	11 (30)	10 (28)	12.3	10.8	11.0	9.3 (21)	10.2 (23)
gly	7.1 (19)	7.1 (19)	6.8 (19)	7.8	6.8	7.4	6.6 (15)	6.2 (14)
ala	4.1 (11)	4.1 (11)	3.9 (11)	5.4	3.1	3.0	2.2 (5)	2.7 (6)
cys-SH	5.2 (14)	5.2 (14)	5 (14)	5.2[b]	7.7[b]	6.8[b]	6.2 (14)	6.2 (14)
val	4.5 (12)	4.4 (12)	5 (14)	5.4	4.5	3.8	4.4 (10)	4.9 (11)
met	1.9 (5)	2.2 (6)	2.5 (7)	2.3	2.6	2.7	3.1 (7)	2.7 (6)
ile	5.2 (14)	5.2 (14)	6.8 (19)	4.2	5.0	5.5	6.2 (14)	7.1 (16)
leu	13.4 (36)	12.6 (34)	12.8 (36)	13.2	10.5	11.1	15.5 (35)	14.6 (33)
tyr	1.5 (4)	1.5 (4)	2.5 (7)	2.5	2.5	2.5	2.7 (6)	2.7 (6)
phe	6.7 (18)	6.3 (17)	6 (17)	5.4	4.8	5.1	7.1 (16)	7.1 (16)
trp	5.2 (14)	5.2 (14)	5 (14)		6.6[d]	6.5[d]	5.8 (13)	5.8 (13)
gln								
asn								

Table 5. (Cont.)

Amino acid residue	(157) p25 gene[e]	(298) HBsAg	p25	p29	p68
lys	0.88 (2)	2.2	2.5	2.3	2.8
his	0.44 (1)	0.4	0.6	0.7	0.6
arg	2.7 (6)	2.6	3.3	3.3	3.2
asp	3.5 (8)	6.3	6.5	6.2	6.5
thr	7.1 (16)	8.7	8.8	8.4	8.2
ser	10.5 (26)	11.8	11.2	11.4	11.0
glu	4.0 (9)	5.5	6.0	6.2	6.6
pro	9.3 (21)	13.8	12.8	13.6	12.7
gly	6.6 (15)	7.7	8.1	7.9	8.0
ala	2.7 (6)	4.3	4.4	4.5	5.0
cys-SH	6.2 (14)	5.1[b]	3.6[b]	3.5[b]	3.4[b]
val	4.0 (9)	4.3	4.5	4.5	4.9
met	2.7 (6)	1.1	1.8	2.0	1.9
ile	7.5 (17)	4.5	4.5	4.3	4.2
leu	15.5 (35)	13.3	12.5	12.4	12.3
tyr	2.7 (6)	2.6	3.1	3.0	2.9
phe	7.1 (16)	5.8	6.0	5.8	5.6
trp					
gln					
asn					

[a]% amino acid on a molar basis.
[b]Determined as cysteic acid.
[c]ND means not determined.
[d]Tryptophan detected by amino acid analysis following base hydrolysis.
[e]Amino acid composition (and sequence) deduced from nucleic acid sequence of cloned HBV DNA.
[f]Number of residues in p25 indicated by number in parentheses.

one (292, 298) and two dimensions (180, 181, 292) also suggests a high degree of homology among these two major polypeptides. Although the glycosylation of p26-29 may be responsible for the differences seen on this level of analysis, the exact relationship of these components to each other requires further investigation. It is interesting to note that the major polypeptide pair of ground squirrel hepatitis surface antigen (GSHsAg (152)) and woodchuck hepatitis surface antigen (WHsAg (120)), which migrate slightly faster on SDS polyacrylamide gels than the corresponding major HBsAg polypeptide pair, also yielded peptide maps in their respective pairs that shared a large degree of homology (180, 181, 768). Since HBsAg, GSHsAg and WHsAg serologically cross-react with anti-surface antigen immunoglobulins derived from immunized guinea pigs (anti-HBs), mice (anti-GSHs) and from convalescent woodchucks (anti-WHs), one or more antigenic determinants must be shared among these surface antigen particles (181). Peptide mapping of the major "nonglycosylated" polypeptide of each showed that human and animal virus surface antigen particles are structurally related and share at least 25% of their amino acid sequences. Perhaps one or more common neutralizing determinants are within these shared regions of peptide structure. Other investigators have also demonstrated serological cross-reactivity among HBsAg, GSHsAg and WHsAg particles, but the results obtained in each case showed considerable variability related to the immunological reagents used as well as assay type and sensitivity. Several studies suggest substantial cross-reactivity among HBsAg, WHsAg and GSHsAg by immunodiffusion and RIA (368-370). In contrast, while purified WHsAg particles reacted with sera from WHV infected animals by immunodiffusion (ID) or counterelectrophoresis (CEP) and observed as immune aggregates by electron microscopy, no cross-reactivity was seen between the same antigens and heterologous antisera in the same assays (299). Passive hemagglutination, however, demonstrated a low level of cross-reactivity (1/1000 of homologous reaction) between these two surface antigen particles. The assay of heterologous antigens by RIA (181, 300), the detection of GSHsAg by immunoelectrophoresis using sheep anti-HBs (180), the fact that the commercially available Ausria II assay for HBsAg also reacts with WHsAg and GSHsAg positive sera (181, 300, 301), and the finding that anti-WHs

and anti-GSHs containing sera could be detected by reagents ordinarily used to assay anti-HBs (300), suggest a substantial serological cross-reactivity among these antigens. Further, the recent establishment of a panel of anti-WHs monoclonal antibodies and the testing of these clones with each of the mammalian surface antigen particles revealed that some of the monoclonal antibodies (3 clones) bound specifically to WHsAg, all of the others cross-reacted with GSHsAg (8 clones), and that one antibody clone in the latter group also cross-reacted with HBsAg (7 of 8 major subtypes), defining a group specific antigenic determinant (301). The monoclonal antibodies described here, in addition to one specific for GSHsAg (180, 181), and several having specificities restricted to HBsAg (302-306), further reflect the antigenic complexity of these particles and provide molecular probes for the dissection of this complexity so that the relationship between neutralizing determinants, serological cross-reactivity and amino acid sequence homology among the major surface antigen polypeptides of each virus could be ascertained. They also suggest the possibility of using a heterologous antigen as a source of human vaccine.

Antigenic and immunogenic properties of HBsAg associated components.

If it is assumed that all of the HBsAg associated polypeptides observed in most studies (see Table 4) are unique molecular entities encoded for by the virus genome, then the amount of genetic information required to encode these polypeptides would far exceed the coding capacity of the HBV genome. To be certain, a number of host derived serum components have been variably detected in HBsAg particles, depending upon the extent of particle purification and the sensitivity of assays used to detect these constituents. Differences in the number and size of many bands observed following SDS-PAGE in addition, may also be due to differences in HBsAg polypeptide solubilization or storage prior to gel electrophoresis. Differences in solubilization, in turn, have resulted in varying degrees of reaggregation, with resultant differences in gel profile (206, 298). Reaggregation is also dependent upon the amount of HBsAg polypeptides subjected to solubilization and tends to increase with increasing amount of polypeptides under the same conditions (792). Further, direct radioiodination subjects polypeptides to both oxidizing and reducing conditions,

which may alter their behavior on polyacrylamide gels. Other factors, including differences in post-translational processing or modification (glycosylation, acetylation, etc.), or the variable effect of proteolytic degradation from blood born leukocyte proteinases (307), may affect the results obtained in analyses of HBsAg polypeptides.

The immunogenicity and antigenicity of HBsAg associated polypeptides were studied using individual components isolated from polyacrylamide gels as immunogens in guinea pigs and mice as well as antigens in serological assays (Table 4). In early work, p22-25, p35 and p40 of subtypes adw and ayw, as well as p19 of adw elicited group specific (anti-a) antibodies in guinea pigs which reacted with intact adw and ayw particles (203, 207). Similar results were independently published (193, 209). Some of the polypeptides used as immunogens also elicited antibodies reacting preferentially with the homologous subtype antigen, indicating the presence of both common and subtype determinants on single HBsAg derived polypeptides (203, 207). These results were later extended, suggesting that virtually all of the HBsAg associated polypeptides share similar immunochemical and antigenic characteristics (202, 208, 209). In another investigation, isolated p22-25, p26-29 and p64-72 reacted with antibodies directed against the individual polypeptides as well as HBsAg (297). General characteristics of antibodies raised against individual polypeptides include (a) induction of lower titers of anti-HBs with each polypeptide than with intact surface antigen, (b) reaction of anti-polypeptide antibodies with the homologous polypeptide more than heterologous polypeptides, (c) the presence of both group and subtype reactivity in antibodies raised against most of the HBsAg polypeptides, and (d) the indication from titration curves using anti-polypeptide antisera that p64-72 contains one or more other components in addition to the one reacting with anti-HBs.

Observations that reduced but unalkylated polypeptides were resolved by gel electrophoresis and that both reduction and alkylation were required to destroy both antigenicity and immunogenicity with respect to group specific "a" determinant(s) (239, 240) suggest that the polypeptides used as immunogens partially refolded and perhaps reaggregated to yield complexes behaving as suitable immunogens. Further, the anti-HBs titers elicited by the polypeptides were often

2-3 orders of magnitude lower in titer than using equivalent amounts of HBsAg, again suggesting partial renaturation, but also indicating that the presence of repeating antigenic determinants may be important in elucidating a high titer of anti-HBs (208, 263). Although some studies describe a failure of p26-29, the major glycosylated component, to elicit anti-HBs (203, 207), other studies claim this polypeptide to be the most immunogenic (202, 208, 209, 296, 297). Although the reasons for these differences are not clear, the anti-HBs titers achieved in the studies where the glycosylated component was immunogenic did not differ much from the nonglycosylated counterpart, suggesting that the presence of carbohydrate did not contribute to immunogenicity.

The preferential reaction of anti-polypeptide derived antisera with the homologous polypeptide may reflect variable reaggregation or renaturation discussed above, or it may be a reflection of antigenic determinants present on some polypeptides but not on others. Tryptic peptide mapping of the surface antigen components larger than the major pair of polypeptides in HBsAg, GSHsAg and WHsAg particles indicates a high degree of homology with their respective major components, but also reveals additional unique polypeptide spots (768). These unique spots indicate that at least some of the minor components are not aggregates of HBsAg polypeptides, nor are they host derived, but may be larger, distinct (precursor) surface antigen polypeptides. The genetic organization of HBV, GSHV, DHBV and WHV DNA and the recent size of HBV specific transcripts seen in cells transfected with recombinant HBV DNA as well as those recently isolated from the PLC/PRF/5 hepatoma cell line suggest that partially unique polypeptides larger than the major component demonstrated in each surface antigen could be present.

The presence of antibodies to common determinants elicited by individual HBsAg polypeptides do not distinguish between "a" and "Re" specificities; and this distinction is an important one to make, since the nature of the immune response against the envelope antigens of Dane particles or on the surface of infected cells may considerably alter the outcome of infection. For example, cell mediated immunity in guinea pigs injected with individual HBsAg polypeptides and measured by macrophage migration inhibition (296), or lymphocyte transformation in response to individual polypeptides or intact surface antigen (208),

may be directed to one or more conformation insensitive determinants, as suggested by an early study in which reduced and alkylated HBsAg induced a delayed type hypersensitivity response in the absence of an anti-HBs response (239), and because antigenic recognition in a cellular immune response is less dependent upon protein conformation than is a humoral immune response (308, 309). The finding of anti-Re in some patients' sera (241, 243) and the induction of a cell mediated immune response in guinea pigs inoculated with Triton X-100 solubilized HBsAg (310) suggest the possible importance of conformation insensitive common determinants in the induction of a cellular response by intact antigen as well as the possible blocking of such induction by antibodies of the anti-Re specificities.

Host components in HBsAg particles.

One of the major components often seen in the gel profile of HBsAg polypeptides is p64-72 (Table 4). It possesses an amino acid composition very similar to each of the major polypeptides of HBsAg (291, 298, Table 5), and recent digestion of p64-72 with papain yielded p22-25, p26-29 and a small amount of p44 by gel electrophoresis (298). While p44 may have been a dimer of p22-25, the actual constitution of p64-72 may be one molecule of p26-29 and two molecules of p22-25 in a non-covalent association. However, when p64-72 was used to inject guinea pigs, and the resultant antisera used to immunoprecipitate purified, radiolabeled p22-25 or particulate HBsAg, the antibody titration curve for p64-72 was not parallel to the curves generated from antisera against the other individual polypeptides in the preparation, suggesting a different antibody avidity in the antisera raised to p64-72 (208). Since the presence of a host component at this molecular weight value would lead to such immunochemical results, and since human serum albumin possesses both a similar size and has been found tightly associated with purified HBsAg particles (34-36, 210, 243, 266), it is likely that the molecular composition of p64-72 includes host as well as virus specified components present in variable amounts. The actual presence of human serum albumin as an integral component of HBsAg particles has been demonstrated by its detection in 20-40% of highly purified small spherical HBsAg (36), by its appearance in 1% Tween-80 treated HBsAg serologically negative for albumin prior to detergent treatment (266),

and by its release from Triton X-100 treated HBsAg and subsequent characterization by size and tryptic peptide mapping (210). Further, antibodies raised against human serum albumin precipitate both intact surface antigen and p64-72 isolated from polyacrylamide gels (201), and antibodies raised to reduced and alkylated HBsAg also bind to denatured serum components, including albumin (243). However, the role of serum albumin in surface antigen particles, if any, remains to be elucidated.

The first generation of hepatitis B vaccines consist of purified, inactivated, 22 nm spherical HBsAg particles purified from high titered carrier sera (274-279, 319-321, Figure 1a). Although these have been highly effective in inducing protective immunity in small populations, the detection of HBsAg associated nucleic acid (69, 322-324) in a small proportion of infected patients suggests that if the nucleic acid enclosed is viral, then small amounts of it present in these vaccine preparations may be transferred during vaccination and successfully infect a certain number of individuals so exposed. Further, the presence of many host serum components with highly purified HBsAg particles, including albumin, rheumatoid factor, lactoferrin, beta lipoprotein, $alpha_2$ macroglobulin, IgG, IgM, beta 1a/1c globulin (of complement), and transferrin (3, 32-40, 201, 266, 322-324a-c), many of which are polymorphic, as well as the association of undesirable autoaggressive or autoimmune reactions to some of these constituents further point to possible dangers to individuals exposed to several immunizing doses of the vaccine. Vaccine trials, however, have failed to demonstrate any problems with the presence of these host constituents. In addition to serum components, up to 30% by weight of small spherical HBsAg is composed of polar phospholipid and glycolipid components derived from the hepatocyte plasma membrane (30, 31, 221, 769), which probably accounts for the low buoyant density of these particles in density equilibrium gradients. Detailed characterization of these components was undertaken with the view that such information could be essential to the construction of subunit or synthetic vaccines, since the apparent role of lipids in modifying antigenicity or potentiating the immunogenicity of HBsAg virus encoded polypeptides had already been suggested (770). Several studies have identified phosphatidyl choline (30, 31, 221), sphingomyelin (30, 31), and cholesterol (221) as major

components and phosphatidyl ethanolamine (30), lysophosphatidyl choline (31), and other variably present lipids as minor components. Two nonsialic acid containing glycolipids have been found associated with HBsAg particles, the major one having been identified as dihydrospingosine, and seem to contain at least one determinant specific to surface antigen particles (325). In a more recent study, HBsAg purified from the plasma of two different carriers at different times over a period of several weeks demonstrated a lipid:protein average weight ratio of 0.34 and a phospholipid:cholesterol average molar ratio of 2.1 (769). HBsAg isolated from pooled sera at a single time point yielded similar results. The fatty acid content of total lipids extracted from individual HBsAg isolates were also similar among the isolates and included major components of palmitic, stearic, oleic and linoleic acids, which together accounted for more than 85% of the total fatty acids. Analysis of the major lipid classes in HBsAg demonstrated the presence of phospholipids (67%), free (15%) and esterified (14%) cholesterol, and triglycerides (3%). Phosphatidylcholine made up approximately 90% (by weight) of the fatty acids in the phospholipids; the remainder being divided among phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and other minor components (769). In contrast to other studies, glycolipids were not detected. These results showed that the lipid composition in different HBsAg preparations from pooled carrier sera or from individual carriers at different times was very similar, indicating that HBsAg particles possess a definitive lipid composition. Certainly, the variable presence of serum components with intact HBsAg, the retention of antigenicity following delipidation (204), and the association of antigenic and immunogenic activity with individual HBsAg polypeptides suggest that the important neutralizing determinants of HBV are probably not associated with host determinants. However, the appearance of rare subtype determinants (q, g, n, x, t) and the overall antigenicity or immunogenicity of HBsAg particles in a primary infection may depend to a substantial extent upon the number and amount of certain host derived components associated with HBsAg and whether they are seen as foreign or "self" determinants by antigen specific or nonspecific components of an intact immunological network. The physical and chemical heterogeneity of HBsAg particles, which is undoubtedly greater in

identical experiments, using polyvinylpyrrolidone as control antigen, no differences in the rate of clearance were seen. Since the animals were not primed with either antigen prior to experimentation, it is possible that antibodies cross-reacting with human host components present in HBsAg and/or by macrophages responding to "nonself" determinants on these particles may account for the clearance of HBsAg (and possibly Dane particles) prior to induction of antigen-specific immunity. In any event, a male antigen associated with HBsAg may account for these differences. A recently described serum glycoprotein, referred to as hepatitis B binding substance, has been identified in many animals but not definitively in man, suggesting another route by which HBsAg could be cleared in the absence of a specific immune response (349). The response to HBV infection, therefore, may consist of two major stages insofar as HBsAg is concerned. In the first stage, the rate of HBsAg and Dane particle clearance, which may alter the incubation period prior to clinical disease and the state of the specific immune response when viral replication and clinical disease finally appear, depends upon the antigenicity or "foreignness" of HBsAg with regard to nonantigen-specific immune and nonimmune clearance mechanisms. The effects of specific immunity against HBsAg, in the second stage of response, would depend upon the magnitude, nature (mostly humoral or cellular), and timing of the response with regard to virus replication. For example, it has been suggested that an overwhelming immune response to HBV (very high titers of anti-HBs (346)) in fulminant hepatitis or an improperly regulated immune response (in agammaglobulinemic patients (347)) often leads to fatal hepatitis. In contrast, intact humoral and cellular immunity against HBsAg in acute hepatitis usually results in resolution of clinical disease, while chronic active hepatitis is characterized by the presence of insufficient immunity to completely eliminate the virus and chronic passive hepatitis or asymptomatic carrier state is often associated with no immunological response against HBsAg and/or other HBV gene products (348, Figure 2).

Possible vaccines lacking host components.

In an attempt to produce a vaccine lacking host cell and serum components but having HBsAg polypeptides present in a highly

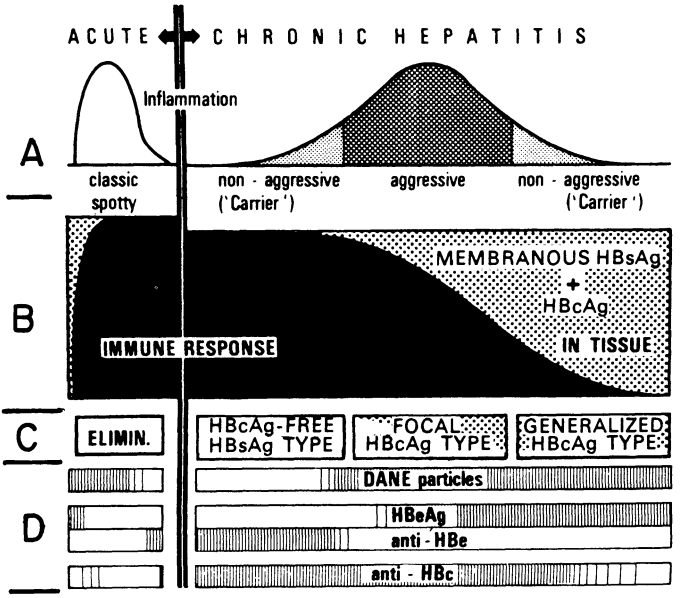


Figure 2. Diagrammatic representation of the four basic reaction types to HBV infection. (A) Type and severity of inflammatory reaction; (B) Extent of nuclear HBcAg and membrane associated HBsAg in relation to efficiency of the immune response; (C) The four proposed reaction types include (1) elimination of infected cells followed by recovery in acute hepatitis, (2) the presence of predominantly liver cell associated HBsAg, (3) focal areas of core antigen expression, or (4) widespread HBcAg expression. Each expression pattern predominates in different forms of chronic hepatitis; (D) HB associated antigens and antibodies in blood. [Reprinted by permission from Bianchi, F. and F. Gudat in Progress in Liver Disease, Vol. 4, Chapter 20, pp. 371-392. Copyright 1979 by Grune & Stratton, Inc. (ref. 348)].

immunogenic form, several polypeptide vaccines consisting of p22-25 and p26-29 isolated from preparative SDS gels (351, 352, 355) or by Triton X-100 treatment of HBsAg particles (350) were used as starting material. Guinea pigs immunized with aqueous polypeptide vaccine yielded antibody titers at least 100 fold less than intact HBsAg under the same conditions and did not show a cell mediated response by inhibition of macrophage migration (352). However, the polypeptide preparation incorporated into liposomes (356, 357) or mixed with alum resulted in both humoral and cellular immunity against HBsAg (352), although the

liposome vaccine was more potent (366). Challenge of susceptible chimpanzees with HBV after immunization with two to three doses (40 μg/dose) of polypeptide vaccine protected them against infection (351), suggesting the feasibility of these adjuvants, which are otherwise safe for human use, for further development of this second generation vaccine. Solubilization of HBsAg particles in Triton X-100, separation of the p22-25 plus p26-29 complex by centrifugation, and reconstitution of this protein complex into micelles, resulted in pleomorphic forms that competed with 22 nm intact HBsAg in a radioimmunoprecipitation assay (350, 367). The competitive ability of these micelles against HBsAg particles was 40%, which may reflect differences in the number, density and conformation of these determinants in relation to HBsAg particles, while injection of alum precipitated micelles into mice resulted in anti-HBs titers higher than those obtained with similarly prepared 22 nm particles. Although the reasons for this enhanced immunogenicity are not clear, the absence of competing immunogenic determinants in host components, which may partially block hepatitis determinants directly and/or result in blocking due to the binding of non-neutralizing antibodies to one or more of these polymorphic host determinants, might favor persistence of the micelle over the 22 nm particles and allow more assured induction of antigen-specific immunity. Further testing with this micelle vaccine in guinea pigs demonstrated a cell mediated response, measured by transformation assay, equivalent to that against 22 nm particles, as well as protection in chimpanzees challenged by HBV (367).

The partial sequencing of p22-25 and p26-29 (264, 292) and identification of the termini of these polypeptides in the nucleic acid sequence of HBV (156-158, 166) have permitted deduction of the total amino acid sequence of the major polypeptide pair encoded for by the S (surface antigen) gene and identification of hydrophilic sequences which may play a role in inducing neutralizing antibodies (Figure 3). The S gene, which encodes a polypeptide 226 amino acids long and 25,400 daltons in size, contains three regions of beta sheet structure included in residues 1-31, 75-109 and 157-226, as well as two regions of relatively hydrophilic beta turns (residues 45-80 and 110-150) separated by a hydrophobic stretch of amino acids (residues 80-110) in the center of

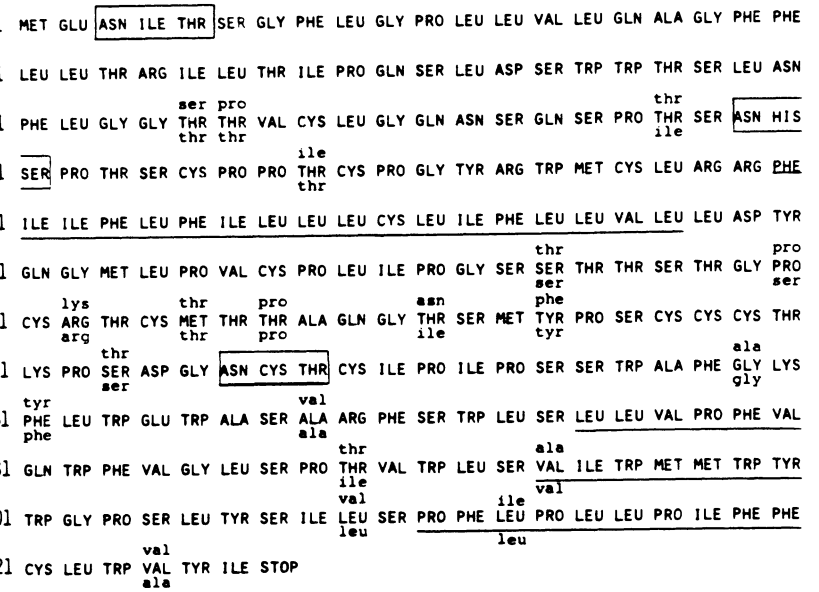


Figure 3. Amino acid sequence of the major nonglycosylated HBsAg polypeptide derived from nucleic acid sequences. [Reprinted with permission from Tiollais, P., et al. in Science 213:406-411. Copyright 1981 by the American Association for the Advancement of Science (ref. 358)].

the polypeptide (263, 293, 294, 358). The contrast between these results, which predict little or no alpha helix content, and those of ORD and CD spectra, which indicate a very high precentage of alpha helical content (242), suggests that although HBsAg polypeptide is composed of beta structure, the folding of these structures with respect to each other in the context of a lipid bilayer may result in globular proteins with various domains (the two beta turns) yielding the spectral data discussed above. Further, protein-protein and protein-lipid associations in intact particles may result in some alpha helical content which is segregated from beta secondary structure along the polypeptide chains. Indeed, this type of structure is characteristic of alpha and beta type proteins, most of which, like HBsAg, are secreted (294). That two hydrophilic domains are separated by an extremely hydrophobic stretch and that both the N- and C-terminal beta sheets are hydrophobic

as well further suggests a very tight packing for HBsAg polypeptides, since it has been shown that chain hydrophobicity and packing density are highly correlated (359). Further, if the sequence 80-110 containing hydrophobic residues represents a local maxima in hydrophobicity, it will tend to bury itself in the core of the folded protein and thereby permit proper juxtaposition of the hydrophilic domain to either side. The presence of a considerable number of cysteine residues within these hydrophilic regions, combined with the previous observation that virtually all cysteine residues are in disulfide bonds, suggest that they may be disulfide bonded together. If this is so, reduction of the disulfide bonds alone would not necessarily reduce antigenicity if the hydrophobic core of the polypeptide maintained close approximation of sulfhydral groups. Under these conditions, one or more antigenic determinants spanning disulfide bond(s) would lose their integrity only after further modification by alkylation or exposure to denaturants such as urea.

Recent experimental evidence discerning the environment around the major surface antigen polypeptides supports many of the theoretical considerations of HBsAg particle structure based upon nucleic acid and deduced amino acid sequences. Using the photoactivatable, hydrophobic probe, pyrenesulfonyl azide (PySA) in its unlabeled or tritiated form, both the major HBsAg associated polypeptides and the lipids associated with HBsAg particles were detected following SDS-PAGE by fluorescence or radioactivity (769). The stoichiometry of labeling the major pair of HBsAg associated polypeptides was the same as that observed for Coomassie blue staining of the same gel, in which the p22-25:p26-29 was 2:1 (769). These results suggest that both polypeptides are associated with the lipid bilayer and that the association of each polypeptide with lipid was similar since their ratio remained unchanged following PySA labeling. It is no surprise that the combination of compact structure and association with lipid renders HBsAg particles totally resistant to trypsinization (771). Mild reduction and alkylation under conditions which did not alter particle sedimentation velocity, buoyant density, electron microscopic image, or CD spectrum, however, resulted in increased susceptibility to trypsinization and the appearance of both soluble and insoluble fragments on SDS-gels. Partial sequencing

Partial sequencing of soluble fragments isolated by high pressure liquid chromatography (HPLC) demonstrated that the tryptic site at residue 122 in either of the major polypeptide pairs was exposed to the environment in the intact particle (771). The tryptic digestion of ^{3}H-PySA labeled HBsAg under these conditions yielded two fragments corresponding to the amino terminal 122 residues and the carboxy terminal 104 residues, which both contained radiolabel, suggesting that regions within both halves of the major polypeptides were buried in the lipid matrix, while the hydrophilic region extending from residues 122 through 150 is exposed on the HBsAg particle (769).

The generation of hydrophobicity curves (359) for surface antigen polypeptide (182-187), combined with the antigenic alterations observed in intact antigen following modification or cleavage at specific amino acid residues, resulted in the selection, synthesis and testing of candidate surface antigen peptides for antigenicity and immunogenicity. In one study a synthetic peptide containing residues 138-149 bound up to 9% of antibodies directed against ad and ay subtypes (184, 362). Since the synthetic peptide remained bead bound, the low percentage of radiolabeled anti-HBs (from Ausria II) binding to the resin may have reflected limited exposure of the peptide on the surface of the bead (360). However, this low amount of binding could be due to the presence of a subset of high affinity antibodies in a heterologous serum binding to a minor determinant or to antibodies binding with low affinity to a subset of the epitope(s) comprising a major determinant (361). Alternatively, the use of guinea pig anti-HBs may reflect major and minor determinants different from those seen in patients. Further antigenicity studies with a smaller "H" peptide (residues 141-146) demonstrated 50-75% absorption of anti-a and anti-d but little absorption to anti-y specific antibodies (187, 363). The injection of mice with H peptide coupled to aldehyde-treated human erythrocytes with glutaraldehyde polymerized peptide, followed by boosting, resulted in up to 50% of the mice producing anti-HBs. Although these experiments demonstrate antigenicity and immunogenicity, the limited ability of the H peptide to absorb antibodies and effectively immunize mice suggests that the conformation of this peptide was different from the same sequences in HBsAg particles. Independent synthesis of peptide 134-146

also resulted in binding to anti-HBs in both hemagglutination and AUSAB (commercial anti-HBs detecting) assays (364). However, binding was observed at levels approximately 10^3 fold less than for p22-25 or p26-29 and 10^6 fold less than intact HBsAg under the same assay conditions. Nonetheless, the presence of eight substitutions in the major hydrophilic domain of HBsAg (residues 110-156), deduced from the nucleic acid sequence of HBV DNA clones published thus far, suggest a basis for "a" determinant heterogeneity and/or the presence of different subtype specific epitopes detected by appropriate specific antisera (187, 363, 364). The alterations in antigenicity of intact HBsAg following various chemical modifications, described in detail above, resulted in prediction of a major antigenic determinant within residues 135-155 in the major hydrophilic region of HBsAg polypeptide (260, 365). In another investigation, 13 peptides from the sequence of the major HBsAg polypeptide were synthesized, coupled to KLH carrier, and injected into rabbits in order to assess the anti-peptide response and to test the binding of these antisera to intact HBsAg (182, 183). Peptides containing residues 221-226, 212-226 and 31-35 failed to elicit anti-peptide responses judged by immunoprecipitation of homologous radiolabeled peptides. A peptide containing residues 140-148, within the area of the major hydrophilic region, yielded only a marginal anti-peptide response which did not immunoprecipitate intact radiolabeled HBsAg. Peptides containing residues 48-81, 2-16, 22-35 and 95-109 elicited relatively high titers of anti-peptide immunoglobulin which also bound to intact surface antigen. These results suggest that antibodies raised against residues 2-16, 22-35 and 95-109 recognize and bind to exposed regions of beta sheet structure in the amino terminal portion of the molecule, as well as to the minor hydrophilic domain of HBsAg polypeptide (residues 48-81), but not to regions in the carboxy terminal portion of the molecule. These results may be indicative of an exposed amino terminus and buried carboxy terminus in intact particles. The finding of anti-peptide immunoglobulin following immunization with residues 38-52, 47-52 and 104-109, which did not bind to intact surface antigen, suggests that these regions are not exposed on the surface of HBsAg; but lack of appropriate peptide conformation might also have contributed to these results. It is interesting that

the peptide having residues 48-81 was successfully used as an immunogen without coupling to carrier protein, suggesting that it may have refolded into a domain-like structure, aggregated and elicited a substantial humoral response. This peptide, being 34 amino acids long, probably elicited at least some antibodies against conformational determinant(s), while the other synthetic peptides, being 15 amino acid residues or smaller in size, probably elicited antibodies directed against only sequential determinants, which may or may not be present as such on exposed areas of HBsAg particles. The synthesis and disulfide bond cyclization of peptides having residues 122-137 and 117-137 have recently been accomplished to examine the effects of "locking in" secondary (beta turn) conformation upon immunogenicity in mice (186). Presentation of either cyclic peptide in complete Freund's adjuvant, liposomes, or in alum absorbed preparations resulted in detectable anti-HBs titers in 50% of the animals after 7 days, independent of the adjuvant used, and with antibody levels comparable to those seen using p22-25 as immunogen. Clearly, in this case, the polypeptides were immunogenic in the absence of carrier protein which, in any event, might sterically hinder the "correct" refolding of any synthetic peptide into a conformation dependent determinant.

A deeper appreciation of the complexity and conformational requirements of the common "a" determinant has been illustrated by the synthesis and immunological characterization of various other synthetic peptides. When a synthetic peptide spanning HBsAg residues 135-155 (p135-155) was coupled to a variety of common carriers and injected into rabbits, anti-HBs was detected by the commercial assay for anti-HBs, AUSAB (777). However, polyclonal rabbit or human anti-HBs as well as most mouse monoclonal anti-HBs did not react with the synthetic peptide, nor did HBsAg inhibit the p135-155 anti-p135-155 reaction. Further, mice immunized with the synthetic peptide in conjugated form failed to show enhancement of their anti-HBs response. In contrast, the majority of both anti-p135-155 and anti-HBs were adsorbed and eluted from an affinity column containing p135-155 coupled to aminohexyl-Sepharose, suggesting that only a subpopulation of anti-p135-155 reacts with HBsAg and that the degree of cross-reactivity was dependent upon the conformation/steric constraints of the synthetic peptide in each conjugate and assay.

Independent synthesis of seven overlapping peptides spanning the same hydrophilic region, from HBsAg residues 122-158, yielded three peptides defined by residues 139-147, 139-158 and 140-158 which partially neutralized human anti-HBs reactivity at concentrations greater than 1 mg/ml (778). These three peptides, coupled to a protein carrier by their amino or carboxy terminus, each yielded an anti-HBs response in rabbits which was low and similar to those obtained when isolated structural polypeptides of HBsAg were used as immunogens. Since different subtypes of HBsAg demonstrated identical behavior, it is likely that the binding involves the common "a" determinant, although whether the low binding was due to strictly conformational differences in comparison to native HBsAg or to the presence of only part of a common determinant which is larger, discontinuous, or both, remained unresolved questions. Reaction of a linear or intrachain disulfide bonded HBsAg synthetic peptide spanning residues 122-137 with a panel of 14 anti-a monoclonal antibodies showed that six of the antibodies bound the cyclic peptide, and that after linearization by reduction and alkylation, only one of the six still demonstrated binding (779). Although on a weight basis, several thousand fold more cyclic peptide than HBsAg was needed to block anti-a activity to the same extent, the differences in antibody binding between cyclic and linear species of peptide are consistent with there being a conformation dependent antigenic determinant possessing group "a" specificity in the cyclic component. Interestingly, three monoclonal antibodies preferentially reacting with the "y" subtype determinant bind equally well to both cyclic and linearized peptide, suggesting that the "y" epitope is sequential. The fact that a majority of the anti-a monoclonal antibodies do not bind either form of the peptide implies that group "a" reactivity may include other epitopes along the HBsAg polypeptide. Significantly, a number of the monoclonal antibodies binding to the cyclic peptide share a common idiotype with anti-HBs antibodies generated in humans naturally infected with HBV, suggesting that the epitope associated with the cyclic peptide is recognized in a natural infection (779-781). That intact HBsAg, micelle reconstituted p22-25 plus p26-29 aggregates, and cyclic p122-137 bind to anti-HBs of a given idioptype, in contrast to reduced and alkylated forms of these species

which do not, indicates that the binding is conformation dependent (781). Partial inhibition of an idiotype-anti-idiotype reaction by these HBsAg forms suggests that the common anti-HBs idiotype is associated, in part, with the antibody combining site, and that regulation of the anti-idiotypic response during infection may significantly alter the progression of disease. The finding of a cross-reacting anti-HBs idiotype in a number of mammalian hosts immunized with HBsAg (e.g., mice), or as a result of natural infection (e.g., chimpanzees), provide opportunities for experimental manipulation of the anti-HBs response (782). Indeed, the injection of anti-idiotypic antibody into mice prior to HBsAg exposure enhances the IgM anti-HBs response, while two injections of anti-idiotype in the absence of antigen exposure yields an anti-HBs IgG response approximately 12 fold over that of similar treatment with pre-immune IgG (783).

The sequence discontinuity of the common "a" determinant(s) was further indicated by the observation that antibodies raised to peptides spanning HBsAg ay p22-25 residues 2-16, 95-109 and 110-139 bound intact HBsAg particles of various subtypes (784). While residues 2-16 compose the amino terminal fragment of p22-25 HBsAg and residues 95-109 include the junction betweeen the central hydrophobic region and the major hydrophilic sequence, residues 110-139 span a portion of the major hydrophilic region of HBsAg proximal to a glycosylation site (residues 146-148) and contain residues which differ among surface antigen subtypes as deduced from nucleic acid sequences. Indeed, reference anti-y but not anti-d sera precipitated radiolabeled residues 110-139, indicating the presence of the "y" determinant on the peptide (784). Further, substitution of amino acids in this peptide deduced from ad derived nucleotide sequences resulted in specific precipitation with anti-d, localizing these subtype determinants to this region of the major polypeptide. In contrast to the cyclic peptides described above, the "a" determinant reactivity in these linear peptides may depend upon local conformation, suggesting that cyclization may not be necessary to elicit the appropriate "a" specificities (784). Injection of peptide 110-139 (coupled to a protein carrier in the presence of adjuvant) into three HBV seronegative chimpanzees, followed by challenge, yielded protection in one chimp, attenuated infection without disease in

another, and typical acute hepatitis in the third animal (784, 785), suggesting the potential for synthetic vaccines.

HBsAg associated carbohydrate.

The binding of ^{125}I labeled small spherical HBsAg particles to Concanavalin A (Con A (237, 372)), the positive reaction of HBsAg in the anthrone reaction (373) as well as by the phenol-sulfuric acid method (195, 252), and the detection of some HBsAg constituents in polyacrylamide gels with the periodate Schiff reagent (PAS (371)) suggest the presence of carbohydrate in HBsAg (31, 195, 196, 201, 203, 208, 210, 292, 325). In order to discern the biological role of the carbohydrate in HBsAg as well as better define the antigenic and structural relationships among the glycosylated and nonglycosylated polypeptides, detailed chemical characterization of the associated carbohydrate was undertaken. The binding of HBsAg particles to Con A, followed by their specific elution with N-acetylglucosamine, mannose or glucose, which bind specifically to Con A (374), suggested that each of these sugars was present in HBsAg (372). While the total carbohydrate content of HBsAg was determined to be 3.6-6.5% in one study (195) and 7.6% in another (375), further analysis by gas chromatography showed N-acetylglucosamine as the major component, mannose, galactose and sialic acid as less prevalent sugars, and fucose as a minor constituent (Tables 1, 2 and 4; 237, 375). Glucose was detected only in trace amounts. Similar results were obtained with delipidated HBsAg. Although serological activity was originally attributed to carbohydrate (252) by virtue of periodate sensitivity, substantial evidence presented above indicates no role for carbohydrate in the common "a" determinant(s) of HBsAg; and preliminary evidence suggests that carbohydrate might account for one or more of the minor determinants (325). Treatment of the HBsAg producing human hepatoma cell line PLC/PRF/5 with tunicamycin (which inhibits glycosylation at asparaginyl residues) yielded antigen containing both "a" and "d" activity, further suggesting that carbohydrate is not essential to antigenicity (260). The presence of sialic acid in the carbohydrate analysis of HBsAg suggested that if some of the associated polypeptides were typical sialoglycoproteins, then sialic acid should be the terminal sugar and galactose the penultimate residue. Indeed, HBsAg was adsorbed to insolubilized sialic acid-specific

hemagglutinin derived from the haemolymph of Limulus polyphemus, and sialic acid was released following treatment with neuraminidase (260, 376). Only neuraminidase treated HBsAg incorporated radioactively labeled sialic acid in the presence of sialyl transferase and cytosine monophosphate-^{14}C-sialic acid. Neuraminidase treated HBsAg exposed to galactose oxidase, followed by reduction with tritiated sodium borohydride, resulted in the labeling of the penultimate galactose residue (295). That 82% of the incorporated counts precipitated with TCA and only 1.5% extracted in chloroform-methanol mixtures suggest that the vast majority of the carbohydrate is protein bound and the carbohydrate composition of glycoproteins in HBsAg differed substantially from that of glycolipids. These results also suggest that these carbohydrate moieties are exposed (and thereby sensitive to enzymatic activity) on intact HBsAg. Although these enzymatic treatments did not alter the serological activity of HBsAg particles (295), neuraminidase treatment drastically reduced the half life of circulating HBsAg injected into rabbits some 10-20 fold (376). Neuraminidase treated HBsAg used as an immunogen in rabbits also induced a higher humoral response than untreated antigen and yielded lymphocytes capable of undergoing transformation in the presence of untreated antigen to a greater extent than when untreated antigen was used as immunogen (376). The rapid antigen nonspecific clearance of asialoglycoproteins is a well documented phenomenon in glycoprotein metabolism (377, 378) and may reflect the extent to which 22 nm HBsAg and infectious Dane particles are removed from circulation. The presence of heterogeneously glycosylated surface antigen polypeptides in 22 nm forms or Dane particles could alter the incubation period between infection and the onset of acute hepatitis, the titer of Dane particles (and DNAp levels) in active disease, as well as both the titer and persistence of HBsAg in carriers. If the stimulated humoral and cellular immune responses against HBsAg elicited by asialoglycoproteins occur in infected patients, the immunopathogenesis of HBV infection could be substantially altered. Since an intact cell mediated immune response is important for recovery from HBV infection (348, 382), the presence or absence of terminal sialic acid residues may play a central role in this respect. Alternatively, if the asialic carbohydrate chains are themselves an immunogenic moiety which directs

production of non-neutralizing antibodies, these antibodies may effectively block the binding of neutralizing antibodies to Dane particles and thereby permit persistent infection even in the presence of high neutralizing titers (379). Autoantibodies directed against polymerized serum albumin (380, 381) or other altered host components associated with HBsAg polypeptides in Dane particles or on the hepatocyte cell surface may have similar ramifications upon immunopathology in both acute and chronic hepatitis (348). The integrity of carbohydrate incorporated into any second generation polypeptide vaccine may also be an important parameter to consider in the consistent generation of optimal protective immunity.

Further characterization of HBsAg polypeptide associated carbohydrate by SDS-PAGE and PAS staining revealed a variable number and size of glycoproteins (Table 1; 376). Most studies show that p26-29 is the major glycosylated component of surface antigen, although glycosylated components at molecular weight values of 22,000 (195, 196, 203), 30-32,000 (195, 196, 201, 208, 210), 35,000 (203), 52,000 (201, 208) and 64-68,000 daltons (210, 292) have been reported (Table 4). Considering that the PAS reagent could undergo nonspecific reactions in SDS gels (292, 371), that the molecular weight estimates of HBsAg derived PAS bands do not vary when the extent of gel cross-linking is varied (211) while estimates for known glycoproteins do vary under these circumstances (382a, 383), and that amino acid analysis of the PAS stained components fail to reveal amino acid sugars (211) may contribute to the apparent discrepancy in results among various studies. Therefore, further work focused upon characterization of the carbohydrate attached to the major component, p26-29.

The digestion of isolated p22-25 and p26-29 with trypsin followed by analysis on polyacrylamide gels revealed partial cleavage into lower molecular weight fragments (292, 384). Further characterization of these fragments by amino acid analysis and limited Edman degradation suggested that tryptic cleavage took place at residue 122 in the isolated polypeptides and in intact HBsAg. All of the published nucleic acid sequences of HBV indicate either lysine or arginine present at this position. Carbohydrate staining of these gels revealed a low molecular weight PAS positive digestion product of p26-29 which was

localized to somewhere within the carboxy terminal 104 amino acids of the major glycosylated component. Cleavage of reduced and alkylated HBsAg with trypsin yielded a single PAS positive fragment spanning residues 122-150 in the major glycosylated component, suggesting the presence of glycosylation within this region (771). Introduction of tritium label into the sialic acid component of HBsAg, followed by SDS-PAGE analysis, revealed most of the protein associated label to be in p26-29. Tryptic cleavage of p26-29, followed by separation of soluble peptides by high performance liquid chromatography, yielded a peak of radioactivity corresponding to a peptide spanning residues 140-147 as determined by amino acid analysis (771). Further, the presence of approximately 2 nanomoles of glucosamine/nanomole of peptide 140-147 in samples of HBsAg adw and ayw, in addition to terminal sialic acid, suggests that the asn-x-thr(ser) sequence at position 146-148 is at least one of the sites of glycosylation in HBsAg. Independent studies, in which HBsAg p26-29 isolated from polyacrylamide gels was digested with nagarse and pronase P, yielded a single glycopeptide approximately 15 amino acids in length which sequence analysis showed to begin at HBsAg residue 141 (786). Although the isolated peptide contained two possible sites for asparagine linked glycosylation, peptide sequence analysis localized the carbohydrate attachment point at asn 146. Immunization of mice with the glycopeptide (in adjuvant with or without prior coupling to carrier) yielded antibodies which bound HBsAg p22-25 isolated from several subtypes, indicating that the antibodies bound a common determinant on all HBsAg isolates independent of subtype or presence of carbohydrate (786).

Further evidence suggests the presence of asparagine linked sugars in HBsAg p26-29. The presence of N-acetylglucosamine as a major component in HBsAg associated carbohydrate and the absence of N-acetylgalactosamine suggested that asparagine-N-acetylglucosamine but not serine- or threonine-O-acetylgalactosamine linkages were present in HBsAg (375, 786). HBsAg particles containing tritiated terminal sialic acid and treated with sodium hydroxide plus sodium borohydride did not release any radiolabeled saccharide components, indicating that the carbohydrate linkage involved in the radiolabeled moieties were not through the hydroxyl groups of serine or threonine (260, 385). In

addition, alkaline sulfite treatment of isolated p22-25 and p26-29 yielded equivalent serine and theonine contents for both polypeptides, suggesting that carbohydrate was not attached by O-glycosidic bonds to these amino acid residues (292, 384). Further, treatment of the human hepatoma cell line (PLC/PRF/5) with tunicamycin, a drug which specifically inhibits the dolichol pyrophosphate mediated glycosylation of asparaginyl residues in glycoproteins (386), resulted in 19 fold reduction in the incorporation of tritiated mannose (260), suggesting that a considerable amount of this sugar was in carbohydrate moieties bound to asparaginyl residues. Since the polypeptide sequences of p22-25 and p26-29 are available from the published nucleic acid sequences of HBV DNA clones (155-157) and all sequences contain asn-x-ser(thr) with asn at positions 3, 59 and 146, multiple sites for glycosylation at asparagine residues exist. The isolation of a single peptidoglycan approximately 3900 daltons in size following pronase digestion of ^{3}H-sialic acid labeled HBsAg (260), and the presence of most of the carbohydrate of p26-29 in the carboxy terminal half of the molecule (292) further suggest that the attachment site of this major carbohydrate moiety is at asn 146 (292, 384) within the major hydrophilic domain of HBsAg polypeptide. Although the structure of this carbohydrate is not known at present, the data from carbohydrate composition and analysis suggest a complex, branched structure characteristic of A-type oligosaccharides (295, 387). The large quantities of N-acetylglucosamine and mannose, the presence of two other glycosylation sites within the minor hydrophilic stretch of amino acids (residues 45-80) and near the N-terminus, the occurrence of simpler B type oligosaccharide structures containing these sugars as major components, and the failure to label any B type oligosaccharide with galactose oxidase and tritiated sodium borohydride still leaves open the question of whether other points of glycosylation occur.

The presence of asparaginine linked oligosaccharides in a major HBsAg polypeptide combined with amino acid analysis, sequencing and tryptic peptide mapping suggest that the only difference between p22-25 and p26-29 is glycosylation. Anhydrous hydrofluoric acid treatment of HBsAg followed by polyacrylamide gel electrophoresis resulted in a disappearance of p26-29 and an increase in p22-25 (292, 384). Treatment

of isolated p22-25 and p26-29 with periodate followed by gel electrophoresis, however, resulted in the disappearance of p26-29, while p22-25 remained completely resistant (298). Further, periodate treatment of a mixture of the two resulted in no definite increase in the amount of p22-25, suggesting non-identity among these major polypeptides. Preliminary evidence in another study also questions whether the difference in molecular weight between p22-25 and p26-29 is due solely to the presence of carbohydrate and suggests that p26-29 may contain repeated amino acid stretches not present in p22-25 (388). In addition, the presence of tryptic peptides in the maps of p26-29 but not in the maps of the corresponding p22-25 component could be explained entirely by heterogeneity in glycosylation, but the presence of additional primary sequence would also be consistent with these results (298, 388, 768). Tryptic peptide mapping of the major polypeptide pair in various HBsAg, GSHsAg and WHsAg isolates showed that the larger component of each pair differed from the respective smaller component in 5-30% of its spots, depending upon the isolate (768). In another study, HBsAg particles treated with neuraminidase were chromatographed on a column of immobilized peanut lectin specific for terminal D-galactose residues (204). Approximately 15% of the HBsAg particles did not bind to this column, even after rechromatography, suggesting that neuraminidase-treated particles are not homogeneous with respect to exposed D-galactosyl residues. Whether this result reflects incomplete cleavage of sialic acid despite extensive neuraminidase treatment or the presence of penultimate residues other than galactose is not clear. Only further characterization of the HBsAg bound oligosaccharides will reveal whether heterogeneity in glycosylation among different isolates really exists and whether this heterogeneity plays any role in the outcome of infection.

HBsAg and the receptor for polymerized albumin.

Sera from patients with chronic liver diseases often possess one or more factors which bind human polymerized serum albumin (PSA) but not its monomeric counterpart (510, 850-854). At least some of these factors were identified as antibodies which bound albumin modified by aging or by glutaraldehyde treatment, suggesting that they react with a determinant generated in the aging or polymerization process, and

remove such modified albumin from circulation (381, 510, 850, 855, 856, 860, 866, 870). Although these antibodies were present at low titers in some normal individuals, their titers were increased several fold in patients with hepatic injury, suggesting that they correlate with liver cell dysfunction (381, 857-859). Further characterization has shown anti-PSA to be of the IgG and IgM classes; the latter increasing in titer more than the former in the presence of liver pathology. Interestingly, the affinities of these antibodies were higher in normal sera than in serum derived from patients with liver disease (859), suggesting that there may not be complete clearance of polymerized albumin from diseased patients.

Examination of sera from HBV infected patients also revealed an activity that binds homologous PSA. In these experiments some HBsAg positive sera agglutinated erythrocytes coated with polymerized human serum albumin (381, 510), suggesting that autoantibodies in HBsAg particles directed against polymerized or denatured serum albumin molecules accounted for the binding. Affinity columns using anti-IgG or anti-IgM, however, did not bind HBsAg particles which bound PSA, suggesting the absence of these classes of antibody (317). Unfortunately, the same experiment was not conducted with an anti-IgA affinity column, since IgA containing mononuclear cells are often present in many types of liver disease (863), and since IgA may complex with albumin in an immunologically nonspecific manner (864, 865). Independent observations, however, are consistent with the absence of IgG and IgM and also with the apparent lack of IgA from receptor bearing HBsAg particles (873). Receptor activity was not affected by anti-human IgG, IgM or IgA reagents, indicating no role for these Ig classes in receptor activity (862).

The association of HBsAg and PSA has been independently demonstrated by the finding of HBsAg particles binding to human PSA adsorbed onto a solid phase (35). It has been postulated that HBV infection may interfere with albumin synthesis in hepatocytes, indirectly resulting in the generation of PSA (35, 380, 381). The ability of PSA to bind intact HBsAg and Dane particles (35, 316-318, 380, 868) implies that the generated complexes form an immunogenic array of repeating haptenic determinants in which HBsAg is the carrier and PSA the hapten (35).

Since receptors for polymerized albumin have been detected on both rabbit (507) and human (871) hepatocytes, the potential for such complexes to boost the anti-PSA titer may result in autoantibody production which might clear infectious Dane particles from the serum, thereby reducing infectivity (867), and/or may exacerbate hepatocellular damage by binding PSA present on hepatocyte plasma membranes. Alternatively, if liver damage affects the ability of hepatocytes to bind and remove PSA from the circulation, the accumulation of serum PSA might trigger increased production of PSA autoantibodies (507).

The binding activity of HBsAg for polymerized albumin differs substantially from that expected if adsorbed autoantibodies were responsible. For example, the temperature dependence of the HBsAg-PSA reaction differs from that of HBsAg-anti-HBs (35). The binding of HBsAg particles to human PSA coated erythrocytes in a hemagglutination inhibition assay was blocked by human antibodies against homologous PSA but not by xenogeneic antisera, suggesting that the human antibodies bound to one or more sites at or near the site in which human PSA binds HBsAg. In contrast, the finding that HBsAg did not block the reaction between a variety of xenogeneic antibodies having human PSA reactivity and human PSA coated erythrocytes suggests that these antibodies bound to determinant(s) other than those recognized by the HBsAg receptor for polymerized albumin (867). When various polymeric and monomeric albumins from different species were tested for binding HBsAg, the HBsAg associated receptors for PSA bound only human and chimpanzee PSA (317, 867, 870), suggesting that the HBsAg receptor binds polyalbumin from very few species. Both human and xenogeneic antibodies having human PSA reactivities reacted with glutaraldehyde polymerized albumin from many different species, suggesting that the binding characteristics of PSA antibodies lacked species specificity (867). This species specific binding of human and chimpanzee polymerized albumin to HBsAg (311, 313, 317, 870) correlates well with the restricted host range and organ specificity of HBV, if polymerized albumin bound to the surface of Dane particles or to the hepatocyte cell surface functions as a bridge by which Dane particles could specifically attach to the hepatocyte (315-317, 870). The strong correlation between the presence of HBsAg associated PSA binding sites and high infectivity of HBV carriers (872) independently supports this hypothesis.

The ability of Dane and 20 nm diameter HBsAg particles from e antigen positive sera to bind polymerized albumin to a much greater extent than similar particles from anti-e positive sera (317, 318), combined with the tendency of HBeAg to be associated with albumin (53, 849), indirectly suggest a possible relationship between e antigen reactive material and the receptor for polymerized albumin. Fractionation of HBsAg positive serum containing e antigen through a column of immobilized human PSA demonstrated binding of Dane particles and small spherical HBsAg but not of e antigen (317), suggesting that HBeAg itself does not bind PSA. Ultracentrifugation analyses have shown that HBsAg particles capable of binding polymerized albumin were heavier than similar particles which could not, and that the receptor for polymerized albumin could be destroyed by exposure to proteolytic enzymes under conditions where HBsAg reactivity was unaffected, suggesting that the receptor was protein in nature and that it was discrete from the major determinants of HBsAg (317, 873). Although the receptor for polymerized albumin was present most often in sera containing high HBsAg concentrations and tended to be absent from sera with low HBsAg concentrations, its appearance was most closely correlated with the e antigen/e antibody status and not the concentration of HBsAg per se (869, 873). However, the demonstration of Cl_q binding to polymerized albumin (313) and HBsAg (312), combined with the presence of antibodies against polymerized albumin in HBV infected patients (380, 381, 850), suggest that polymerized albumin-complement complexes on the hepatocyte cell surface or Dane particle may serve as targets for autoaggressive immune responses independent of a patient's e antigen or e antibody status.

The expression of an HBsAg associated receptor for polymerized albumin is an early event in the natural course of acute and chronic HBV infection (861, 862, 869). Serial sera from HBeAg positive patients with clinically acute hepatitis demonstrated a high receptor:HBsAg ratio (862). Upon recovery from acute disease and seroconversion to anti-HBe (within three weeks from onset), the ratio dropped several fold, indicating a shift in receptor expression upon resolution of acute hepatitis (862). Patients with higher-than-average levels of receptor activity in acute disease progressed to chronic hepatitis more often than those who recovered without complications (869).

Sera highly positive for receptor activity were also positive for HBeAg, HBV specific endogenous DNA polymerase activity, and were highly infectious, suggesting that the HBV associated receptor may play a critical role in the life cycle of the virus (861, 862, 869, 871, 872). In natural acute infection, the PSA receptor became undetectable well before seroconversion to anti-HBe and eventual clearance of HBsAg, suggesting that the receptor titers were predictive of coming HBe/anti-HBe seroconversion (869). Interestingly, low levels of receptor activity have been detected in some anti-HBe positive sera (316, 869), and it is possible that this observation correlates with low levels of virus replication and the infectivity of such sera. Patients with persistent HBeAg and high PSA receptor titers presented a more prolonged phase of active virus replication than those with lower titers, suggesting that the receptor may facilitate virus attachment to susceptible hepatocytes and spread throughout the liver (317, 861, 869). As in acute hepatitis, chronic carriers who are HBeAg positive and demonstrate other markers of virus replication also have initially high titers of receptor which decrease over time as virus replication slows down (862). In acute hepatitis this occurs within a few days following onset of disease, while in chronic hepatitis, much the same process occurs over a period of years (862). Although the presence of the receptor correlates with infectivity and viral replication, the ultimate infectivity of an HBsAg positive inoculum depends upon a number of factors including the e antigen/e-antibody status, the HBV PSA receptor titer, as well as the levels of anti-polyalbumin antibodies present in the serum of the normal recipient (508, 509).

To further understand what role, if any, the HBsAg associated receptor for polymerized albumin plays in the life cycle of HBV, a number of studies have focused upon its chemical characterization. The finding of such a receptor on HBsAg and Dane particles from chimpanzees and patients (317) as well as on HBsAg particles isolated from an HBV infected human hepatoma cell line (314), suggests that the receptor is virus encoded. Analysis of receptor activity on individual HBsAg polypeptides separated by SDS-PAGE showed that the surface antigen particles from e positive sera contained more than 10 fold more p31 and p35 than particles derived from anti-e positive sera. The same species

specificity in PSA binding was observed with these isolated polypeptides as seen in intact HBsAg (874). Further, this activity was not susceptible to heat or reducing conditions, suggesting that the receptor was sequential and not conformational in nature. The amino terminal amino acid in p31 was methionine, while the carboxy terminal sequence was -val-tyr-ile; the latter being identical with the carboxy terminal tripeptide of HBsAg p22-25. Combined with amino acid composition data on p31, the finding of N-terminal methionine suggests that p31 is encoded for by the S gene and included 55 amino acid residues adjacent to the S gene in the pre-S region (874). This suggests that the HBsAg associated receptor for polymerized albumin might be encoded for by sequences mapping to the pre-S gene region of HBV DNA, since p22-25, encoded only by the S gene, does not demonstrate binding activity (874). The finding of relatively large quantities of HBsAg p31 on Dane particles is also consistent with the hypothesis that its function may be that of a virus receptor (317). The suggestion that one or more HBsAg associated polypeptides are partially encoded for by pre-S sequences has gained further support by independent analyses of these components by 1- and 2-dimensional peptide mapping (768, 876). Small spherical HBsAg particles isolated from viremic carriers were separated into constituent polypeptides on SDS-gels (876). Individual bands further digested with trypsin or V8 protease and analyzed on similar gels showed several bands in p33 and p36 digests corresponding in size to fragments which would have been generated based upon the deduced amino acid sequence of the pre-S region (876). In a larger study, two dimensional tryptic peptide mapping of surface antigen associated polypeptides from HBsAg, GSHsAg, WHSAg and DHBsAg particles demonstrated that HBsAg p33, p36 and p43; GSHsAg and WHsAg p39; and DHBsAg p38.5 were probably unique translation products encoded for by both pre-S and S-gene sequences (768). Recent amino terminal sequencing of HBsAg p33 and p43, as well as DHBsAg p38.5, prove that these large minor polypeptides are initiated within the pre-S region of their respective virus genomes (792).

The original finding that HBsAg particles from e antigen positive sera differed in density and PSA receptor binding properties from similar particles in anti-HBe positive sera suggests that there are two populations of HBsAg particles (317, 873). One population, from HBeAg

positive viremic donors, often contains HBsAg p33 and p36 as the most prominent of the minor components (875). The other population, from anti-HBe positive donors, often demonstrates little or no p33 and p36 (875), again suggesting a strong correlation between the appearance of these polypeptides and the receptor for polymerized albumin. If the virus uses a polymerized albumin bridge to successfully infect hepatocytes, then the ratio of these HBsAg populations should be variable among carriers and within a single carrier. Variable protein composition of HBsAg particles has been documented among different donors (875), and two populations of HBsAg separated by size on Biogel A5M also differ with respect to p33, p36 and p43 from single carrier sera (792). If these same surface antigen populations are described in one or more of the animal models infected with a HBV-like virus, then direct biological experiments could be carried out to discern whether their respective receptors for polymerized albumin alter the infectivity of an inoculum or the course of acute or chronic hepatitis.

The realization that polymerized albumin may act as a bridge between HBV and susceptible hepatocytes suggests that antibodies to polymerized albumin might block formation of this bridge and thereby alter infectivity or the natural course of infection. Certainly a large variety of animals resistant to HBV infection possess a hepatitis B binding substance (349); usually an IgM antibody reacting with polymerized serum albumin (877, 878). IgM with polymerized albumin binding characteristics has also been documented in HBV associated acute hepatitis, chronic persistent hepatitis, and primary biliary cirrhosis (868). Although one study suggests that the IgM antibodies from various animals possess anti-HBs subtype reactivities (877), characterization of the IgM from HBsAg-IgM complexes in some acute and chronic patients' sera found no anti-HBs reactivity (879, 880). Indeed, the blocking of the HBsAg-IgM reaction by human PSA is consistent with these antibodies having anti-PSA reactivities (879, 880). In natural infection these antibodies were observed transiently in nearly all patients with acute hepatitis, in approximately one quarter of those that progressed to chronicity, and in nearly half of those with inactive and asymptomatic chronic infection. Although these antibodies provided a marker for transition to chronicity, their presence did not

correlate with markers of viral replication (879, 880). In case reports, low levels of apparently anti-HBs IgM (800, 801), much like those levels of anti-HBs IgM documented in animals (877, 878), failed to protect individuals from acute hepatitis B following exposure. Although it has not been demonstrated, if these antibodies are against polymerized albumin, then they were clearly not protective under these circumstances. The finding of anti-HBs in the absence of anti-HBc in other groups of people (881, 882) suggests that they may not be immune to HBV in all cases, and that those susceptible individuals experiencing acute hepatitis have a greater chance of progressing toward chronicity (879, 880).

DANE PARTICLE "SPECIFIC" ANTIGENIC DETERMINANTS

It is generally recognized that the determinants of HBsAg are shared among small spherical HBsAg, variably long filamentous forms, and Dane particles (19). However, there is accumulating evidence indicating that Dane particles and, to some extent, filamentous forms also possess distinct antigenic determinants not found on small spherical surface antigen. For example, the serum from some patients with acute hepatitis B contained antibodies which specifically precipitated radiolabeled Dane particles (389). A high frequency of individuals having self-limited acute HBV infection (71% of 31 patients) possessed this type of antibody specificity, which usually disappeared in those cases progressing to chronicity. Further, 42% of asymptomatic carriers with normal liver histology and 13% of those having chronic hepatitis demonstrated this type of specificity (390). Aggregates composed almost entirely of Dane particles have been observed by electron microscopy in HBsAg positive sera in the absence of anti-HBs (391-394). Treatment of Dane particles with 0.01% mucasol, chloroform, or 0.25% deoxycholate resulted in greatly increased aggregation, suggesting that most if not all of these specific antigenic sites are cryptic in nature and that they become exposed following partial denaturation of the envelope components (391). Independent observations have demonstrated that native Dane particles show a line of identity with 22 nm HBsAg spherical particles by immunodiffusion, while a distinct precipitation line was formed following mild Tween 80 treatment of Dane particles

but not after like treatment of small spherical HBsAg (395). The antibody specificities against Dane particle determinants were further shown not to be cross-reactive with any known reactivities in the nucleocapsid of HBV, including HBcAg and HBeAg (395). At least some of these antibodies have recently been shown to bind the Dane particle associated receptor for polymerized albumin (973).

ANTI-HBs MONOCLONAL ANTIBODIES

The generation of monoclonal IgM antibodies against small spherical HBsAg particles has resulted in the establishment of a highly sensitive assay for the detection of surface antigen (302, 305). Further studies with this assay soon revealed a high binding activity in the sera of HBV infected individuals which was unreactive with conventional anti-HBs in standard assays (396). Characterization of the material by immune electron microscopy and SDS-PAGE revealed surface antigen-like properties (396), suggesting that the determinant recognized by IgM monoclonal antibody 5D3 was different from that recognized by hetero-specific anti-HBs raised in guinea pigs. Isolation of 5D3 binding "HBsAg" by affinity chromatography was accomplished from the sera of patients showing acute and chronic inflammatory liver diseases without evidence of HBV markers and from the sera of asymptomatic individuals suspected of transmitting acute hepatitis (397). This material weakly cross-reacted with HBsAg following 100-fold concentration (by volume) and using heterospecific anti-HBs. These results indicate that HBV infected patients possess additional HBsAg-like particles virtually lacking antigenic reactivity with reagents used to detect the common "a" determinant(s) of HBsAg. Whether these particles are an altered form of HBsAg or represent an HBV associated but distinct infectious agent present in HBsAg negative acute and chronic liver disease is still to be determined.

The association of HBsAg with the membrane of infected hepatocytes in chronic HBV carriers often demonstrating one or more markers of viral replication suggests that HBsAg may serve as a target for specific humoral immune responses, leading to the eradication of at least some HBV infected cells (348, 452, Figure 2). Anti-HBs mono-clonal IgM 5D3, IgG2a or IgG1 antibodies were individually tested for

their ability to specifically bind to and lyse target cells in the presence of complement (787). The target cells used were from the human hepatoma cell line PLC/PRF/5, which possesses membrane associated HBsAg (788, 789). Although each of these monoclonal antibodies bound to different antigenic determinants on HBsAg particles, and all bound specifically to PLC/PRF/5 cell membranes, only IgM and IgG2a monoclonal anti-HBs specifically lysed the cells in the presence of complement. Balb/c male nude mice injected intravenously with IgM anti-HBs or intraperitoneally with IgG2a anti-HBs, followed by challenge with PLC/PRF/5 hepatoma cells, resulted in suppression of tumor growth or formation in more than half of the mice (760). Although nearly all of the control mice injected with PLC/PRF/5 cells grew large tumors, suggesting that the effects of antibody injection were statistically significant, the mechanism(s) by which these monoclonal antibodies altered tumor growth remain(s) to be elucidated. Interestingly, lymphocytes from HBsAg carriers produce anti-HBc but not anti-HBs in pokeweed mitogen stimulated cultures (791). Further investigation of this result using mixed cultures containing control or carrier B or T cells demonstrated an apparent specific B lymphocyte defect in anti-HBs production in some cases and a defect in T helper function in others (791). Since the serological profiles of many chronic carriers are positive for HBsAg and anti-core, a partial or total shutdown in anti-HBs production might permit persistence of scattered groups of infected cells which could eventually expand into clones of preneoplastic or neoplastic tissues.

Monoclonal anti-HBs have been made that detect different determinants on HBsAg particles and are being used to better resolve the antigenic complexity of surface antigen. In one study, for example, clone BX182 anti-HBs reacted with the "d" subtype determinant, clones BX259 and CN324 reacted with the group specific "a" determinant(s), and clones BX248 as well as DN296 reacted with the "w" subtype determinant (303). Since the group specific "a" determinant is complex in nature, and monoclonal antibodies possessing anti-"a" reactivity probably vary in their neutralizing capabilities, it is possible that production of an antibody subset during infection could partially determine whether virus is cleared from circulation or whether persistence leading to

chronic disease results. Indeed, a proposed explanation for the simultaneous presence of HBsAg and anti-HBs in chronic carriers is that such carriers suffer from an inadequate antibody response or a defect in T helper function (382, 791, 793), resulting in progression toward chronic liver disease (794). Perhaps some of the same immunological alterations are present in those individuals unresponsive to the commercial vaccine as well as those having anti-HBs but still susceptible to HBV infection (800, 801). The finding of heterotypic antibodies in chronic carriers of HBV (795) as well as those with HBsAg positive fulminant hepatitis (796) further suggests a qualitative deficiency in anti-HBs response whose missing reactivities may eventually be defined by a panel of human monoclonal anti-HBs from vaccinated individuals or those having resolved infection.

DANE PARTICLES AND HEPATITIS B CORE ANTIGEN (HBcAg)

The discovery of 42 nm diameter, double-shelled HBsAg (Dane) particles in the sera of some HBV infected patients (19, 20, 392) consisting of an outer envelope of HBsAg determinants, an inner 27 nm diameter nucleocapsid possessing antigenically distinct HBcAg (20), and a small circular, partially double-stranded DNA genome (42, 46) suggests the Dane particle as a putative virion of HBV. Some of the physical properties of Dane particles are listed in Table 6. In addition, Dane particle cores possess an endogenous DNA polymerase activity (48), a protein kinase activity (54, 55, 409), a protease activity (54) and e antigen activity (401, 408). In attempts to characterize each of these HBV associated activities, various methods have been employed to separate and purify Dane particles free from serum contaminants and from the other HBsAg forms. Most studies have reported the existence of at least two major populations of Dane particles. Density equilibrium centrifugation in cesium chloride (CsCl) has resulted in Dane particle peaks at 1.20 and 1.25 g/ml (194). DNA polymerase activity was only found associated with the heavier of these two peaks, suggesting the presence of DNA in the heavier peak and its absence in the lighter Dane particle fractions. Attempts to demonstrate DNA polymerase activity by addition of exogenous templates have yielded mostly negative results, making it impossible to assess the presence or absence of polymerase

Table 6. Some physical properties of Dane particles.

Parameter measured	ref(s)	result
1. Particle diameter	19	42 nm
2. Particle density (gms/ml); CsCl	194	1.24-1.25
	198	1.26-1.27
	41	1.24, 1.27
	225	1.24
	418	1.23-1.24
major peak	419	1.28
minor peak	419	1.25
3. Particle sedimentation coefficient	225	58.5S
4. Particle isoelectric point(s) (pH)	198	3.82

in light Dane particles (410). Examination of sera from HBV carriers exhibiting low levels of polymerase activity in their blood has resulted in the finding of frequently high numbers of "empty" Dane particle cores, suggesting that the low DNA polymerase activity of these sera was due to the absence of nucleic acid in these particles (271, 410-412). Independent observations have shown light and heavy Dane particle peaks at buoyant density values of 1.22 g/ml and 1.24 g/ml respectively (410, 412). Similarly, Dane particles have been detected at densities 1.24 and 1.27 g/ml (41). Two Dane particle populations have also been detected by rate zonal sedimentation (406). When partially double-stranded core associated HBV DNA was made fully double stranded in the presence of radioactive nucleoside triphosphates by the endogenous DNA polymerase reaction (48, 49, 557), labeled DNA was found only in the Dane particle fraction having greatest density. The variations in density values for light and heavy Dane particle peaks among these studies prompted closer examination of the associated nucleocapsids.

Removal of the HBsAg containing envelope from purified Dane particles by mild detergent treatment (20, 41, 226, 228, 399, 406, 410, 413) or by shearing forces (398) releases 27 nm diameter core particles, which possess an overall hexagonal shape and apparent icosahedral

symmetry by electron microscopy (Figure 4, Table 7; 226, 228, 715). Evidence from electron microscopy further suggests that approximately 180 densely packed capsomers, each composed of a single core polypeptide, make up the core particle (715). The larger capsomeric structures, characteristic of pentamer-hexamer clusterings, were not observed. Cores released from Dane particle preparations and analyzed by density equilibrium centrifugation resulted in populations at 1.30 g/ml and 1.36 g/ml (229). Examination of each population by electron microscopy showed that the lighter cores were penetrated by stain, suggesting they were "empty" and, therefore, lacking DNA, while heavy cores did not take up stain and probably contained viral DNA (228). Further, light cores showed smooth surfaces and reacted with human anti-HBc by immunodiffusion, while heavy cores showed a ragged surface and did not form a precipitin line. It is possible that the ragged surface of heavy cores may be due to the presence of incompletely removed envelope components, to the presence of bound anti-HBc, or to the presence of as yet uncharacterized "matrix" protein which may block HBcAg reactivity in immunodiffusion (228, 229). The presence of anti-HBc in moderate to high titers in the sera of HBV carriers (414-417) and the lack of ragged surface structure on core particles isolated from an immunosuppressed chimpanzee liver (226) suggest that at least some of the density heterogeneity in core particle populations isolated from Dane particles or liver under varying conditions could be due to the presence of bound anti-HBc. The presence of variable levels of DNA polymerase activity in light core gradient fractions, then, may be related to aggregation of some fraction of heavy cores with anti-HBc and their migration to the light core peak during centrifugation (229). In another study, DNA polymerase positive heavy Dane particles (1.27 g/ml) yielded a single peak of radiolabeled core particles at 1.38 g/ml in density gradients, while lower buoyant density, polymerase negative Dane particles (1.24 g/ml) yielded two peaks of core particles at 1.38 g/ml and 1.325 g/ml (41). Although the appearance of a heavy core population (1.38 g/ml) from light Dane particles (1.24 g/ml) is in contrast to that observed elsewhere, where only a single population of light cores resulted from light Dane particle solubilization (410) and the polypeptide profiles of both heavy and light cores were reported

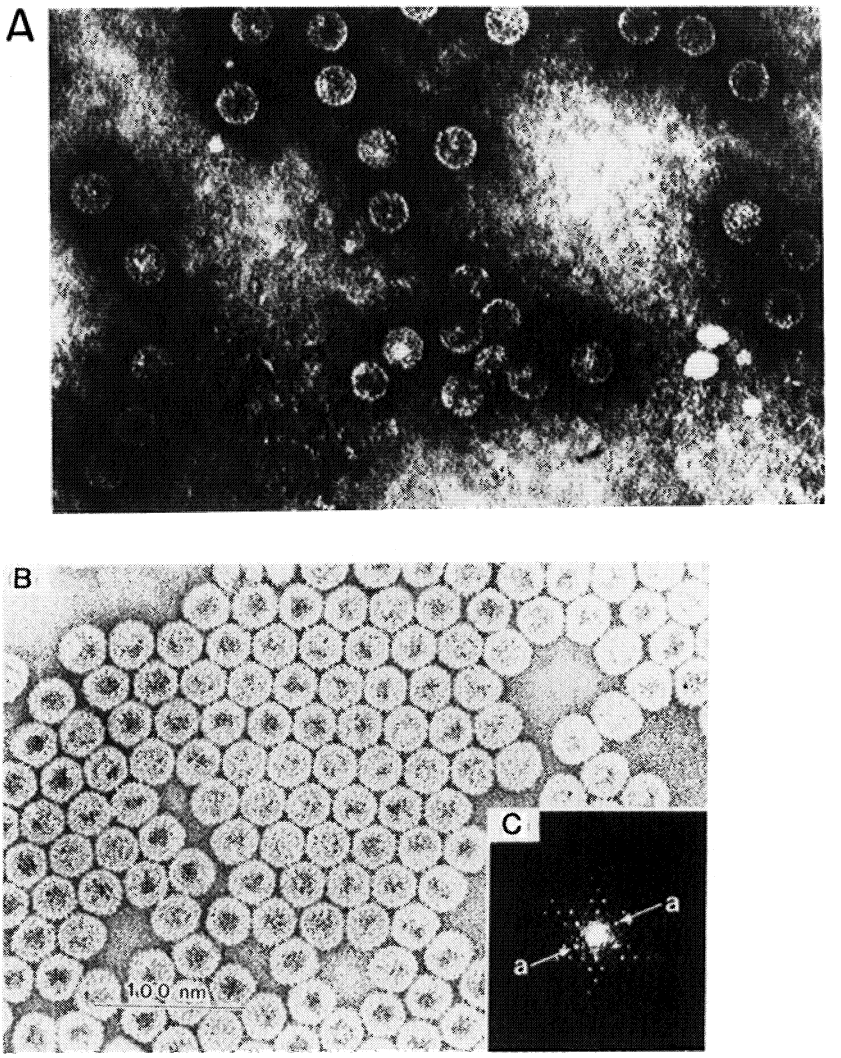


Figure 4. (A) Electron micrograph of Dane particle derived cores. [Reprinted with permission from Robinson, W. S. in Comprehensive Virology 14:471-526 (ref. 974). Copyright 1979 by Plenum Press]; (B) Electron micrograph showing fine structure in a crystalline array of liver derived core particles; (C) The first order diffraction spectra of the crystalline array in (B) shows a center-to-center (between arrow tips) particle distance of 28.3 nm [copyright 1980 by Alan R. Liss, Inc. (ref. 715)].

Table 7. Some physical properties of core (HBcAg) particles.

	Parameter measured	ref(s)	result
1.	Particle diameter; (E.M., liver)	57	21-25 nm
	- intranuclear	715	28.3 nm
	- intranuclear	400	27-30 nm
	- intranuclear, extracellular	19, 20, 226, 636	27-28 nm
	- capsomer diameter	715	4.0-4.2 nm
2.	Particle molecular weight;	437	1.3×10^{-17} gms
	- by Sepharose 4B	45	$8.5\text{-}9.0 \times 10^{6}$ daltons
3.	Particle density (gm/ml); from liver	636	1.30
		45	1.33
	- liver, heavy cores	414a	1.358
		44	1.32
		636	1.30
	- liver, heavy cores	135	1.35-1.36
	- liver, light cores	135	1.30-1.33
	- from Dane particles	419	1.36
		45	1.28-1.32
	- from e positive serum	43	1.28-1.30, 1.358
	- from e negative serum	43	1.28-1.30
	- from Dane particles at 1.24 gms/ml	41	1.325, 1.38
	- from Dane particles at 1.27 gms/ml	41	1.38
	- from Dane particles	421	1.30
		410	1.30, 1.36
		228	1.28-1.30
		194	1.31
		229	1.28-1.32, 1.35-1.36
4.	Particle sedimentation coefficient;	48	110S
	- Dane or liver cores	45	124S
	- from e positive serum	43	70S, 110S
		44	110S
5.	Particle isoelectric points; (pH)	43	4.0-5.8
	- major peak	198	4.15
	- minor peak	198	4.7
	- from Dane particles	419	4.4
	- radiolabeled liver/Dane particles	45	4.0
	- from liver	45	3.7

Table 7. (Cont.)

	Parameter measured	ref(s)	result
6.	Particle symmetry	226, 228, 400, 715	icosahedral
	- number of capsomers	715	180-190
	- T value	715	3
	- rotational symmetry	715	5 fold axis
7.	Half life; in sucrose (4°C)	44	22 days
8.	Width of core shell	715	3.5 nm

the same by Coomassie blue staining (41), one or more minor polypeptides having polymerase activity may be absent from the population of light Dane particles, resulting in polymerase negative cores having high density. The analysis of cores released from Dane particles purified away from anti-HBc by repeated pelleting through sucrose gradients resulted in three populations of core particles banding at 1.355 g/ml, 1.33 g/ml and 1.30 g/ml in CsCl density equilibrium gradients (413). Both heavy and intermediate density core particles contained DNA polymerase activity. However, the intermediate HBcAg population demonstrated a higher HBcAg:DNA polymerase activity ratio than was observed in heavy cores, suggesting a greater number of HBcAg polypeptides or determinants in the intermediate population in comparison to the heavy core population. Despite these differences in buoyant density, DNA polymerase positive populations of HBsAg (1.355 g/ml and 1.33 g/ml) sedimented together in rate zonal analysis, while DNA polymerase negative light cores (1.30 g/ml), making up the dominant peak in equilibrium centrifugation, sedimented at a slower rate. Analysis of the sedimentation coefficient of HBV DNA radiolabeled by the endogenous polymerase reaction and quantitation of the incorporated radioactivity indicated that the differences in density between the two polymerase positive peaks were not due to major differences in the characteristics of the nucleic acid. If, as the HBcAg:polymerase ratios suggest, the intermediate HBcAg population is composed of a larger number of polypeptides and/or antigenic determinants than heavy cores, and cleavage,

followed by release of some of this material as soluble peptides, results in a shift from intermediate to heavy cores, then the difference among these populations may be one of polypeptide assembly. The recent finding of cysteine as the carboxy terminal amino acid residue of the major core polypeptide (716), combined with the suggestion that each core particle associated capsomer is a single core polypeptide (715) which does not resolve on SDS-gels in the absence of reducing conditions (792) implies that the capsomers are held together by disulfide bonding. If an intermediate HBcAg population is the immediate precursor of heavy core particles, then its conversion to fully mature core particles would be accompanied by cleavage and release of core associated polypeptides or polypeptide fragments having e antigen activity. Treatment of heavy Dane particles with 0.1% sarkosyl and 0.1% 2-mercaptoethanol, for example, resulted in the release of core particles and soluble e antigen material (406). Further exposure of these core particles to stronger conditions (1% sarkosyl and 0.1% 2-mercaptoethanol) resulted in further release of HBeAg. Indeed, part of the difference between intermediate and heavy density core particles may be the presence of larger numbers of e reactive polypeptides in the intermediate population compared to the heavy HBcAg population, as suggested by the separation of two polymerase positive core peaks by rate zonal sedimentation (406). The purification of heavy core particles by affinity chromatography using anti-HBc IgG-conjugated Sepharose 4B or by density equilibrium centrifugation in CsCl, followed by sedimentation in CsCl, resulted in the release of e antigen activity which was located at the top of the gradients (414a, 792). HBeAg activity was also released from intact cores following incubation in 2.5 M CsCl, pH 7.5, or by treatment of intact cores with 6 M urea. Both the morphology and HBc antigenicity were retained following these treatments (846, Table 8). Further, the finding of both heavy (1.358 g/ml) and light (1.28-1.30 g/ml) core populations in e antigen positive sera, the presence of only light cores (1.28-1.30 g/ml) in e antigen negative sera (43), the detection of e activity in heavy but not light cores, the release of further e antigen activity to the top of the gradient upon resedimentation of heavy cores (414a) and the identification of some of these released constituents as core related polypeptides by

Table 8. Effects of various treatments upon the integrity of HBcAg particles.

Treatment		Morphology	Antigenicity HBcAg
1. heating;	37°C, 10 hrs	- (846)	+ (846)
	37°C, 20 hrs	+ (400)	+ (400)
	56°C, 4 hrs	- (846)	+ (846)
	60°C, 20 hrs	+ (400)	+ (400)
	100°C, 20 min	- (400)	- (400)
2. freezing/thawing (10 cycles)		+ (400)	+ (400)
3. storage; -20°C, 1 yr		- (400)	- (400)
4. u.v. irradiation, 3 hrs		+ (400)	+ (400)
5. enzymes;	trypsin, 37°C, 90 min	- (400)	± (400)
	trypsin, 37°C, 24 hrs	- (400)	- (400)
	pronase, 37°C, 90 min	- (400)	± (400)
	pronase, 37°C, 24 hrs	- (400)	- (400)
	lysozyme	- (400)	- (400)
		+ (57)	+ (57)
			+ (43)
	lipase	+ (400)	+ (400)
		+ (57)	+ (57)
	DNase	+ (57,400)	+ (57,400)
	RNase	+ (57,400)	+ (57,400)
	diastase	+ (57,400)	+ (57,400)
	neuraminidase	± (400)	+ (400)
		+ (57)	+ (57)
	hyaluronidase	+ (57)	+ (57)
	phospholipase C	+ (57)	+ (57)
6. pH changes;	pH 1.8-2.9	- (400), + (846)	- (400,846)
	pH 2.5	+ (846)	- (846)
	pH 3.0	± (400)	+ (400)
	pH 4.5	+ (846)	- (846)
	pH 5.5-10.5	- (846)	+ (846)
	pH 10-11.8	+ (400)	+ (400)
	pH 11.5	± (846)	+ (846)
7. reduction/alkylation;	2-ME	- (400,846)	+ (400,846)
		+ (57)	+ (57)
	- iodoacetic acid	± (400)	- (400)
	- 2-ME, then iodoacetic acid	- (400)	- (400)

Table 8. (Cont.)

Treatment	Morphology	Antigenicity HBcAg
8. denaturant; 1% SDS	+ (57)	+ (57)
	± (400)	± (400)
- 1% sodium deoxycholate	+ (400)	+ (400)
- 1% Tween 80	+ (57,400)	+ (57,400)
- 8M urea	± (400),	+ (400),
	+ (846)	- (846)
- 3M NH_4SCN, pH 4.5	+ (846)	- (846)
- 3M NH_4SCN, pH 6.5	± (846)	+ (846)
- 6M urea	+ (846)	± (846)
- 0.18-2.93 M formaldehyde	+ (846)	- (846)
- 2.5 M CsCl, pH 7.5	- (846)	+ (846)
- 0.15% SDS		- (846)
- 0.05% sarcosyl		- (846)
9. Other treatments; 4% formalin	+ (400)	+ (400)
- chloroform:methanol (2:1 v/v)	- (400)	- (400)
- 50% diethyl ether	+ (400)	+ (400)
- 50% acetone	+ (400)	+ (400)
- 50% butyl alcohol	- (400)	- (400)
- 50% ethanol	- (400)	- (400)
- 50% methanol	- (400)	- (400)
- sonication		± (414a)

+ = property retained following treatment

± = property decreased following treatment

- = property destroyed following treatment

tryptic peptide mapping (792) support the conclusion that core particles undergo slow disintegration accompanied by the antigenic conversion of core to e and finally by release of soluble e polypeptides from Dane or core particles. This conclusion is further supported by recent results which clearly demonstrate the loss of HBc antigenicity with the morphological disintegration of core particles (846, Table 8). The detection of morphologically aberrant Dane particles containing structurally abnormal or incomplete core particles (415a-c) is particularly relevant in this context, since in the cases where serum e antigen was assayed, it was usually found in high titers. If the conversion of core to e antigenic activity is a consequence of HBcAg maturation, as suggested above by the presence of an intermediate density HBcAg particle population, and/or occurs during uncoating of HBcAg, then the presence of e antigen would correlate most highly with viral replication. Further, the presence of "empty" Dane particles, low serum DNA polymerase, and anti-e suggest that polymerase negative Dane particles may be defective and interfering. That HBsAg determinants on Dane particles are involved directly or through a receptor for polymerized albumin in binding to susceptible hepatocytes further suggests that small spherical and variably long filamentous HBsAg forms also act as defective interfering particles. The establishment and persistence of chronic disease including the asymptomatic carrier state may be a consequence of the titers of these particles and the immune response against them.

The recent indication that at least some of the steps in viral DNA replication occur within an immature core particle (188, 189) suggests that a more detailed examination of the core associated constituents of HBV and related viruses might help to elucidate the molecular details of viral DNA replication. Early studies have indicated that the core particles isolated from infected liver and from serum Dane particles are serologically identical (226, 229) with respect to HBcAg activity even though core particles resolve into a number of populations by density equilibrium and rate zonal analysis. The sensitivity of HBcAg activity and particle morphology to various physical, chemical and enzymatic treatments indicates that the determinant(s) is protein in nature (400). Treatment with lysozyme or neuraminidase, however, has

resulted in conflicting results as to the presence of carbohydrate (57, 400, Table 8). While the effects of reduction upon morphology and antigenic integrity are minimal, reduction and alkylation of HBcAg particles destroyed both (400), suggesting that HBcAg activity is conformationally dependent involving intact sulfhydryl groups. Further, the destruction of antigenic activity, morphology or both by heat, acid pH, and exposure to several denaturants (400), indicate that hydrogen or hydrophobic bonding might also play important roles in core antigenic activity. The apparent resistance of tissue bound core particles to proteolytic and mucolytic enzymes as well as to SDS and 2-mercaptoethanol (57), in contrast to free core particles, indicates that more work of this type is needed to adequately define HBcAg determinant(s) on the molecular level.

Analysis of both liver and Dane particle derived HBcAg particles by SDS-PAGE resulted in the appearance of a major polypeptide component approximately 16-22,000 daltons in size (41, 43, 44, 56, 177, 405, 407, 414a, 420, 432) with few exceptions (45, 325, Table 9). The apparent discrepancy in molecular weight values reported for the major HBcAg polypeptide were resolved by the finding that this polypeptide (20,600 daltons) could be cleaved upon incubation of intact core particles with anti-HBc containing sera, yielding components 14,700 and 6000 daltons in size (54). The recent deciphering of the amino acid composition of the major core associated polypeptide indicates a high percentage of arginine (12.9%) and leucine (11.9%) residues, frequent appearance of proline, glutamic acid and serine residues, and predicts a polypeptide with 177 amino acids and having a molecular weight of 20,079 daltons (432, Figure 5, Table 10). Nucleic acid sequencing of cloned HBV DNA isolates and identification of the various open reading frames reveals one which would encode a polypeptide 183-185 amino acids in length and having a molecular weight of 21,042 daltons (177). While the first 140 amino acid residues deduced from this sequence are not remarkable, the carboxy terminal 40 residues demonstrate a protamine-like structure with repeated arginine residue runs separated by serine and proline (358, 434, Figure 5). Although there is no amino acid sequence data on core polypeptide, which would define its exact encoded region in the genome of HBV, a number of studies using subgenomic fragments of HBV DNA

Table 9. Apparent molecular weight of polypeptides associated with hepatitis B core antigen particles[a]

	(414a)[d]	(43)[e]		(45)[c]		(41)[d]	(44)[c]	(325)	(177)
Source:	liver	Dane		liver	Dane	Dane/liver	liver		recombinant expression
		light	heavy						
						200			
				88	88				
						80			
				79	79				
						70			
		68	68						
				59	59			59	
					56				
					54				
	43							53	
			42						
		39					35		
		22							
	20[b]	20				19			19
							17		
		16	16						
		5	5						

Table 9. (Cont.)

Source:	(56)[e] liver	(420)[c] Dane	(405, 407, 432)[d] Dane	(716)[d] liver
			45	
	40			
		37		
	27.5			
		25		
	22			
				21-21.5
	19		19	
		18		

[a]Molecular weights are reported x 10^{-3} daltons.
[b]Underscoring indicates a major HBcAg associated component.
[c]Polypeptide profile deduced from ^{125}I labeled components using chloramine T or lactoperoxidase procedures.
[d]Polypeptide profile deduced from Coomassie blue staining.
[e]Polypeptide profile deduced from ^{125}I labeled components radioacylated by the Bolton-Hunter reagent (267).

Table 10. Amino acid composition of the major HBc/HBe reactive polypeptide.

Amino acid	from hydrolysis mol (432)	from hydrolysis % (716)	from DNA (156)	from DNA (177)	from DNA (536a)	nearest whole no. of residues - hydrolysis (432)	nearest whole no. of residues - DNA (177)
lys	1.2	2.0	1.1	1.1	1.1	2	2
his	2.3	2.3	2.3	2.3	2.2	4	4
arg	12.9	13.1	13.7	13.7	14	22	24
asx	7.3	8.0	6.3	7.4	7	12	13
thr	5.6	5.8	7.4	8.0	7	9	14
ser	10.3	8.4	10.3	9.7	9.7	17	17
glx	9.6	9.9	8.6	8.0	8.1	16	14
pro	10.2	7.6	9.7	9.1	9.2	17	16
gly	5.1	6.0	4.0	4.0	4.3	9	7
ala	6.3	6.9	5.1	6.3	4.9	11	11
cys-SH			2.2	2.2	2.2		4
val	5.8	6.0	6.9	6.9	6.5	10	12
met	1.5	1.5	1.7	1.1	1.1	2	2
ile	3.7	3.9	3.4	2.9	3.2	6	5
leu	11.9	11.2	12.0	12.0	11.3	20	21
tyr	2.4	3.6	2.9	2.9	2.7	4	5
phe	3.9	3.8	4.6	4.6	3.8	7	8
trp			2.2	2.2	2.2		4
total amino acids			183	183	185		

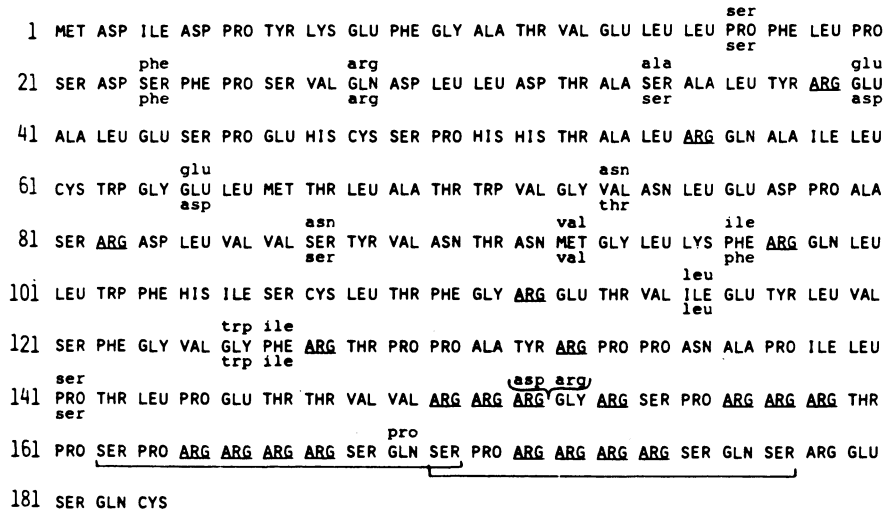


Figure 5. Proposed amino acid sequence of the major core antigen associated polypeptide (p22) derived from nucleic acid sequences. [Reprinted with permission from Tiollais, P., et al. in Science 213: 406-411. Copyright 1981 by the American Association for the Advancement of Science (ref. 358)].

including this region have successfully resulted in the production and detection of core reactive material (167, 172, 176-179, 435, 436, 797-799). The finding of 4 cysteine residues in core polypeptide (432), that one of these residues may be the carboxy terminal amino acid (177, 358), and that core polypeptide does not resolve on SDS-gels if HBcAg is not reduced (792) suggest the presence of both intra- and intermolecular disulfide bonds in intact HBcAg responsible for both morphological and antigenic integrity. The proposed carboxy terminal protamine structure of core polypeptide also suggests that it may possess DNA, RNA or both binding capabilities, although this has not yet been demonstrated. In addition to the CD spectra of intact or disrupted antigen (716), when each of the amino acids in the proposed sequence of the major core polypeptide is assigned a value defining its tendency to form or disrupt secondary protein structure (155, 177, 293), the amino terminal portion of the molecule shows considerable alpha helical conformation, while the carboxy terminal 40 amino acids seem to show

mostly nonhelical-random coil structure. The center of the molecule seems to be best characterized by beta sheet conformation, as deduced from CD spectra (716), suggesting that core polypeptide may possess several distinct structural domains. Further studies of the like carried out to define some of the HBsAg determinants on the molecular level will undoubtedly bring about a better understanding of core antigenic activity and its relationship to the other activities of the HBV nucleocapsid.

SDS-PAGE of the components present in purified cores from liver or Dane particles has resulted in a variable number of minor polypeptide components both larger and smaller than the major component described above (Table 9). Two studies have described minor high molecular weight bands at 25-27,500 daltons in size (56, 420). Although this is the molecular range of immunoglobulin light chain, which is often present in preparations of core particles, tryptic peptide mapping of this band has shown it to be highly related to the major core polypeptide and not to IgG light chain (56). Identical analysis of GSHcAg associated polypeptides also revealed a band in the same region of the gel (26,000 daltons) having a similar relationship to its major core polypeptide (20,500 daltons) (56). In both viruses, these minor higher molecular weight polypeptides shared approximately two thirds of the spots on their peptide maps with that of their respective major core components, suggesting unique sequences in these higher molecular weight constituents. Further, a larger number of studies describe a minor component between 35,000 and 43,000 daltons (43, 44, 56, 414a, 420, Table 9). Although it is not known whether these values represent a single molecular entity, tryptic peptide mapping of a core associated polypeptide with an apparent molecular weight of 40,000 daltons showed that it was highly related to both the 27,500 dalton band and the major (22,000 dalton) HBcAg polypeptides. A 37,500 dalton band in the GSHsAg profile was similarly related to its core components (56). Further, the 40,000 dalton and 37,500 dalton bands of HBcAg and GSHcAg, respectively, shared only 60% homology with their respective major polypeptides and also include many of the spots unique to the 27,500 dalton and 26,000 dalton bands of HBcAg and GSHcAg, respectively, when each was compared to its major polypeptide. Since the variably appearing

polypeptides greater than 40,000 daltons resulted in peptide maps suggesting aggregation, it has recently been proposed that these pair of higher molecular weight components are unique translation products of the core antigen gene of each virus (56). The implications of these results upon the genetic organization of the core antigen gene will be discussed below.

ANTI-HBc

Although a direct role for one or more of the core antigen associated polypeptides in viral replication has not yet been defined, the presence of anti-HBc in acute and chronic hepatitis has been found associated with both infectivity and replication. Several studies suggest that blood containing high titers of anti-HBc is infectious (438, 439, 441, 458). High titer anti-HBc of the IgM class has been found in patients with acute hepatitis at approximately the same time that serum e antigen, transient DNA polymerase and clinical disease are present (61-64, 414, 440, Figures 2 and 6). The high titer of HBsAg particles during acute hepatitis and the prevalence of cytoplasmic surface antigen and nuclear core antigen in the preclinical stage of acute disease (348) suggest that replication in the preclinical stage is followed by production of infectious virions and a vigorous anti-HBc response resulting from HBcAg synthesis and maturation. The presence of these antibodies in acute HBV infection, however, does not signal the beginning of recovery from clinical disease, indicating that anti-HBc possesses no eliminating or protective function (414). The frequently rapid decline in HBsAg titers to nondetectable levels following acute disease and the variable appearance of anti-HBs during this same period means that anti-core may well be the only serological marker indicating HBV infection following acute hepatitis (444). Indeed, the screening of surface antigen- and anti-HBs-negative sera for anti-core following acute viral hepatitis has indicated the presence of HBV in these cases (62, 63, 442). Following acute viral hepatitis with HBV, the titers of IgM anti-core often decrease over many months or years and may disappear, indicating little or no HBcAg synthesis and suggesting cessation of viral replication (443, 448). Alternatively, the persistence of high titers of anti-core IgM

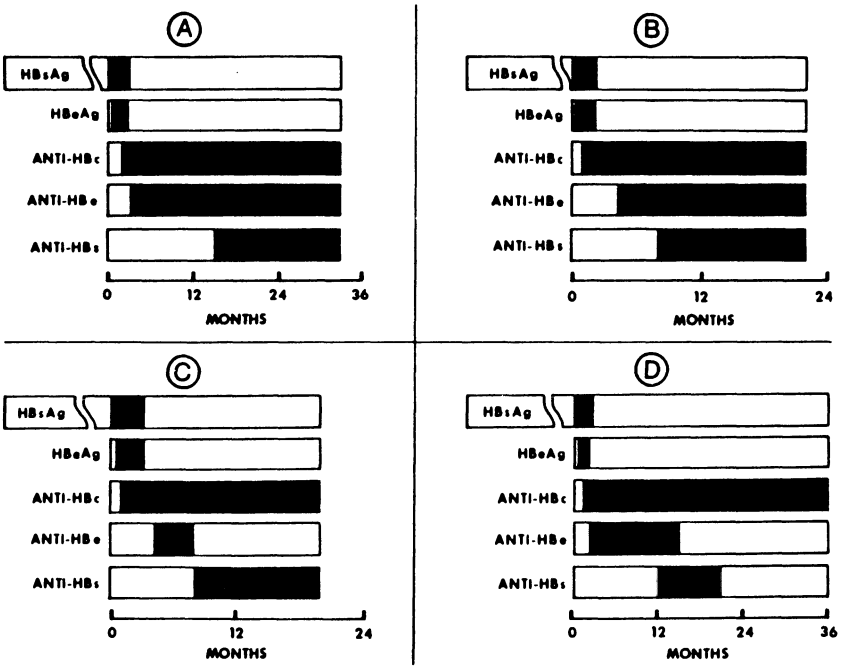


Figure 6-1. Four serologic profiles of hepatitis B virus markers during acute infection. (A) HBeAg seroconversion to anti-HBe without delay; (B) As in (A), except that seroconversion to anti-HBe was delayed; (C) Short-lived anti-HBc and anti-HBs; (D) A demonstration of long-lived anti-HBc.

following acute hepatitis is often associated with continued expression of one or more viral antigens in liver, continued viral replication, and progression to chronic disease or carrier state (61, 63, 440, 445, Figure 6). In chronic aggressive hepatitis (CAH), high titers of anti-core IgM have been found with large numbers of Dane particles, high DNA polymerase activity and e antigen in the sera of infected individuals (348, 446, 447), suggesting continued high levels of replication and infectivity. Most cases of chronic passive hepatitis (CPH) and some asymptomatic carriers also possess high titers of anti-core IgM along with persistently high concentrations of circulating HBsAg particles and few Dane particles (348). In those carriers possessing e antigen, DNA polymerase elevated serum enzyme levels and HBsAg concentrations

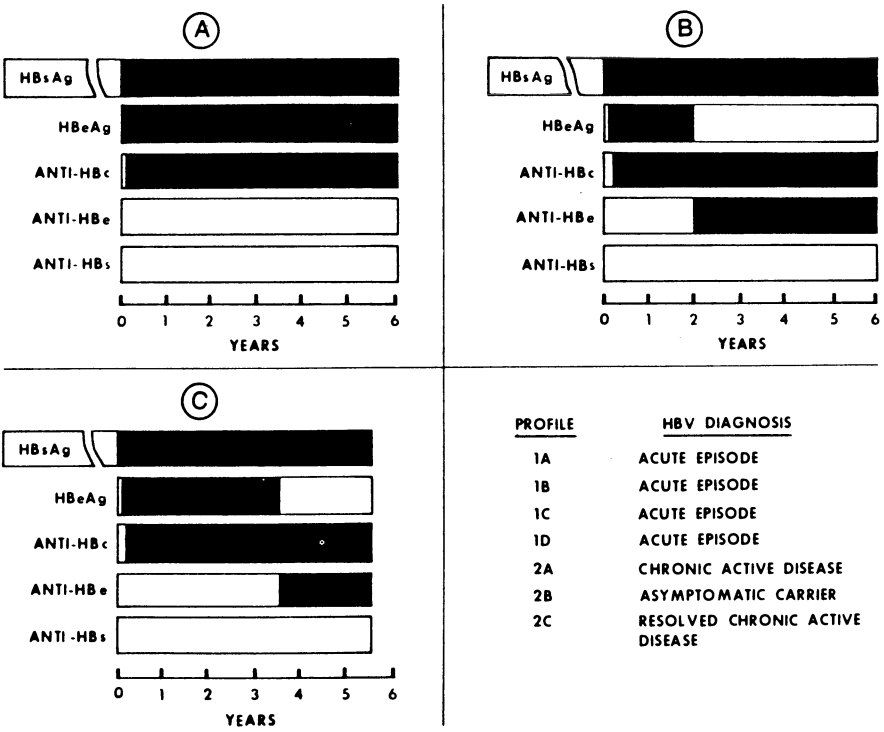


Figure 6-2. Three serologic profiles of hepatitis B virus markers during chronic infection. (A) Persistence of HBsAg and HBeAg; (B) Persistence of HBsAg and anti-HBe; (C) A long persistence of HBeAg followed by emergence of anti-HBe.

greater than 80 μg/ml, over 90% also possessed anti-HBc IgM (417). In comparison, a substantially lower percentage of carriers (60-70%) possessing anti-HBee, no DNA polymerase activity, normal liver enzyme levels and HBsAg concentrations less than 80 μg/ml also had detectable anti-core IgM (417). These results suggest that while anti-core IgM may reflect viral replication under most circumstances, its finding together with anti-HBe suggests that this is not the case. In asymptomatic chronic carriers, several studies have consistently demonstrated low titers of anti-HBc IgM (62, 63, 442, 809), while others have found no anti-HBc IgM or insignificant titers in similar groups (802, 807, 808, 813). While the significance of these findings is not clear, the presence of anti-HBc IgM in chronic hepatitis seems to correlate best

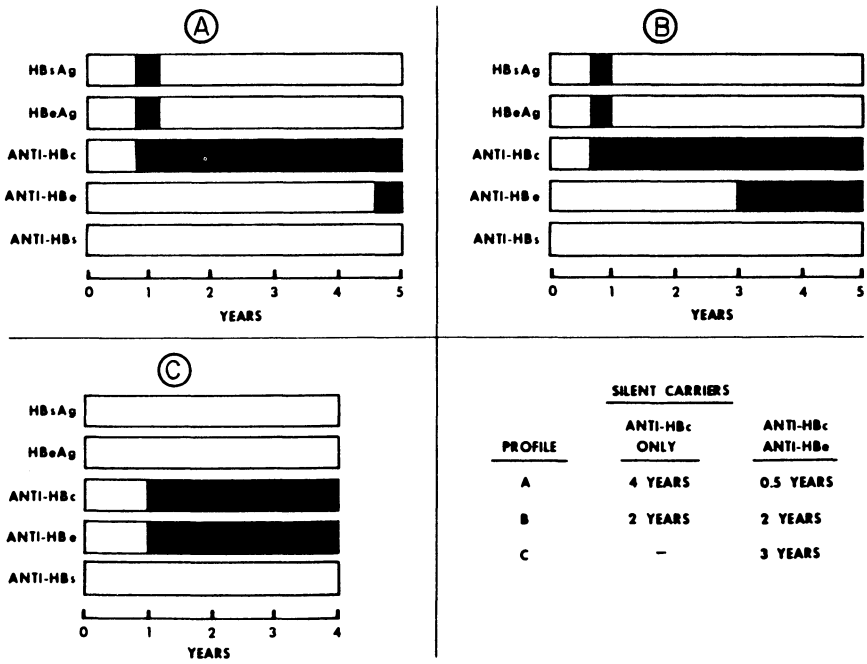


Figure 6-3. Three serologic profiles of hepatitis B virus markers of "silent carriers." (After disappearance of HBsAg and HBeAg, there was a long delay before seroconversion to anti-HBe.) (A) 4-year window; (B) 2-year window; (C) Anti-HBe and anti-HBc appeared without detection of the corresponding antigens. No anti-HBs was detected in any of the cases. [Figure 6 reprinted from L. Overby, in Viral Hepatitis: 1981 International Symposium, Szmuness, W., et al., eds. Copyright 1982 by permission of the Franklin Institute Press (ref. 975)].

with severe liver disease independent of markers indicative of viral replication (802, 803). Further, it has recently been suggested that in carriers demonstrating normal liver functions and low infectivity, persistent production of HBcAg might not be synonymous with continued synthesis of infectious HBV and that another component, possibly e antigen, may be necessary for complete particle assembly (416). Since the major core antigen polypeptide in intact HBcAg particles is cleaved in the presence of anti-core to a polypeptide having similar characteristics to soluble e antigen, the presence of persistently high anti-core titers in carriers and those with chronic hepatitis may play a negative role in favoring persistence of infectious virus in which the generation of e antigen is an essential part.

Although it is well established that anti-HBc IgM represents part of the primary response against HBV in acute infection (62, 63, 802-810), there is some confusion concerning its presence and significance in the asymptomatic carrier state. Infection with HBV, followed by acute hepatitis, often results in high anti-HBc IgM titers which decrease to low or undetectable levels within months after recovery from acute disease. High titers of anti-HBc IgM have also been observed in cases of HBV associated fulminant hepatitis and have diagnostic value, especially in cases where patients are HBsAg seronegative (814). Interestingly, if anti-HBc IgM is a marker of recent HBV infection, then the absence of this marker in 51 HBV infected children born to HBsAg seropositive carrier mothers suggests that infection could take place at birth from contact with contaminated maternal blood and not from transplacental transmission in utero (806). Although HBV markers have been found in cord and venous blood of neonates born to HBsAg carrier mothers, and a number of HBsAg seropositive neonates have remained as chronic carriers (815-817), the disappearance of HBsAg after several weeks in some infants followed by the appearance of clinical hepatitis several weeks later (815, 818) suggests that both transplacental transmission or transmission during birth might be operative in different individual cases (806). The significance of the anti-HBc response is that it may identify infants infected at birth who would benefit from immediate vaccination and thereby reduce the number of childhood chronic HBV carriers (806). The measurement of anti-HBc IgM in acute hepatitis is also useful in discerning etiology, since it is present in acute hepatitis associated with HBV infection, but absent in acute hepatitis due to other factors (802, 805). Importantly, the absence of this antibody in HBsAg carriers experiencing an episode of acute hepatitis suggests that the acute disease is due to infection with an agent other than HBV (802, 804, 805) and will provide an assay useful in differential diagnosis of type B and non-B acute viral hepatitis.

In addition to anti-core IgM, the humoral immune response to HBcAg also results in the production of anti-core IgG, which has become a marker of past infection (448). Anti-core IgG has recently been eluted from the hepatocyte plasma membrane in cases of CAH, and its presence has been found associated with active virus replication (449-451).

Antibodies against other HBV specificities or liver cell membrane components have not been found. Although HBcAg has been found associated with hepatocyte membranes by immunofluorescence (58, 452), further studies by electron microscopy have demonstrated HBcAg particles only in the intracytoplasmic submembraneous region (58), suggesting that core particles are not directly exposed to the exterior of the hepatocyte (453) but does not exclude the presence of individual core reactive polypeptides in the plasma membrane. The latter alternative is unlikely, however, because enveloped viruses do not usually express one or more nucleocapsid components on the plasma membrane of infected cells. Further characterization of the bound immunoglobulin has revealed only IgG_1 and IgG_3 subclasses, which bind complement and could potentially serve a cytolytic function. However, the lack of correlation between the presence of anti-core IgG and severity of liver lesions (454) argues against a cytopathologic role and suggests that these antibodies bind through an Fc receptor on the hepatocyte cell membrane. Cytophilic antibodies could bind soluble antigen and antigen-antibody complexes and result in attachment of Fc receptor bearing non-antigen specific cytotoxic lymphoid cells to infected hepatocytes. If anti-core penetrates the infected cells and forms immune complexes with nuclear core particles, cytolysis of such cells would rapidly occur, releasing Dane particles which may further chronic infection, especially under circumstances where anti-core titers are high and anti-HBs titers are low or non-existent (725). Alternatively, the binding of such antibody to hepatocytes could result in the blocking of one or more antigenic sites vital for recognition and elimination of infected cells. Finally, the binding of cytophilic antibody might induce or suppress expression of one or more HBV genes, thereby affecting the course of viral replication. Indeed, immunologically mediated alterations in viral gene expression have been documented for several systems (455-457), and the likelihood of this being the case in HBV can only be answered with further work, perhaps making use of the animal models (120, 151-154, 370).

HEPATITIS B e ANTIGEN (HBeAg)

HBeAg and infectivity.

A third antigenic specificity associated with HBV infection, designated as e antigen or HBeAg, was found in the sera of some individuals with acute or chronic hepatitis as detected by immunodiffusion (51, 52, 459). The screening of HBsAg positive sera from hemodialysis patients for this antigen revealed a close correlation between the presence of e antigen and infectivity (51, 52). The transmission of HBV from e antigen positive sera through needlestick exposure (462, 463, 819) or by injection of such sera into susceptible chimpanzees (464, 465) confirmed this close association. Further, the transmission of HBV among sexual partners (466) and from mother to newborn (467-470, 720, 816, 817, 824, 825) has also demonstrated a similar relationship. HBeAg was also found in greater association with HBsAg positive chronic than with acute hepatitis (460, Figure 6) and found as well in a subset of chronic carriers demonstrating persistently abnormal liver function tests and severe clinical disease (460, 461, 469, 473, 477, 486, 819-821). Although associated with chronicity, the presence of HBeAg did not always correlate with the type or severity of liver disease after HBV infection, nor with elevated serum transaminase levels (473). Occasionally, HBeAg has been found in the serum of asymptomatic carriers with no liver involvement (402, 473), while anti-e has been found in some patients with chronic liver disease (476-478). In assessing the role of HBV markers in acute and chronic disease, resolution of acute hepatitis was often characterized by the rapid elimination of HBeAg, the slower removal of circulating HBsAg and the sequential appearance of anti-core, anti-e and anti-HBs (Figure 6). In comparison, progression to chronic hepatitis is often accompanied by persistence of both surface and e antigens in the sera of infected individuals, suggesting that the long-term persistence of HBeAg is associated with development of chronic liver disease and a poor prognosis, while seroconversion to anti-e following acute hepatitis often results in a favorable prognosis and recovery (479). Indeed, when the e antigen/anti-HBe status of 25 chronic HBV carriers were serially assayed over a period of 1 to 6 years, nearly half spontaneously seroconverted from HBe to anti-HBe. This occurred in rough parallel

with decreased aminotransferase levels and disappearance of markers indicative of viral replication as well as decreases in HBsAg titer (823). The disappearance of clinical and biochemical evidence of active liver disease in those who seroconverted to anti-HBe stood in contrast to those who did not spontaneously seroconvert, suggesting that anti-HBe or antibodies associated with it are important in the resolution of chronic active hepatitis (823). The risk of virus transmission, due to the presence or absence of e antigen, has since suggested the division of HBsAg carriers into two groups: one having e antigen and being highly infectious, and the other demonstrating no e antigen or having anti-HBe and being much less infectious (471, Figure 7). That anti-HBe containing sera could transmit infection at a much lower frequency than e antigen positive sera, however, has also been documented (464, 475).

The relationship between HBeAg and infectivity, established above, not only suggests ways in which important sources of transmission could be eliminated through vaccination but also suggests that e antigen is in some way correlated with the presence of actively replicating HBV. The sera of asymptomatic carriers having anti-HBe contained few if any Dane particles and no detectable DNA polymerase activity (471-474). However, sera from a sick carrier and from five of six hemodialysis patients tested had e antigen and all had relatively large numbers of Dane particles as well as high serum DNA polymerase values (471). Similarly, 21 of 22 HBeAg positive carriers were also polymerase positive, 13 e antigen negative patients were polymerase negative, and three of four patients seroconverting to anti-HBe showed a loss of DNA polymerase activity (472). Similar results were obtained elsewhere (428). Assessment of Dane particle morphology by electron microscopy in HBsAg positive, persistently infected patients, either positive or negative for e antigen, showed the presence of mostly complete (DNA polymerase positive) or full Dane particles in HBeAg positive sera, and incomplete (DNA polymerase negative) or empty Dane particles in sera containing anti-HBe (478). This was especially true of patients with CPH and CAH. The finding of DNA polymerase negative, presumably non-infectious, empty Dane particles in anti-HBe containing sera (478), the disappearance of membrane bound IgG anti-core following seroconversion

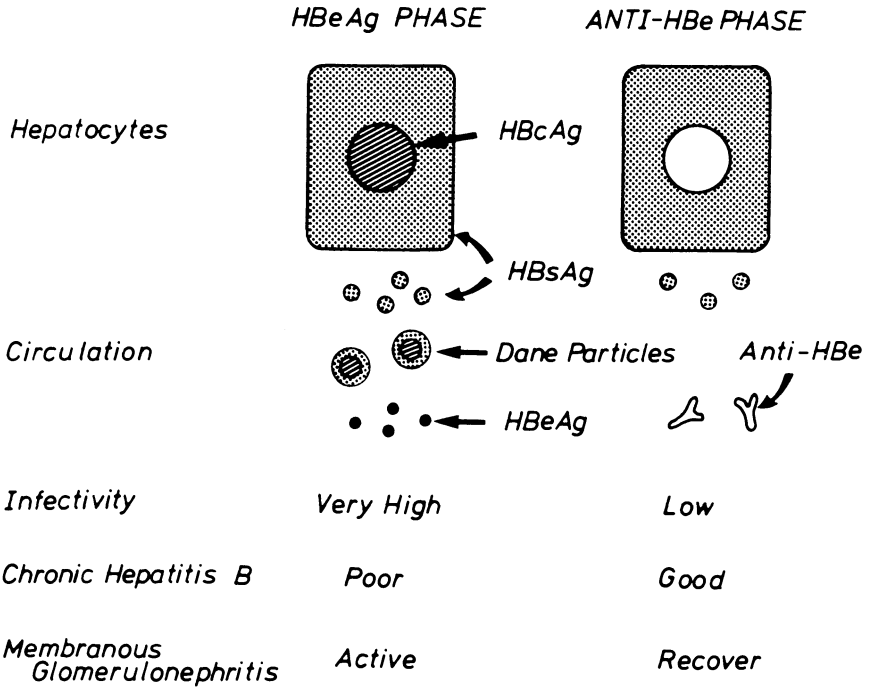


Figure 7. HBeAg and anti-HBe phases of HBV infection. HBV-related antigens in the hepatocyte and serum, as well as some epidemiologic and clinical aspects of the two phases of HBV infection, are illustrated. [Reprinted from Miyakawa, Y. in Viral Hepatitis, 1981 International Symposium, Szmuness, W., et al., eds. Copyright 1982 by permission of the Franklin Institute Press (ref. 437)].

to anti-HBe (449), and the possible role of empty Dane particles as defective interfering particles (481) suggest that the appearance of anti-HBe results in a specific immunological elimination or suppression of one or more components associated with HBV DNA replication. Indeed, the persistence of e antigen in serum is not only associated with complete Dane particles and 20 nm HBsAg, but also nuclear HBcAg, nuclear HBeAg, cytoplasmic HBsAg (348, 437, 480) and with detectable serum HBV DNA (826-828). In comparison, serum containing anti-HBe usually also has 20 nm HBsAg and may or may not have empty Dane particles. Although the cytoplasm of hepatocytes still contains HBsAg, their nuclei are

devoid of both HBcAg and HBeAg (437, Figure 7). As discussed above with anti-core, anti-HBe may bind to the surface of infected hepatocytes in an antigen nonspecific manner and modulate expression of these polypeptides. Indeed, anti-HBe has been reported being eluted from infected hepatocytes (512). However, if the modulation of antigen expression occurs through Fc receptor binding, then it is not clear why anti-core binding correlates with virus replication and anti-HBe binding does not. Alternatively, HBV may undergo an average number of replication cycles in hepatocyte nuclei before enough defective interfering genomes or genome fragments prevent the synthesis of enough core particle precursors in the nuclei to yield a positive result by immunofluorescence. If partial genomes are packaged in some "empty" Dane particles, and these behave as defective viruses, the presence of anti-HBe may signal the end of complete virus replication or may play a role in maintaining a persistent infection (410, 421, 473, 481). Interestingly, active liver disease is often found in carriers with anti-HBe, since one or more markers of viral replication is found in hepatocytes, serum or both (827). This observation may explain the lower but present infectivity of some HBsAg and anti-HBe positive sera. Other carriers with anti-HBe and normal liver histology demonstrate little or no evidence of viral replication, suggesting that the presence of chronic liver disease depends upon the state of HBV replication (827). In fact, detectable serum HBV DNA and intranuclear HBcAg in hepatocytes correlated more closely with viral replication than the HBeAg status (827). Further, carriers with chronic hepatitis and liver disease often have elevated serum aminotransferase levels as well as free, replicating HBV DNA in the liver, while chronic carriers without accompanying liver disease have normal or near normal serum aminotransferase levels and often demonstrate only integrated HBV DNA (105, 826). The transition from chronic hepatitis to the asymptomatic carrier state accompanied by seroconversion from e antigen to e antibody represents, then, a shift from replicating to nonreplicating phases in the HBV life cycle. Examination of the events surrounding seroconversion in a longitudinal study with HBeAg positive patients having clinicopathologically verified chronic hepatitis demonstrated an abrupt elevation in serum glutamic pyruvic transaminase and histological changes consistent with

exacerbated chronic hepatitis in 13 of 20 (65%) patients within three months prior to spontaneous HBeAg clearance (830). Although HBeAg seroconversion occurs frequently in chronic hepatitis associated with HBV (823, 831, 832), and the abrupt clinical as well as histological changes preceding seroconversion have been independently observed (833, 834), the explanation of these events still remains speculative. Significantly, HBeAg was also cleared from a group of immunosuppressed patients, although the clearance was uneventful in that the abrupt changes described above occurred in very few of the patients (830). These results suggest that exacerbated chronic hepatitis preceding seroconversion may be immunologically mediated and that the response is directed against hepatocytes replicating HBV in which core and e antigens are present (826, 830). Recently, core antigen has been shown to be preferentially expressed in association with the cell membranes of hepatocytes in chronic active hepatitis (450, 632). If viral replication is shut down by immunomodulation, or cells replicating virus are destroyed, then only cells expressing HBsAg remain. Since disease often resolves after seroconversion, cells expressing HBsAg alone do not induce further immunologically mediated cell damage under these circumstances (826-828, 830).

Although seroconversion to anti-HBs often results in the resolution of HBV replication and chronic hepatitis, spontaneous reactivation of the virus in asymptomatic anti-HBe positive chronic carriers could occur (836). Although reactivation of HBV has been documented during or following cancer chemotherapy (837-839), or by immunosuppression following transplantation operations (840, 841), a clear role for the immune system leading to spontaneous reactivation is not apparent (836). Long-term carriers of HBV who are HBeAg seronegative or anti-HBe positive could also develop cirrhosis, hypoalbuminemia and other sequelae of chronic liver disease in the absence of viral replication, suggesting that active viral replication represented an early stage in the progression to advanced liver disease (842). Further, 8 out of 19 (42%) patients with HBsAg positive liver cirrhosis developed primary hepatocellular carcinoma (PHC) while only 7.8% of patients with HBsAg negative liver cirrhosis similarly developed PHC. Again, many of those patients who were HBeAg seronegative or anti-HBe positive progressed

onto PHC within a three-year follow-up period, suggesting that active HBV replication became increasingly defective or suppressed during progression to chronic disease and liver cancer (843).

Chemical characterization.

The apparent central role of HBeAg in the life cycle of HBV has brought about widespread interest in its isolation and molecular characterization. This has prompted the development of more sensitive assays for the detection of HBeAg, including counterelectrophoresis (486), reversed passive hemagglutination (482) and solid phase radioimmunoassays (425, 426, 483-485). It became evident during early characterization that HBeAg was protein in nature (52) and that it behaved as a family of polypeptides demonstrating heterogeneity with respect to both physical and chemical properties (51, 52, 487, Table 11). Serum derived HBeAg reactive material exhibited multiple parallel precipitation lines by immunodiffusion against homologous sera (51, 52), resulting in the designation of subspecificities e_1 (269, 487), e_2 (269, 487) and e_3 (488, 489). In studying the possible clinical or pathological significance of these subspecificities, two studies found variable concentrations of e_1 and e_2 in a series of HBeAg positive sera (487, 491) while another consistently found e_1 and variably present e_2 (490). Independent observations of HBsAg positive hemodialysis carriers indicate that 95% contain e_1, while 80% of asymptomatic carriers also carry this subspecificity. The small percentage of asymptomatic carriers possessing both e_1 and e_2 (17%) also had higher titers of HBsAg, HBcAg and anti-HBc than sera from like carriers possessing e_1 specificity only. In hemodialysis patients, e_1 antigen appeared early after transfusion and did not correlate with onset of higher liver transaminase levels in the serum, while the appearance of e_2 occurred at or just before the rise of these enzyme levels, suggesting that e_2 may be of value as a diagnostic marker for viral activity in the liver (494). This data indicated that e_1 and e_2 were distinct physical molecules, and further characterization showed that they differed in size and isoelectric electric point values (424). In addition, physical heterogeneity has also been documented within the e_2 antigen subspecificity in sera from asymptomatic carriers when compared to sera from hemodialysis patients (424). Similar heterogeneity was not

Table 11. Some physical properties of HBeAg.

	Parameter measured	ref(s)	result
1.	density; (gm/ml) in CsCl	52	1.29
	of e_1	52, 427	1.3
2.	molecular weight; $(NH_4)_2SO_4$ ppted (e_{1+2})	423	2.2×10^5
	(in daltons) - e_2	424	$0.7 - 3 \times 10^5$
	- e_1	424	1.5×10^5
	- HBeAg	429	3.24×10^5
	- by Sephadex chromatography (e)	425	3.5×10^4
	- by Sepharose 4B-CL (HBeAg)	403	3.5×10^4
	- e_3 by exclusion chromatography	426	2.5×10^4
	- e_1 by Sephadex	427	3×10^5
	e_1	52	3.4×10^5
	- e_{1+2} by gel filtration	431	3.39×10^5
	- size after 3M KCNS treatment (e_1)	427	3×10^4
	- liver derived HBcAg	430, 849	3×10^4, 9×10^4
	- serum derived HBeAg	430, 849	3×10^4, 9×10^4, 2.4×10^5, 4×10^5, 5.4×10^5
	- IgG free HBeAg (e_1); gel filtration	430	3×10^4, 9×10^4
	- IgG bound HBeAg (e_{1+2}); gel filtration	430, 849	2.4×10^5, 4×10^5, 5.4×10^5
3.	sedimentation coefficient	52, 429	11.6S
4.	isoelectric point(s); (pH)		
	IgG free HBeAg (e_3)	422	4.8
		424	6.4 - 7.2
	e_1	427	4.5 - 5.0
	IgG bound HBeAg (e_{1+2})	422	5.7
		423	4.9
		424	4.5 - 8.2
	HBeAg (serum)	428	5.4
5.	electrophoretic mobility;		
	- IgG free HBeAg (e_3)	422, 430	α globulin
	- IgG bound HBeAg (e_{1+2})	422, 430	β-γ globulin
6.	solubility in $(NH_4)_2SO_4$;		
	- IgG free HBeAg (e_3) in 1.33M	422, 430	soluble
	- IgG bound HBeAg (e_{1+2}) in 1.33M	422, 430	insoluble

Table 11. (Cont.)

Parameter measured	ref(s)	result
7. Percent e subspecificities in		
- serum derived antigen;		
IgG associated	849	50% e_1, 50% e_2
IgG free, albumin associated		80% e_1, 20% e_2
- liver derived antigen	849	80% e_2, 20% e_2
Other properties;		
- reduction + alkylation in 8M urea	425	-*
- 8M urea	425	+
- 8M urea + iodoacetic acid	425	+
- 10 mM DTT (e_1)	427	±
- heat; 56°C, 30 min (e_1)	427	±
- trypsin (e_1)	427	±
- pronase (e_1)	427	±
- e_1 binding to phenyl-Sepharose (hydrophobic support)	427	yes
- pH 4.5 (HBeAg)	431	±

*+ = antigenicity intact
± = antigenicity decreased
- = antigenicity destroyed

observed, however, with e_1 (424). The study of HBeAg subspecificities in a group of hemodialyzed HBsAg carriers revealed that, in addition to e_1 and e_2, 44% of the patients had e_3 antigen subspecificity as well (489). Immunoelectrophoretic characterization of these subspecificities indicated that e_1 and e_2 behaved like beta-gamma globulins, while e_3 migrated as an alpha globulin (489, Table 11). When the distribution of these subspecificities was ascertained with regard to different categories of clinical disease, asymptomatic carriers, CAH, CPH or hemodialyzed patients positive for HBeAg were shown to possess high frequencies of e_1 antigen alone, while a substantially smaller percentage of these individuals had both e_1 and e_2, and only a few percent had all three. Although it has been reported that HBeAg positive asymptomatic carriers have higher titers of HBsAg than e antigen negative or anti-HBe positive carriers (486), patients within the other clinical categories

mentioned above show the same trend (489). Asymptomatic carriers possessing e_1 antigenic activity alone also had significantly lower titers of HBeAg than carriers also possessing additional subspecificities. Further, HBsAg positive sera containing e_1 antigen only had the fewest number of Dane particles in comparison to sera having e_1 and e_2. The largest number of Dane particles were detected in sera positive for all three subspecificities, suggesting that the presence of multiple subspecificities, especially e_3, is associated with high levels of virus replication and production of complete Dane particles (489).

While at least a portion of the heterogeneity or polymorphism of HBeAg may be due to the unique molecular nature of each subspecificity, the nature of anti-HBe or other host components binding to HBeAg might also substantially contribute. The finding of HBeAg precipitating IgM and IgG in some sera and only anti-HBe IgG in other sera (51), the presence of an IgM rheumatoid-like factor in some anti-HBe sera (492, 493) directed against e_1 only (491), and the sequential appearance of rheumatoid-like factor against e_1 followed by IgG against e_2 (491) suggest that these subspecificities elicit different types of immunological response at various times after the appearance of HBeAg. A possible pathological role for at least some of the antibodies in the anti-HBe response can be appreciated from the close association of the HBV carrier state with HBsAg negative membranous glomerulonephritis in children (495). Using affinity column purified anti-HBe for conjugation with fluorescein, HBeAg was detected on the renal glomeruli of some patients with glomerulonephritis (496). Analysis of serum derived HBeAg by SDS-PAGE revealed the presence of HBeAg and IgG polypeptides, suggesting the presence of e_2 determinants in immune complexes on renal glomeruli. The role of anti-HBe IgG in this process is also consistent with the observation that seroconversion from e antigen to e antibody is accompanied by resolution of the glomerulonephritis.

The chemical characterization of serum HBeAg has been hampered by its strong association with a variety of host components and the molecular heterogeneity resulting from such associations. The immunoelectrophoretic mobility of e antigen in early studies suggested that it shared some properties with IgG (52, 429). Independent observations

indicated that HBeAg might be an immunoglobulin of the IgG_4 subclass bearing an idiotypic determinant capable of eliciting an anti-idiotypic response (anti-HBe), suggesting that HBeAg may be made in response to HBV infection and that it blocks an effective host immune response necessary to inhibit virus proliferation (497). The presence of IgG heavy and light chains in HBeAg by SDS-PAGE, the binding of HBeAg to Sepharose CL-4B linked protein A, the immunoabsorption of e antigen to rabbit anti-human IgG, and the size estimate of HBeAg being larger than monomeric IgG collectively suggest that HBeAg may be a dimer of IgG or a determinant associated with such a dimer (429). The relationship between HBeAg and IgG was clarified by the finding of two physiochemically distinct molecular entities of e antigen in serum (422, 499). One form was soluble in 1.33 M ammonium sulfate, was smaller than monomeric IgG, migrated as an alpha globulin in electrophoresis and was not bound by a column of anti-Ig. The other form of HBeAg precipitated in 1.33 M ammonium sulfate, was larger than monomeric IgG, migrated as beta and gamma globulins in electrophoresis and was bound to an affinity column of anti-IgG. The smaller form, corresponding to e_3 antigen subspecificity above, probably corresponds to free circulating HBeAg; while the larger form, corresponding to e_1 and e_2 subspecificities above, probably represents the IgG bound immune complex of the smaller form (422). In a patient with laboratory acquired HBV infection, in five patients with acute hepatitis, and in a chimpanzee experimentally inoculated with HBV, free HBeAg appeared first, followed by a gradual shift into IgG bound forms as the appropriate specificities of anti-HBe were generated (498). The detection of e antigen in a complex of one or more molecules of IgG, then, indicates a relatively rapid seroconversion to anti-HBe, lower infectivity and generally better prognosis than detection of free e antigen. Further characterization of free HBeAg by isoelectric focusing revealed a single acidic component having a size of approximately 35,000 daltons, which yielded a single component of 17,000 daltons after analysis on SDS-gels, suggesting that serum derived e antigen may exist as a dimer (425). Reduction and alkylation of purified e antigen in the presence of urea destroyed antigenicity, while exposure to 8 M urea or alkylating reagent had no effect, suggesting that disulfide bonds are essential for reactivity and that secondary

and tertiary protein conformation, which are crucial for the integrity of HBcAg, play no major role in one or more of the e antigenic determinants (425). Analysis of e antigen reactive material from a 130,000 dalton complex by SDS-PAGE yielded polypeptides approximately 66,000 and 17,000 daltons in size (423). Although the serological specificity of the 17,000 dalton component was not assessed due to difficulties in its radiolabeling, the 66,000 dalton polypeptide bound anti-HBe, and migrated on SDS gels slightly faster than albumin and slower than the heavy chain of IgG, suggesting that it may be a multimer or precursor of a smaller e antigen component. A thermolabile, sulfhydryl-sensitive, e antigen reactive complex having a molecular weight of 300,000 daltons was isolated from appropriate sera under nondenaturing conditions (427). Exposure of this material to chaotropic ions resulted in HBeAg reactive material about 30,000 daltons in size, and when the latter was analyzed further by SDS-PAGE, a band of 17,000 daltons was resolved (427, 844). These data again suggest a dimer of e antigen polypeptide from serum, and that the molecular heterogeneity observed during purification may be due to the binding of HBeAg to various host cell or serum components. If HBeAg polypeptide really exists as a dimer in serum as suggested (427) and intact disulfide bonds are important in maintaining e antigen reactivity, then it is possible that at least one of the subspecificities is composed of amino acid sequences spanning an intermolecular disulfide bond, while the other subspecificities are present on molecular domains maintained by intramolecular disulfide bonds and/or sequences present on either side of these disulfide bonds. The differing susceptibilities of intra- and intermolecular disulfide bonds to intracellular or serum proteases could result in a loss of the disulfide bonded dimer, with simultaneous loss of one serological subspecificity and the persistence of e antigen reactive monomers in either free or IgG bound form. Further cleavage of the most susceptible intramolecular disulfide bond in the monomer might result in the loss of yet another subspecificity. In the presence of a persisting e antigen dimer possessing all of the subspecificities described above, sequential loss of e_3, e_2 and finally e_1 would inversely correlate with their frequenices in various clinical states (489) and would be consistent with the decreasing titers of complete Dane particles observed in

the absence of e_3 and the even greater effect in the absence of e_3 and e_2. Since e_3 antigen is the subspecificity most closely correlated with complete virus replication, and since e antigen is closely related to the major polypeptide of core antigen (see below), which may be a DNA and/or RNA binding protein, the integrity of the HBeAg dimer, signaled by the presence of e_3, may play a vital role in directly stimulating one or more aspects of viral replication. The destruction of the dimer, signaled by the destruction of e_3, may result in nonfunctioning or weakly functioning monomers of HBeAg. The binding of several anti-HBe monoclonal antibodies to isolated e antigen polypeptide revealed two antigenic subspecificities (437, 845). The relationship between these subspecificities, designated a and b, and the three subspecificities described above has not been clarified by these studies. Indeed, antibodies directed against e_1, e_2 or e_3 individually generate specific precipitation lines by immunodiffusion, while both anti-a and anti-b were required to form a precipitation line in the same assay. The fact that the original serological subspecificities were defined with antibodies in whole sera, while the latter analyses were carried out with monoclonal antibodies, suggests that either the subspecificities assayed in these two circumstances are different or that antibodies directed against e_1, e_2 or e_3 in whole sera really consist of a family of distinct antibodies directed against each subspecificity. The presence of two e antigen subspecificities on a single monomeric polypeptide, however, is consistent with the model outlined above.

The basis for the physiochemical heterogeneity of HBeAg has been the starting point for its further characterization. Although e antigen activity has often been found at 1.3 g/ml on density equilibrium gradients (52), a substantial amount of activity has been found at 1.15 g/ml (792, 414a, 427); and in one study e_1 antigen subspecificity has been identified at this lower density (427). These results imply that the density heterogeneity is due to the binding of a subpopulation of HBeAg molecules to lipid and that HBeAg may possess some hydrophobic character. In addition to its specific binding to anti-HBe IgM or IgG, HBeAg has also been found associated with lactic acid dehydrogenase (LDH) isoenzyme 5 (500). The finding that Dane particles and variably long filamentous forms of HBsAg are absorbed to insolubilized anti-HBe

columns, that addition of anti-HBe to these large HBsAg particles results in agglutination, and that immunization of rabbits with these particles or e antigen yields antibodies which agglutinate Dane particles and filamentous HBsAg, suggest that these forms possess exposed e antigen or e antigen-like determinants (395, 501). Small spherical HBsAg particles did not possess similar determinants when assayed by the same procedures. HBsAg particles purified from a single HBeAg positive chronic HBsAg carrier chimpanzee were tested for the presence of HBeAg before and after exposure to 1% Tween 80 (502). Intact Dane particles and variably long filamentous forms showed little or no HBeAg reactivity. However, a strong reactivity of the e_1 antigen subspecificity was detected following detergent treatment, suggesting a cryptic association of e antigen in HBsAg. Small spherical HBsAg from the same serum demonstrated similar characteristics following detergent treatment. Similar particles isolated from anti-HBe positive sera did not contain detectable HBeAg reactivity in the presence of Tween 80, suggesting that e antigen polypeptide from serum could become embedded in HBsAg particles after the latter are released from hepatocytes, or that e antigen becomes associated with HBsAg particles during surface antigen biosynthesis. The cryptic association of HBeAg with these particles is also consistent with the finding that Dane particles could not be precipitated with anti-HBe containing sera and a second antibody (503, 504). Alternatively, e antigen might not have been associated with HBsAg in these studies. Although the presence and nature of HBsAg associated e antigen requires further work to resolve the apparent discrepancy, it is possible that the exposure of cryptic determinants in damaged HBsAg particles or the presence of anti-HBe rheumatoid-like factor in some isolates may account for these differences (403). Exposure of small spherical HBsAg to 0.1% sarcosyl also resulted in the increased detection of particle associated e antigen-like reactivity (505). HBeAg-like reactivity remained associated with 20 nm diameter HBsAg through further purification steps and persisted in levels unrelated to HBsAg concentration or serum HBeAg titers, suggesting that these determinants were not acquired by simple adsorption from serum. Further detergent treatment of 20 nm diameter HBsAg particles resulted in their disintegration and release of HBeAg reactive material (502,

505). Characterization of this released material suggests the presence of polypeptides related to albumin and IgG by affinity chromatography and SDS-PAGE (502, 505). Neither study demonstrated the presence of monomeric or dimeric e antigen polypeptide by SDS-PAGE.

HBeAg activity in Dane and core particles.

The association of HBeAg with the various physical and biochemical markers of Dane particles suggests that e antigen may be part of the structure of these particles and correlate with infectivity and viral replication for that reason (401, 402, 462, 469, 471, 472, 474, 476). Early evidence, cited above (395, 501), suggested HBeAg reactive determinants on the surface of Dane particles. Further studies have failed to confirm this observation and instead suggest that HBeAg may be associated with one or more of the internal components of Dane particles (401, 503, 504). Serological testing of core particles derived from the nuclei of hepatocytes with anti-core and anti-HBe revealed only core antigenic activity. Treatment of these core particles with 2-ME and SDS resulted in a disappearance of core antigenic activity and the appearance of e-antigenic activity (404), suggesting that HBeAg reactivity exists in core particles in a cryptic form covered with HBcAg reactive determinants. Similar treatment of liver cores resulted in immunodiffusion precipitin lines that fused with a standard serum HBeAg, and further analyses indicated the presence of e_1 and e_2 subspecificities (511). When intact core particles are treated with 2.5 M CsCl at pH 7.5, HBeAg became detectable (846). Analysis of HBcAg particles so treated by CsCl sedimentation density gradient centrifugation resulted in two peaks of e antigen reactivity. A broad peak centering around density 1.38 gm/ml, corresponding to core associated HBeAg reactivity, was identified as e_2 subspecificity. The other e antigen reactive peak, centering around density 1.19 gm/ml and probably representing released e antigen reactive polypeptides, was identified as e_1 specificity. Incubation of core particles in 0.1% SDS and 0.1% 2-mercaptoethanol at 37°C for increasing time periods, resulted in the appearance of e_1 followed by the appearance of e_2, suggesting that the former is released more quickly from core particles than the latter subspecificity (846). Further, core particles depleted of e_1 were more morphologically liable than native cores, suggesting that e_1 is

essential to the structural integrity of HBcAg particles (846). Pronase treatment of core particles also resulted in the detection of e_1 and e_2 subspecificities, although in this case e_1 components were found to be immunochemically identical in all preparations tested while e_2 resulted in an immunodiffusion spur, indicating only partial identity with serum HBeAg (511). The partial identity reaction suggests that e antigen reactive polypeptides may not arise directly from the degradation of Dane particles but may be synthesized in excess and released from infected hepatocytes.

In addition to liver derived cores, HBeAg has been detected in the cores of disrupted Dane particles. An early investigation revealed HBeAg reactivity by rheophoresis following treatment of Dane particles with 0.5% Tween 80 (408). Subsequently, a Dane particle rich sera treated with 0.1% sarkosyl and 0.1% 2-ME resulted in the release of intact core particles and soluble e antigen as determined by reverse passive hemagglutination (406). Further treatment of these core particles with 1% sarkosyl resulted in the release of additional soluble HBeAg reactivity. As mentioned above, only DNA polymerase positive cores yielded soluble e antigen activity, suggesting a possible role of e antigen in the maturation of Dane particle cores. Independent observations in which Dane particles were treated with NP40 and 2-ME failed to yield HBeAg reactivity on the surface of the released cores (405). However, additional treatment of these disrupted Dane particles with pronase, followed by sucrose gradient ultracentrifugation, revealed soluble e antigen reactivity at the top of the gradient and intact core particles in the appropriate density range. Treatment of the HBcAg positive fractions with SDS and 2-ME revealed cryptic HBeAg which, upon sucrose gradient ultracentrifugation, again showed soluble e antigen activity at the top of the gradient (405). Similar results were obtained elsewhere in which immunoprecipitates of Dane particle cores with anti-HBc IgG were chromatographed on a Sepharose 4B CL gel filtration column in the presence of 3M NaSCN and the fractions assayed for surface, core and e antigens (403). A peak of intact core particles was detected at the void volume, while a small peak of HBeAg reactive material approximately 35,000 daltons in size followed some time later. Both of these studies suggest a strong association between HBeAg and

the core of Dane particles and that e antigen could be released from Dane particle cores. When Dane particles were treated with 0.1% NP40, both HBcAg and HBeAg reactivities were present, while treatment of Dane particles with 0.5-3 M guanidine-HCl yielded only HBeAg reactivity (848). The commercial assay used for HBeAg detection in NP40 treated Dane particles was blocked by anti-HBc, whereas the activity in guanidine treated Dane particles was only inhibited by anti-HBe. These results suggest that e antigen activity is associated with cores after NP40 treatment, but that it is released from core particles following treatment with guanidine (848). The finding of Dane particle associated e antigen in 8 of 45 (18%) HBsAg, anti-HBe carriers with chronic liver disease, but not in 10 carriers with normal liver histology, suggests that some anti-HBe positive carriers are potentially infectious and continue replicating virus over a long period of time. SDS-PAGE of disrupted Dane particles revealed HBeAg reactivity in a pair of polypeptides at 19,000 and 45,000 daltons, the former component being the most dominant and probably corresponding to the major or a cleavage product of the major core antigen polypeptide (405). Analysis of the HBeAg reactive material at the top of liver derived core antigen gradients by SDS-PAGE and tryptic peptide mapping has revealed a pair of major bands at 19-20,000 and 40,000 daltons having peptide maps related to the major core antigen polypeptide (792). The lower molecular weight band has a peptide map very similar to that of the polypeptide cleaved from the major core component, and the larger band (40,000 daltons) with that of a unique high molecular weight core antigen associated polypeptide of the same size (56). A number of other core related polypeptide bands were observed being released from core antigen particles. If each of these core related polypeptides possess HBeAg reactivity, they may very well add to the physiochemical heterogeneity of serum e antigen described above. Small fragments of HBcAg particles generated by exposure to NP40, 2-ME and pronase, followed by analysis on SDS-gels, resulted in the appearance of three HBeAg positive bands at 31,000, 16,500 and 15,000 daltons (407). Heating of the 31,000 dalton band resulted in a shift to 15,500 daltons, indicating dimerization of the lower molecular weight component. These results may well be analogous to those above in which a 17,000 dalton serum

derived HBeAg positive polypeptide is also often found in a dimeric state. Indeed, the same study showed that serum derived HBeAg polypeptides also consisted of a low molecular weight pair at 15,500 and 16,500 daltons, suggesting that at least some physiochemical forms of serum HBeAg may derive from the release of cleaved or uncleaved Dane particle polypeptides. The finding of serum e antigen reactive polypeptide in the molecular weight range 15-20,000 daltons by many independent investigators suggests that a component or family of core derived components in this size range (Table 12) represents the basic unit of HBeAg reactivity (407, 423, 425-431, 433). Further, the molecular heterogeneity of HBeAg, dependent upon self aggregation as well as aggregation with host components, has recently resulted in a model proposing the basis for this heterogeneity (430, Figure 8). Interestingly, SDS denatured core polypeptide from core antigen has been shown to bind serum albumin (716), demonstrating a shared property with serum derived HBeAg (849). Further, many of the polypeptide bands observed on SDS-gel analysis of HBcAg and HBeAg polypeptides fall into multiples of this basic 15-17,000 dalton e antigen polypeptide, and at least some of the differences in gel profiles obtained among studies could be the consequence of differences in aggregation of these basic components.

The presence of HBeAg reactivity in the major core polypeptide (19-22,000 daltons) suggested in the discussion above has been further substantiated by results showing that such a polypeptide could be immunoprecipitated with anti-HBe (405, 433). Injection of solubilized core polypeptides into a rabbit failed to give rise to anti-HBc in an early study (41). Although anti-HBe was not assayed for here, an independent investigation of the immunogenicity of the major core polypeptide demonstrated the presence of both anti-core and anti-HBe in comparable titers from the sera of injected rabbits (432, 437). If e antigenic determinants are located on the major core polypeptide as suggested (437), the major core polypeptide is 19-22,000 daltons in size, and an open reading frame on the sequenced genome of HBV is sought to encode such a polypeptide, then a region downstream from the termination of the surface antigen gene just beyond the nick in the long strand may be the core and e antigen encoding gene (156).

Table 12. Apparent molecular weight of polypeptides associated with e antigenic activity*.

(423)[d]	(425)[e]	(407)[d]	(426)[c]	(427)[c,e]	(428)[d]
2.2×10^5 or 1.3×10^5	3.5×10^4 daltons Sephadex chrom.	IgG bound or free	free		
					> 100
80					80
66					
					45
			21		
17[b]	17		17	17	
		16.5			
		15.5			
			6-9		

Table 12. (Cont.)

(429)[c]	(430)[d]		(792)[e,f]	(431)[c,d]	(433)[c]
3.24×10^5 daltons	liver	IgG bound serum	liver cores		IgG bound or free serum
	90				
		80			
			59		
55					
		43			
			40.5		
				38	
			30.5		
	30				
			25		
24[g]					
22					
		21			21
				20	
			19.5		
					17

*Superscripts a-e here are the same as those in Table 8, except that underscoring (b) indicates the major HBeAg associated component.

[f]Polypeptides derived from the top (d = 1.15 - 1.24 g/ml) of CsCl equilibrium density gradients used to isolate intact core particles from liver.

[g]Band size deduced from molecular weight of the 3.24×10^5 dalton complex containing a dimer of IgG.

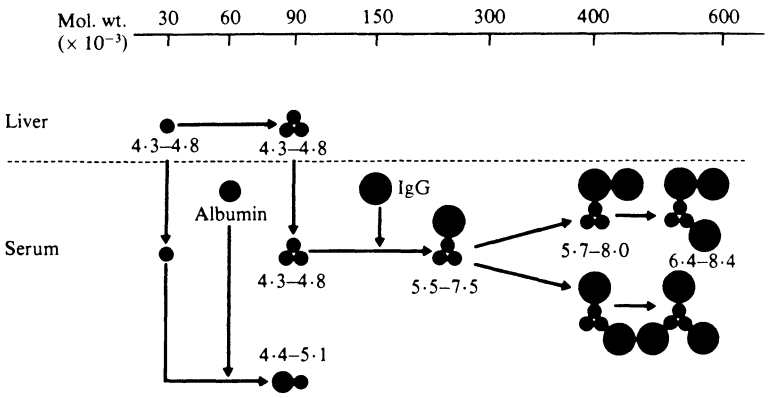


Figure 8. A hypothetical scheme to explain the heterogeneity of HBeAgs. Small circles represent HBeAg, medium circles represent albumin and large circles represent IgG. The pI ranges are indicated [Copyright 1981 by the Society for General Microbiology (ref. 430)].

Expression of the HBV fragment encompassing only the putative core polypeptide in E. coli yielded bacterial extracts positive for core antigen by immunodiffusion, proving that core antigen reactive material could be generated from this genome fragment (172). HBcAg was resistant to treatment with 0.1% pronase but sensitive to treatment with 0.1% SDS alone or SDS plus 0.1% 2-ME. Exposure of this extract to 0.1% pronase plus 0.1% 2-ME resulted in the partial breakdown of core reactivity and the appearance of HBeAg by immunodiffusion, showing that on the molecular level HBcAg could be converted to HBeAg by denaturation and proteolysis. If e antigen polypeptide is the component remaining after digestion of the protamine-like carboxy terminal portion of the major core polypeptide (172), as suggested by tryptic peptide mapping (56, 792), the release of such a component or family of components would explain the presence of soluble e antigen but not core antigen activity in the plasma of HBV carriers and patients.

Recent studies have further substantiated the relationship between polypeptides possessing core and e antigen reactivities. A panel of monoclonal antibodies raised against serum derived HBeAg polypeptide, p15.5, react with the major HBcAg component in radioimmunoassay (845). The pattern of reactivities indicates that serum and core derived

polypeptides each possess one copy of two independent determinants (845). Further, purified liver cores treated with 10% SDS, 10% 2-ME and 8 M urea prior to SDS-PAGE yielded a major polypeptide component (p21.5) in gels which reacted with anti-HBeAg/1 and anti-HBeAg/2 following transfer to nitrocellulose (846). Although these sera contained anti-HBc, blotting with e positive sera having anti-HBc only did not show reactivity. Using monoclonal antibodies directed against two different antigenic determinants on Dane particle derived cores, it was shown that intact cores and, to a much lesser extent, the isolated major core polypeptide reacted in a solid phase sandwich radioimmunoassay (847). Surprisingly, when anti-HBe or anti-HBc monoclonal antibodies were immobilized, and the antibody of heterologous specificity was used as the radiolabeled tracer, both the cleavage product of the major core polypeptide (p15.5) and serum e antigen polypeptide (p15.5) were detected (847). When both coating and tracer antibody were a monoclonal anti-HBc, these components as well as the major core component were not detected, suggesting the monomeric (single copy) nature of the determinant being assayed. The detection of these polypeptides on plates coated with polyclonal anti-HBc and radiolabeled monoclonal anti-HBe suggest that in natural infection anti-HBc of some specificities react with HBeAg reactive components (847). Further comparison of HBeAg p15.5 from serum with the similar sized major core polypeptide cleavage product by amino acid composition suggests a high degree of relatedness. Carboxy terminal sequencing of these polypeptides with carboxypeptidase A yielded a C-terminal -thr-thr-val-val from serum derived HBeAg p15.5 and -thr-thr from the cleaved major core polypeptide (847). Attempted N-terminal sequencing was unsuccessful, suggesting that these termini were blocked (716, 847). However, cleavage of the major core polypeptide or HBeAg p15.5 with formic acid, which cleaves at asp-pro linkages, resulted in a number of smaller fragments whose sizes corresponded to those predicted from the deduced amino acid sequence of HBcAg major polypeptide derived from nucleic acid sequence data (847). Combined with the data on carboxy terminal sequencing, which map to a unique position in the core gene, and the results using monoclonal anti-HBc and anti-HBe, it is likely that core and e antigens derive from the same gene. The carboxy terminus of

HBeAg p15.5 and the cleaved core polypeptide position the site of core cleavage at residues 149 and 147, respectively. This means that the carboxy terminal 34-36 residues of the major core polypeptide, containing the highly basic polyamine structure, should be missing from HBeAg p15.5 as well as the major core polypeptide cleavage product. Indeed, tryptic peptide mapping of the major core component and its cleavage fragment demonstrates that cleavage results in the removal of the highly basic tryptic peptides present in the major HBcAg polypeptide (56).

THE PROTEIN KINASE ACTIVITY IN HBcAg AND THE PROPOSED NUCLEIC ACID BINDING PROPERTIES OF CORE POLYPEPTIDE

Protein phosphorylation has become an important post-translational modification vital to the regulation of intermediate metabolism, hormone activity, neurotransmission, gene expression and a variety of other cellular processes (513, 514). Phosphorylation occurs through the action of protein kinases, which are enzymes capable of catalyzing the transfer of the gamma-phosphate of ATP (or of other nucleoside triphosphates) to the hydroxyl groups of serine and threonine residues (513, 514). Although other chemical groups in proteins could be phosphorylated (e.g., carboxy, amino or aromatic hydroxyl), their frequencies in normal cells are relatively low and their specific biological roles not firmly established. While many cellular protein kinases are characterized as being stimulated in the presence of cyclic nucleotides, a number of others are not stimulated any further under these circumstances (513, 514). Protein kinase activities copurifying with many viruses have almost uniformly been resistant to any further induction upon addition of cyclic nucleotides, indicating that these enzymes are probably cyclic nucleotide independent. The recent finding and partial characterization of a protein kinase activity in the core of HBV (54, 55, 409) has revealed a number of properties analogous to like activities associated with vaccinia (515, 517, 518), frog virus 3 (515, 519, 523), rabies (515, 520), vesicular stomatitis (515, 520-522), RNA tumor viruses (524, 525), herpes (526, 527), Sindbis (515, 528), Semliki Forest (515, 528), corona (516), as well as with other enveloped viruses (515, 529, Table 13). A strong protein kinase activity has been

Table 13. Comparison of the HBV associated protein kinase activity with those of some other animal viruses.

Virus	protein(s)	phosphorylated	exogenous acceptor	nucleic acid binding
HBV	core polypeptides	+, at multiple sites	-	+ proposed
CMV	51 K daltons	+		+, basic protein, binds SS DNA
SV40	large T, 95 K daltons, polymers	+, autophosphorylation, mult, sites	+, casein; -, IgG other host	+
	VPI	+		+, to DNA protein core
VSV	NS	+, core proteins only	+	
Rabies	N-protein in nucleocapsids, virions	+	-	+, binds tightly to nucleocapsid
Frog viruses	virus proteins	+	+, histones +, phosvitin +, casein	
Ad	72 K daltons	+		+, SS DNA only

Table 13. (Cont.)

cyclic nuct. stimulation	phosphorylated amino acid in vivo	phosphorylated amino acid in vitro	nature of phosphorylated proteins	nonionic detergent stimulation	cellular location
-		ser, thr	structural, nucleocapsid	+	nucleus
			first of late nonstructural proteins		nucleus
-	ser, thr	ser, thr	early, nonstructural	NA	nucleus
			late, structural	NA	cytoplasm, virion
	ser, thr differs in sensitivity to alkaline phosphatase		subset of structural proteins	+	nucleus
	ser, thr localized on one end of N-protein	ser, thr	structural, nucleocapsid	+	
-		ser, thr	structural proteins of virion	+	
-	ser	ser	early, nonstructural	NA	nucleus

Table 13. (Cont.)

proposed functions
?
protection, replication, and packaging of viral DNA
initiate DNA synthesis, regulation of transcription, transformation, helper function, DNA binding varies with phosphorylation
transcription regulation via differential phosphorylation
maturation; kinase probably host in origin; kinase activity also found in virion envelope
maturation; N-protein may be inactive protranscriptase--activated by phosphorylation and/or cleavage
maturation; kinase apparently of virus origin--phosphorylates host and virus proteins
DNA replication; down modulation of early proteins; ts mutant affects the adeno associated virus maturation on level of translation regulation

Table 13. (Cont.)

Virus	protein(s)	phosphorylated	exogenous acceptor	nucleic acid binding
Rauscher MuLV	gag 12 K daltons	+, heterogeneously phosphorylated		+, to viral genome
AMV	p 15 K daltons phosphorylated polymerase	+, p 15 is an acidic phospho-protein		+

Table 13. (Cont.)

cyclic nuct. stimulation	phosphorylated amino acid in vivo	phosphorylated amino acid in vitro	nature of phosphorylated proteins	nonionic detergent stimulation	cellular location
	ser	ser	early, structural		nucleus
-			reverse transcriptase		

Table 13. (Cont.)

proposed functions
transcription regulation; increased phosphorylation of p12 = decreased binding to RNA genome; kinase(s) may be cellular in origin
increase reverse transcriptase activity; affects replication and integration; kinase(s) may be cellular in origin

(See text for appropriate references)

HBV: hepatitis B virus (54, 55, 409)
CMV: cytomegalovirus (584-586)
VSV: vesicular stomatitis virus (520-522, 529, 554, 558-561)
AMV: avian myeloblastosis virus (529)
Ad: adenoviruses (577, 579-583)
SV40: Simian virus 40 (534, 566-578, 582a)
MuLV: murine leukemia virus (525, 529, 563, 564)
rabies (520, 584)
frog viruses (519, 523, 529, 553)
NA: not applicable

demonstrated in both serum derived Dane particles (54) and liver derived core particles (54, 55, 409). This activity has been shown to remain associated with liver cores even after extensive purification, indicating an intimate association with HBcAg (409). Treatment of intact, phoshorylated cores with excess alkaline phosphatase resulted in no loss of ^{32}P, suggesting that the substrates of the kinase activity, as well as the activity itself, reside within the core particles (409). In contrast, a substantial portion of the ^{32}P label was removed by excess alkaline phosphatase following treatment of the cores with 2% SDS at 100°C (409). Analysis of protein kinase activity in Dane particles purified by density gradient ultracentrifugation in CsCl showed the presence of kinase and polymerase activities in the same fractions (54). However, similar analysis of these activities and HBcAg reactivity in gradients containing liver derived core particles showed that most of the kinase activity was associated with light cores, while only a small fraction remained associated with DNA polymerase positive heavy cores (55, 409). Steric hindrance, present in heavy cores containing DNA, and absent in light cores lacking DNA, may account for the apparent inverse relationship between the presence of kinase activity and DNA (409). However, a number of studies discussed above have already demonstrated both morphological disintegration and antigenic conversion of heavy core particles during the course of purification (406, 414a). Further, CsCl density equilibrium centrifugation of liver derived core particles as a first step in purification resulted in comigrating polymerase and kinase peaks, while rebanding the same core particles in later steps of purification resulted in a shifting of kinase activity to polymerase negative core particles at a lighter density (55).

SDS-PAGE of the protein kinase reaction products showed the major core polypeptide of Dane particles and liver derived cores to be the major phosphorylated component (54, 55, 409). Liver derived GSHcAg, which also possesses a copurifying protein kinase activity, also yields an SDS-gel pattern in which the major core polypeptide is the major but not only phosphylated component (55). In one study with liver derived HBcAg, the major core polypeptide was the only component phosphorylated (409), while in another study, additional bands at 68,000, 53,000,

48,000, 40,000, 34,500, 30,500 and 27,500 daltons, in addition to several bands smaller than the major core polypeptide, were observed (55). Although a number of these high molecular weight bands may be aggregates, the results from peptide mapping of ^{125}I-labeled core polypeptides showed that p27.5 and p40 were unique high molecular weight core components and that p40 was often the most dominant of the high molecular weight bands. Indeed, phosphorylated p40 was the most intense of the higher molecular weight bands larger than the major core polypeptide (55). Independent analysis of Dane particle phosphorylated polypeptides resulted in larger bands at 63,000, 52,000, 48,000, 43,000 and 38,000 daltons; the latter band often being more prominent than the others (54). Identical analysis of phosphorylated liver derived core particles, in contrast to Dane particles, showed a single band in the position of the major core polypeptide without any of the higher molecular weight bands (54), in agreement with some results (409) but not others (55). The discrepancy may lie in the fact that p27.5 and p40 are not disulfide bonded to the core particle (792) and that they may be retained or lost in liver cores depending upon the protocol used for HBcAg purification. Further, their retention in Dane particles and cores immediately released from Dane particles suggests that the Dane particle envelope is important in retaining these high molecular weight minor polypeptides so that the full structural and/or functional integrity of the Dane particle is maintained.

SDS-PAGE and Coomassie blue staining of liver derived HBcAg yielded a major polypeptide component at 19,700 daltons prior to the protein kinase reaction (54). Identical analysis of core polypeptides after the protein kinase reaction revealed some remaining 19,700 dalton component and the appearance of two new polypeptides at 20,600 and 14,700 daltons. The failure to completely convert the 19,700 dalton component to the slower migrating 20,600 dalton component even under conditions of extensive phosphorylation suggests that only a fraction of the former is phosphorylated during the protein kinase reaction (54). Measurement of the kinetics and extent of phosphorylation showed an initial rapid phase of ^{32}P incorporation followed by a much slower rate of incorporation which did not plateau after several hours, suggesting that one group of receptor sites is accessible to the

enzyme while another group is much less so or that there are two protein kinase activities, or both (409). Calculation of the amount of ^{32}P covalently attached to a known quantity of core antigen suggests that only one core polypeptide in several hundred becomes phosphorylated (409). Based upon the size, specific gravity and weight of a core particle, and taking into account the weight of the enclosed DNA and DNA polymerase, the approximate number of core polypeptides per intact core particle is about 300 (437). If this is so, then a core particle would be expected to be phosphorylated at approximately one site by a single phosphate molecule in the in vitro reaction. Phosphopeptide mapping, however, reveals multiple sites of phosphorylation in both the major polypeptides of human and ground squirrel core antigens (55). Virtually all of the points of phosphorylation were conserved in labeled polypeptides both larger and smaller in size than the major phosphoprotein of each virus. Further, many of the peptides seen on the maps of the major HBcAg polypeptide were also observed in the major GSHcAg polypeptide, indicating that many of the same sites are phosphorylated in both core particles (55). However, it is probable that individual core particles are phosphorylated at different conserved sites within multiple polypeptides and that further analysis of a population of phosphorylated cores would yield a group of heterogeneously phosphorylated polypeptides. The amino acids phosphorylated within these conserved regions of polypeptide are of some interest since many tumorigenic viruses possessing a protein kinase often phosphorylate tyrosyl residues (530-535) and since there is considerable evidence linking HBV with primary hepatocellular carcinoma (89-91, 94, 95, 102, 122, 536-538). Partial hydrolysis of the product(s) of the protein kinase reaction showed that serine was the major phosphorylated amino acid (55, 409). In one study, threonine was also found phosphorylated to a minor extent in both human and ground squirrel liver derived core particles (55). Whether the low levels and restricted specificity of phosphorylation reflect enzyme specificity and/or substrate availability is not known. The origin of this activity, be it viral or host, is also unknown. Animal viruses encoded protein kinase enzymes have been demonstrated in vaccinia (554, 555) and frog 3 viruses (519, 553) but not in any of the other viruses mentioned above.

Phosphorylation of viral proteins often affects their nucleic acid binding characteristics, and whether this is the case with core antigen related polypeptides or other components of the core is not known. It has not been demonstrated that the substrate specificity of the protein kinase reaction in vitro is the same as that in vivo because there is no suitable tissue culture system for HBV, nor has it been ascertained whether the core particles obtained from liver or Dane particles are phosphorylated prior to the in vitro reaction. Finally, it has recently been proposed that the large open reading frame in the HBV genome which covers all of the surface antigen and some of the core antigen but in a different reading frame (536a), potentially encodes a 90,000 dalton polypeptide possessing polymerase, kinase and nucleic acid binding capabilities (409).

Comparison of the HBV and GSHV protein kinase activities with those in other animal viruses suggest possible functional role for the phosphorylated polypeptides of these hepatitis viruses (Table 13). The phosphorylation of a subset of nucleocapsid structural proteins has been recently described in vesicular stomatitis virus (VSV), and an increase in phosphorylation of the NS protein, a minor nucleocapsid component which is the major phosphorylated product of the kinase reaction, seems to be correlated with an increase in the rate of transcription in the VSV ribonucleoprotein core complex (554a, 558). Only the most highly modified forms of NS, phosphorylated at both serine and threonine residues, in combination with a minor nucleocapsid structural protein L and the major nucleocapsid structural protein N, reconstitute in vitro transcription (554a, 559, 560). Additionally, it has been proposed that the extent of NS protein phosphorylation might regulate the switch between transcription and replication (561). In rabies virus, the major nucleocapsid species, or N-protein, is also the major phosphorylated component following the protein kinase reaction. It has been postulated to be an inactive transcriptase (proenzyme) which could be activated by phosphorylation or proteolytic cleavage, or both, after uncoating (520). Further, the findings in Rous sarcoma virus (RSV) (556) and in avian myeloblastosis virus (AMV) (562) of an RNA dependent DNA polymerase (reverse transcriptase) activated by phosphorylation, suggest that one of the consequences of phosphorylation may be an

alteration in the endogenous DNA polymerase activity or reverse transcriptase-like activity (188, 189) associated with all or at least one of the hepadna viruses, respectively. The separation of protein kinase positive from DNA polymerase positive liver derived cores during the course of HBcAg and GSHcAg purification in CsCl density equilibrium gradients suggests that in vitro protein phosphorylation is not required for the expression of DNA polymerase activity and that the polymerase enzyme is probably not phosphorylated in this kinase assay. The localization of most radioactive phosphate in the cleaved 14,700 dalton component from the major HBcAg polypeptide, and relatively little in the 6000 dalton component (54), suggests that phosphorylation occurs nonuniformly along the length of the major core associated polypeptide. Peptide mapping of the larger cleaved, ^{125}I labeled polypeptide showed that virtually all of the highly charged spots observed on the map of the major core component were absent (56). Since the nucleic acid sequence proposed to encode core polypeptide would yield a major component having a protamine-like structure near its carboxy terminus, most of the phosphorylated sites are probably localized on the amino terminal portion of the major core polypeptide. These results are consistent with those observed in rabies virus, in which phosphorylation has been found localized to a small fragment of the N-polypeptide. The limited number and distribution of phosphorylated sites in rabies, HBV and GSHV suggests that phosphorylation occurs following assembly of the nucleocapsids in each case. In hepatitis B virus liver derived core particles there does not seem to be a relationship between in vitro phosphorylation and cleavage, since cleavage products of the major core polypeptide possessing e antigen activity have been found as free polypeptides at the top of CsCl density equilibrium gradients during core purification (414a, 792). Whether cleavage precedes phosphorylation or has an effect upon it or DNA polymerase activity are still unanswered questions.

An important relationship exists between the state of nucleocapsid polypeptide phosphorylation and nucleic acid binding with a number of viruses. This property need not be considered mutually exclusive from the changes in enzymatic properties discussed above, since the regulation of DNA or RNA synthesis may be partially dependent upon nucleic

acid binding. For example, the p12^{gag} of Rauscher murine leukemia virus, which is a structural protein residing in the nucleocapsid of the virus, is heterogeneously phosphorylated at serine residues (563, 564). Phosphorylation of this protein has been shown to be inversely proportional to its RNA genome binding capability. Hypophosphorylated p12 binds most tightly to the virus genome, while more highly phosphorylated species bind with the same specificity but to a lesser extent (564). A phosphorylated subpopulation of VP1 from SV40 or polyoma has been shown to bind tightly to their respective nucleoprotein cores (565, 566). Large T antigen of SV40, although an early, nonstructural protein, is heterogeneously phosphorylated and also varies in its nucleic acid binding characteristics dependent, in part, upon phosphorylation state (534, 567-574, 582a). Other viruses possessing an associated protein kinase activity yielding phosphoproteins whose state of phosphorylation is related to their nucleic acid binding properties are listed in Table 13. The phosphorylation of core polypeptide and the suggested localization of most phosphorylated sites away from the carboxy terminal portion of the molecule suggest at least two types of nucleic acid binding. The carboxy terminal basic sequences would bind the nucleic acid genome in the core particle nonspecifically in a manner analogous to the way tobacco mosaic virus coat proteins coat the genome of that virus. The amino terminal portion of the molecule, being heterogeneously phosphorylated at one or more sites, may bind one or more regions of the genome specifically. Since the highly basic amino acid sequences in core polypeptide are absent in its cleaved component, since the cleaved component carries most of the phosphate attached in the protein kinase reaction, and since this polypeptide has e antigen activity (792), it is possible that the generation of e may provide a free polypeptide capable of specific nucleic acid binding properties in the Dane particle and/or nucleus of the infected hepatocytes responsible for, at least in part, the regulation of virus gene expression. As discussed above, e antigen isolated from blood is an acidic polypeptide approximately 17,000 daltons in size (425). An acidic polypeptide in this size range is also obtained if the cleavage of the major core polypeptide is predicted to take place just amino terminal from the protamine-like basic sequence in the carboxy terminal

35 amino acid residues (358). Further, the apparent association of e antigen polypeptide with itself to form a dimer or higher multimers (425) and its association with many other host components is somewhat analogous to the situation with SV40 large T antigen, whose nucleic acid binding properties depend upon its state of phosphorylation, aggregation (567, 570, 572, 574), and tight binding to at least one host cell component (575-577). The correlation of serum e antigen with high DNA polymerase and high concentrations of both Dane particles and HBsAg is consistent with a specific role for e antigen polypeptide in HBV replication. Indeed, several adenoviruses (Ad) encode a 72,000 dalton protein which could be phosphorylated and bind to single-stranded DNA (580, 582, 583). A temperature sensitive mutant of Ad5, H5ts125, synthesizes a 72,000 dalton polypeptide that does not bind the genome at restrictive temperatures, which effectively inhibits initiation of new rounds of DNA replication (529, 581-583, 587, 588). A single-stranded DNA binding protein has also been isolated from human cytomegalovirus infected cells (585). It is 51,000 daltons in size, phosphorylated, and found in the nuclei of infected cells shortly after infection (585, 586). The Epstein-Barr virus associated nuclear antigens (EBNA) are a small family of phosphorylated peptides possessing chromatin and nucleic acid binding properties (529, 589-592). Similarly, a group of herpes simplex virus (HSV) related antigens bind chromatin or synthetic poly dA - poly dT, reducing the melting temperature of the latter helix (529, 598, 594), and suggesting that a possible function of these polypeptides is unwindase-like activity necessary for initiation of virus replication. The binding of phage T4 gene 32 product at the replication fork of DNA is not only responsible for local unwinding of the single-stranded template, but has also been found to stimulate the T4 DNA polymerase (529, 595). Three major DNA unwinding proteins isolated from normal calf thymus also stimulate homologous DNA polymerase alpha activity, again suggesting an important role in the initiation of DNA replication (529, 595a, 596, 597). Whether native or cleaved core antigen polypeptide(s) play an analogous role in the initiation of virus replication is not known. Despite the lack of a suitable tissue culture system, some of the in vitro approaches employed in the cases mentioned above in conjunction with recombinant DNA technology can be used to approach some of these questions.

THE DNA POLYMERASE ACTIVITY OF HBV

Characterization and relation to other polymerases.

Pellets made from sera highly positive for surface antigen were found to contain a weak endogenous DNA synthesizing activity (DNAp) by incorporation of tritiated precursors into acid insoluble radioactivity. In contrast, an RNA synthesizing activity was not detected in these same preparations as measured by lack of tritiated UTP incorporation (50). Some of the properties of HBV associated DNA polymerase in comparison to other polymerases are listed in Table 14. The sensitivity of the endogenous reaction product to deoxyribonuclease (DNase), but not ribonuclease (RNase), suggested that the product of the polymerase activity was DNA. However, predigestion of the pellets with RNase abolished incorporation of tritiated thymidine, suggesting DNA synthesis from an RNA template. Addition of the synthetic nucleic acid polymer rA • dT, which stimulates oncornavirus reverse transcriptase activities (598), did not enhance HBV associated DNAp; nor did the addition of N-dimethyl-rifampicin or 4-N-benzyldimethyl-rifampicin, which strongly inhibit reverse transcriptase activities, have any effect upon HBV DNAp (541, 577, 599). This endogenous DNA polymerase activity did not directly correlate with HBsAg complement fixation titer, with HBsAg subtype (48), nor with highly purified small spherical or variably long HBsAg particles (48, 600, 601). Sucrose gradient sedimentation of crude serum pellets followed by DNAp assay and electron microscopic examination of the fractions revealed polymerase activity associated with Dane particles (48). Similar pellets pretreated with NP40 and mercaptoethanol resulted in a peak of polymerase activity in gradients comigrating exclusively with Dane particle derived cores (48). Treatment of these particles with DNase or RNase did not alter incorporation of tritiated thymidine, nor did addition of exogenous templates capable of stimulating reverse transcriptase activities enhance DNAp, suggesting that the polymerase activity and product of that activity were sequestered inside core particles (48). Further evidence for this tight association was demonstrated by immunoprecipitation of polymerase activity with anti-HBs (and not with anti-HBc) prior to NP40 treatment of Dane particles and immunoprecipitation with anti-core (and not with anti-HBs) after such treatment (49).

Table 14. Comparison of HBV associated endogenous DNA polymerase activity with other polymerases

classification	hepadnaviruses	cellular[550a]	cellular[550]	cellular/mitochondrial[529,550]	retroviruses
examples	HBV, HBV-like				leukemia, sarcoma viruses
polymerase	DNA dependent DNAp[48-50,557]	α	β	γ	reverse transcriptase
proposed function	DNA repair/ replication	replication	repair[529]	replication-mt DNA	replication, provirus formation
size (K daltons)	90?	120-220[551,552]	30-50[551,552]	150-300[551,552]	70-160
subunits	?	50-70, 155[529]			α 65-69, β 105-110
location	virion core[48,49]	cytoplasm, nucleus[551,552]	nucleus[551, 552]	cytoplasmic + nuclear membrane[552]	virion core
pH optimum	7.5[48]	7.2	8.5	8.0[529]	7.5-8.5
4 dNTPs	required	required	required	required	required
ara-ATP	- [549]	-	∓	∓ [529]	
ara-CTP	- [549]	- [529]	∓	∓ [529]	
DTT	+[48,540]	+			
p-hydroxymercuribenzoate	- [540]	-	∓	-	-
N-ethylmaleimide	- [540]	- [551,552]	∓, 0[551,552]	- [551,552]	-

Table 14. (Cont.)

Mn^{++}	0^{540}, $+^{48}$		+	+	+
Mg^{++}	$+^{48}$	+	+	+	+
monovalent cations + Mg^{++}	$+^{48}$	- 551	+551	+551	
ethidium bromide	- 541,544,547	- 529	∓ 529	-	-
rifampicin	0^{48}				0
phosphonoformate	- 545, 546, 885	∓ 886	∓ 886	∓ 887	-885
phosphonoacetate	$0^{545,546}$		∓ 529		
d(AT)[b]	- 48				+
poly rA·oligo dT[b]	0^{48}	± 552	+552	+552	+
DNA, activated[b]	0^{48}	+552	+552	+552	+
endonuclease act.	none	none[529]	none[529]		
exonuclease act.	none	none[529]	none[529]		RNase H

[a]Reference number above classification term indicates the origin of virtually all the data in the column. Additional references are located near the individual pieces of data to which they refer.

[b]It should be noted that in assays where exogenous templates are added, the activity measured is for soluble enzymes with the exception of that within HBV core particles.

+ = stimulation of enzyme activity
0 = no effect
- = inhibition
± = weak stimulation
∓ = weak inhibition

Pronase digestion, phenol extraction and analysis of the radioactive nucleic acid product by density equilibrium centrifugation suggested that the product labeled was DNA. Further, the extracted product was DNase sensitive, RNase resistant, and alkali stable (48). The finding of DNAp activity in a subpopulation of liver or serum derived cores suggests that a frequent majority of the Dane particles in any given isolate lack the DNA genome and may behave as defective interfering particles. Further, a large proportion of incomplete Dane particles may release soluble polymerase as well as other internal core components into the bloodstream of infected patients. Indeed, an explanation for the apparent RNase sensitivity of the polymerase activity in crude serum pellets and the stimulation of such an activity by the addition of poly d AT (50) might be related to a polymerase activity derived from disrupted Dane particles or a host contaminant copurifying with the crude pellets. One study has shown that sera high in DNA polymerase activity can be stimulated by an exogenous DNA primer, inferring the presence of a soluble enzyme in some hepatitis B and non-B hepatitis patients, and that a number of sera containing antibody to HBeAg inhibit this activity (539). Preliminary characterization of the serum component involved indicates that it may be antibody (539). Independent observations have also documented the presence of antibodies to a DNA polymerase in acute hepatitis B (604). Another study has described the presence of a Dane particle associated DNA polymerase activity activated by calf thymus DNA and NP40 which precipitated upon addition of anti-HBs to crude surface antigen positive pellets and whose reaction product in intact Dane particles was DNase resistant. Since the activity was not found in purified core particles, it is likely to be a copurifying component located between the nucleocapsid and envelope of Dane particles (398). Antibodies directed against single-stranded DNA have also been detected in the sera of patients with chronic hepatitis (602). Further characterization of the HBV related polypeptides will undoubtedly reveal other antigen-antibody systems derived from the internal components of the core particles.

A soluble DNA polymerase activity has been isolated and partially characterized from a number of HBsAg positive sera or plasma (603). Interestingly, this activity was best detected only upon several fold

dilution of sera, suggesting that reversible inhibitors may have prevented detection in earlier attempts. Soluble polymerase activity was detected in approximately one third of randomly chosen HBsAg containing sera. No correlation was seen between the appearance of soluble and particle associated activities, nor between the appearance of soluble activity and other markers of HBV. Purification and molecular characterization of this activity yielded a molecule differing substantially from the endogenous DNA polymerase associated with HBV. The pH optimum of the soluble enzyme is 9.2, while that for the endogenous activity is 7.5. The optimum Mg^{++} concentration for activity varied greatly; the soluble enzyme being inhibited in high salt while the endogenous counterpart is stimulated in high salt (398, 540, 557, 603). The necessity of sulfhydryl groups for activity of the endogenous enzyme is without question; yet the soluble polymerase is not inhibited by treatment with reagents which block sulfhydryl groups. In addition, the approximate molecular weight of the soluble enzyme exceeds the coding capacity of any open reading frame in HBV DNA and the capacity of the entire genome, unless splicing of the genetic information derived from two different reading frames is translated into a single polypeptide. Although the soluble polymerase differs substantially in its characteristics compared to the common mammalian DNA polymerases (550-552, 603), repeated failures in attempted solubilization of the endogenous DNAp activity associated with Dane particle cores has made direct comparison impossible.

Further characterization of the HBV associated endogenous DNA polymerase activity and its relationship to other DNA polymerases has been carried out using various reagents which stimulate or inhibit known polymerase activities (Table 14). The requirement of the endogenous reaction for the four deoxyribonucleoside triphosphates and its inhibition by treatment with daunomycin or actinomycin D, both of which inhibit DNA dependent DNA or RNA synthesis, suggest that the endogenous activity is one of DNA synthesis on a DNA template (48, 189, 541). By synthesizing radiolabeled DNA in an extensive DNAp assay, it was shown that between 25% and 50% of the Dane particle genome was copied in this reaction, as determined by the amount of acid insoluble radioactivity or complexity measurements (Cot $\frac{1}{2}$) of the newly synthesized DNA from

different experiments (605). The similar degrees of complexity found in the newly synthesized DNA (605), the finding of radioactive restriction fragments corresponding to a similarly limited portion of the genome following the endogenous polymerase reaction (47), as well as the conversion of the partially single-stranded to mostly double-stranded circular molecules following the polymerase reaction--as assayed by electron microscopy (606) and gel electrophoresis (607)--suggest that the DNAp activity is responsible for repair synthesis in which the genome is made fully double stranded.

Unlike most characterized DNA polymerase activities, the endogenous DNAp associated with Dane particles is stimulated by much higher concentrations of monovalent cations than other polymerases (603) in the presence of magnesium ions (Mg^{++}), suggesting that in high salt an assay specific for HBV could be conducted in the presence of contaminating polymerases (540, 608). In the presence of NP40 concentrations up to 10% at constant ionic strength, the polymerase activity remained activated at a stable, higher level than in the absence of detergent (398). Measuring the differences in enzyme activation in the presence of increasing concentrations of potassium chloride (KCl) with or without NP40 showed that at low KCl concentrations a difference existed which disappeared as the monovalent cation concentration increased. Alternatively, in the absence of monovalent cations, the polymerase activity showed a 110% increase in the presence of NP40 compared to the activity measured in the absence of detergent. Addition of increasing KCl to the assay decreased the percentage of increased activity, but the differences seen in the presence or absence of detergent at high KCl concentrations were still large, indicating a different mode of polymerase activation. In separate experiments, the presence of NP40 resulted in stimulation of enzyme activity 280% which was decreased to relatively low levels at high KCl concentrations. These three different patterns of polymerase activation may be due to differences in the permeability of the Dane particle coat, since the process by which nonionic detergents activate viral polymerases likely involves permitting access of deoxynucleoside triphosphates and other reactants to the core protected DNA and DNA polymerase (398). However, if the polymerase enzyme is heterogeneously phosphorylated, perhaps by <u>in vivo</u>

protein kinase activity, distinct activation patterns of polymerase activity may have a structural basis. Although the in vitro phosphorylation of reverse transcriptase in some oncornaviruses correlates with the level of polymerase activity (556, 562), any role that phosphorylation or other post-translational modification(s) play in the differential stimulation patterns of the endogenous DNAp activity of Dane particles remains to be elucidated.

While the DNAp activity in Dane particle cores is stimulated by the presence of high concentrations of monovalent cations and Mg^{++}, activity is inhibited upon addition of various divalent cations either in the presence or absence of Mg^{++} (540), suggesting that other divalent cations do not substitute for Mg^{++} and that they may compete with Mg^{++} (or bind to an allosteric site) in the polymerase molecule. The failure of zinc chloride to stimulate the reaction (54), as well as the failure of the chelating agent 1, 10 phenanthroline [an inhibitor of many reverse transcriptases which are zinc containing metalloenzymes (609, 610)] to inhibit endogenous DNAp activity, suggest that Zn^{++} plays no role. The association of zinc with terminal deoxynucleotidyl transferase (611) and a number of DNA polymerases (612) demonstrate similar differences between these enzymes and the endogenous DNAp in HBV. Other inhibitors of the endogenous activity include calcium elenolate (543), a potent inhibitor of reverse transcriptases (613); heating at 60°C for 1 hour, treatment with sodium hypochlorite, ethanol, diethyl ether, or concentrations of SDS greater than 0.1% (548). Inactivation by ethanol and sodium hypochlorite increased with increasing concentrations of these compounds while heat inactivation was time dependent and showed two inactivation rates. Seventy-five percent of the DNA polymerase activity was lost in 1 hour at 60°C ($t_{\frac{1}{2}}$ = 11 minutes), while the remainder of the activity was lost more slowly in continued incubation at the same temperature ($t_{\frac{1}{2}}$ = 100 minutes). Whether this heterogeneity in stability to heating is a consequence of differences in one or more post-translational modifications or due to the presence of two HBV associated polymerases, as discussed above, awaits further study into the molecular basis of the polymerase enzyme.

Chemotherapy.

The production of almost fully double-stranded DNA by the polymerase reaction in Dane particles is thought to be the first step

in replication of the virus genome (189). Several studies have been directed toward using a series of drugs, some in clinical use, to determine whether they could inhibit DNAp activity and thereby curb ongoing virus replication. Of the intercalating agents used, ethidium bromide was the strongest inhibitor of DNA polymerase activity in vitro (541, 544, 547, Table 14). Quinine, chloroquine, quinacrine, primaquine, hydroxychloroquine, chloropromazine and methylene blue alone or in various combinations resulted in different degrees of DNAp inhibition (544, 547). In general, the extent of inhibition in drug combinations was usually not greater than the inhibition shown by the more active drug alone. In a few combinations where inhibition was increased, the increase was modest. The accumulation of at least some of these intercalating agents in the liver to very high concentrations similar to those resulting in substantial inhibition of DNAp activity in vitro, combined with the known property of these agents to eliminate bacterial plasmids (614, 615), might effectively "cure" hepatocytes containing episomal or replicative forms of HBV DNA (544). However, their effectiveness in patients and toxicity at effective levels have not been demonstrated. Another group of compounds which include phosphonoacetic acid (PAA) and phosphonoformic acid (PFA) are pyrophosphate analogous that manifest antiviral activity by inhibiting viral polymerases at the pyrophosphate binding site and effectively prevent chain elongation even after initiation (529, 616-618). PAA is effective against replication of a number of herpesviruses (529, 616, 618) at concentrations having relatively little inhibitory effect on cellular DNA polymerases (529). When these compounds were tested in vitro with HBV, it was found that PAA had little inhibitory effect while PFA markedly inhibited the activity (545, 546, 618). PFA inhibition seemed much more selective for the HBV DNA polymerase activity when compared to cellular DNA polymerases (885-887). Although both PAA and PFA inhibited the polymerase activities of several viruses, only Dane particle and retroviral polymerases were inhibited by PFA alone (885). Synthesis of pyrophosphate analogs then defined the structural requirements for PFA inhibition (885). The fact that WHV DNA polymerase activity was also inhibited by PFA in vitro suggested their use in WHV infected woodchucks (883). However, administration of PFA to chronically infected

woodchucks had no effect upon the *in vivo* levels of DNA polymerase activity even after two weeks of treatment (883). The inhibitor has most recently been used to specifically assay for HBV DNA polymerase activity *in vitro* in 0.4 M KCl in the presence of a variety of other similar activities (884). Treatment of purified Dane particles with 9-β-D arabinofuranosyladenine (ara-A), a carcinostatic drug active against HSV (619, 620), varicella, vaccinia and many oncogenic RNA viruses (529), inhibited DNAp activity only at high concentrations (549). A similar compound, 1-β-D arabinofuranosylcytosine (ara-C), did not inhibit the reaction. The triphosphates of these drugs, ara-ATP and ara-CTP, each inhibited the *in vitro* polymerase assay competitively with respect to their appropriate deoxynucleoside triphosphates (549). Inhibition was also noted when the triphosphates were added after initiation of the DNAp reaction, and was reversible, indicating that ara-ATP or ara-CTP did not become incorporated into the DNA as chain terminators (549). Studies with uninfected mammalian cells and HSV infected cells have shown that ara-A is converted intracellularly to ara-ATP which binds much more strongly to HSV DNA dependent DNA polymerase than to cellular polymerases with similar activities (529). Interestingly, the analog can become stably incorporated into cellular DNA but tends to inhibit HSV replication by premature termination (529). *In vivo* administration of ara-A or ara-AMP to a number of patients having chronic hepatitis with detectable serum DNAp and HBeAg values reduced and eliminated these serum markers in some patients but not others, suggesting that under certain circumstances viral replication is inhibited (621-623). In another study, six patients treated with ara-A yielded only one which became permanently DNAp negative, and only four of 16 patients treated with human leukocyte interferon alone (a well-known antiviral drug) responded likewise, while those treated with both resulted in a much larger proportion of the group becoming DNAp negative (623). Inoculation of post-treatment sera into susceptible chimpanzees from patients who permanently lost DNAp activity as a consequence of ara-A and/or interferon chemotherapy was proven to be noninfectious, indicating the value of these treatments in at least some patients (624, 625).

The effects of corticosteroid treatment upon viral replication has also been examined, since it has been used in the treatment of chronic hepatitis (626, 627). Discontinuation of immunosuppressive therapy, however, has resulted in the reduction or loss of DNAp or HBeAg from the serum, suggesting that immunosuppression often potentiates viral replication (628). Discontinued immunosuppression has also resulted in seroconversion from HBeAg to anti-HBe in two patients (629), and similar results, including reduction of HBsAg titer, have also been reported (630a, 631). Further, patients undergoing steroid treatment do not respond well to concurrent chemotherapy with ara-A and/or interferon, suggesting that at least part of the effects of chemotherapy may be to restore the cellular immune responses suppressed by chronic HBV infection (623). If this is so, then the state of immunological responsiveness may modulate virus gene expression at the surface of infected cells and thereby alter replication. Interestingly, T lymphocytes from the blood of chronic hepatitis B cases seem to demonstrate core antigen reactivity in a microcytotoxicity assay using autologous hepatocytes, since the cytotoxicity could be blocked with anti-HBc but not anti-HBs (632). The net levels of HBV replication in chronic hepatitis may be a balance of anti-HBc binding (449-451) which may promote replication by blocking T cell cytotoxicity and/or by direct modulation of gene expression, as in HSV (630).

HBV GENOME STRUCTURE AND EXPRESSION

General physical characteristics.

Characterization of the HBV associated DNA polymerase activity and the nucleic acid product before and after the reaction resulted in the finding of a small circular DNA molecule which served as both primer and template (46, Figures 9 and 10). The ability of AMV reverse transcriptase to successfully introduce radioactive nucleotides into isolated Dane particle DNA suggests the presence of a single-stranded region, since the added DNA polymerase lacks the exonuclease activity required to introduce radioactive precursors into a fully double-stranded DNA molecule (47). Electron microscopy showed that the circular molecules before the DNAp reaction were about 0.8 μm in length which corresponds to a molecular weight of 1.6×10^6 daltons or 2300

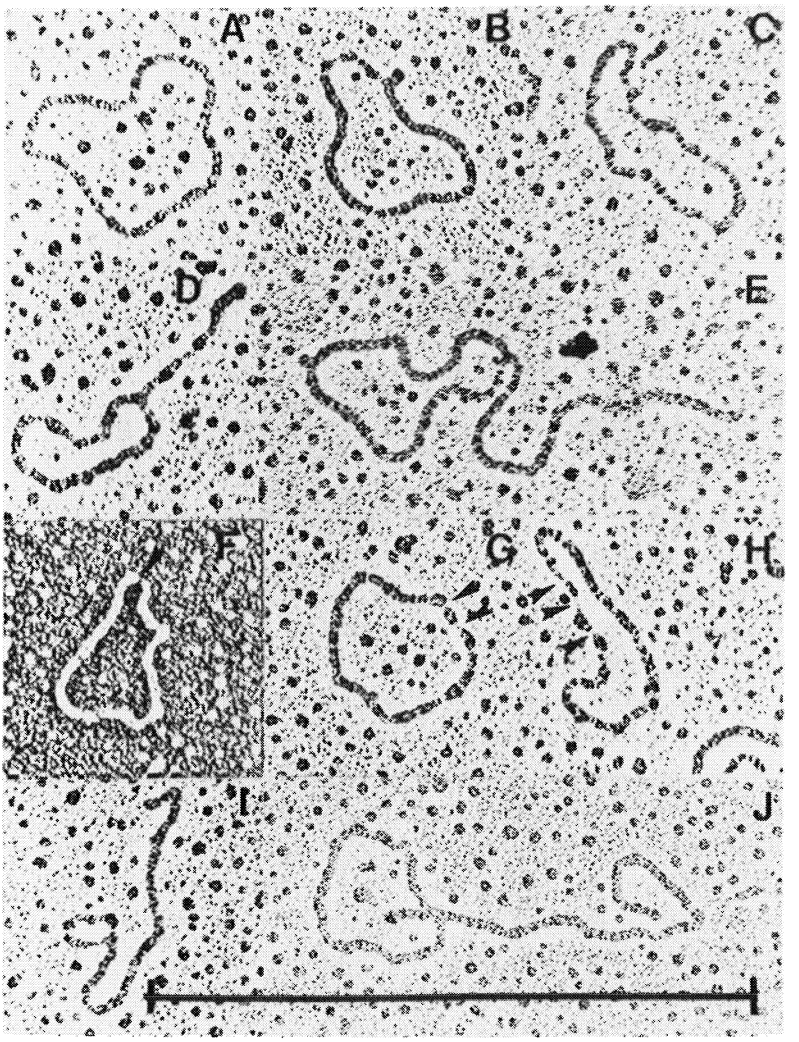


Figure 9. Electron micrographs of Dane particle DNA forms. DNA was extracted from Dane particles not subjected to DNA polymerase reaction conditions and spread by the formamide technique. (A) A 1 μm length plain circle; (B-E) circles with one tail; (F) circles with one small gap, (G) two gaps, and (H) multiple gaps; (I) a small circle with a tail; and (J) a circle with two tails emanating from the same site on the circle. The black arrows in F-H denote the position of gap regions. The solid bar at the bottom represents 1 μm on the electron microscope grid. [Reprinted with permission from Hruska, J.F., et al. in Journal of Virology 21:666-672. Copyright 1977 by the American Society for Microbiology (ref. 606)].

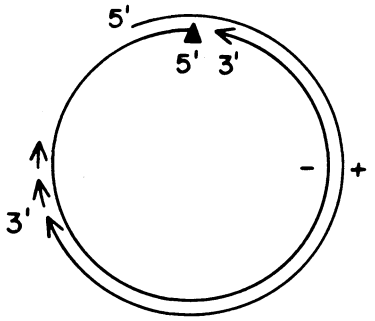


Figure 10. Genome structure of hepatitis B like viruses. (▲) Covalently bound protein. [Reprinted with permission from Summers, J. and W. S. Mason in Cell 29:403-415. Copyright 1982 by M.I.T. (ref. 189)].

nucleotide base pairs (bp) (46). After the endogenous DNAp reaction, the single-stranded regions, detected by DNA spreading in formamide, were closed; the mean length increased to 1.06 μm, and the molecular weight was approximately 2.1 x 10^6 daltons or 3150 bp of double-stranded DNA (42, 46, 606). Analysis of Dane particle DNA by gel electrophoresis and ethidium bromide staining has resulted in an electrophoretically heterogeneous smear resolving into a region of double-stranded linear molecules 1700-2800 bp in length following S1 nuclease treatment (607). Identical analysis following the polymerase reaction resulted in elongated molecules approximately 3200 bp long in both nicked circular and linear (form III DNA) conformations. One study has shown the presence of a short linear tail of DNA attached to some circular molecules of Dane particle derived nucleic acid which is elongated following the polymerase reaction (632a). These results suggest that the mode of HBV DNA replication may be similar to that of bacterial rolling circles (633) in which a closed, circular negative strand serves as a template for positive strand synthesis and that tail elongation requires complementary fragments be synthesized from the growing positive strand (632a). Failure to observe similar tailed DNA structures by electron microscopy or to observe molecules sedimenting in a gradient faster than unit length after different durations of the DNA polymerase reaction, however, suggests that the DNA does not replicate by the rolling

circle model (606). Further, treatment of Dane particle DNA with S1 nuclease following extensive DNAp reaction yielded products sensitive to nuclease activity, suggesting that the circle still possessed a nick or small gap in both strands (607, Figure 10). HBV DNA made double stranded by the endogenous DNAp reaction was converted from circular to linear configuration by heating, indicating that closed circular molecules were not present (634). This change was reversible, while recircularization was blocked following treatment of linearized DNA with S1 nuclease or AMV reverse transcriptase, indicating the presence of single-stranded, complementary nucleic acid sequences at each end of the genome. Further, these "sticky ends" were 270-300 nucleotide base pairs in length as determined by contour length in electron microscopy and by the size of restriction fragments generated from these regions of the genome following the incorporation of radioactive precursors into these end regions by reverse transcriptase (634). The fact that discrete terminal restriction endonuclease fragments were labeled under these conditions also indicates that the discontinuity in each strand on the circular DNA molecule is at a unique site (634). Similar studies support this conclusion but suggest the presence of sticky ends only 200-210 base pairs long (164, 889). The circular configuration of the genome, then, is maintained by hydrogen bonding created by complementary sequences on either end of the DNA. Although the function of these sequences is unclear, they may play a role in circularization of newly synthesized Dane particle DNA for correct packaging into nascent core particles. Alternatively, or additionally, if the HBV genome becomes linearized in infected hepatocytes and the sticky ends are made fully double stranded, then terminal repeats similar to those recently characterized in retroviruses would be formed and may function analogously to long terminal repeats (LTRS) in preparing the genome for both expression and integration (635, 639).

The HBV DNA in different DNA molecules within a population of Dane particles from a single source possesses a single-stranded region 1700-2800 nucleotides in length (47, 606, 607, 888, 889). Combined with experimental evidence that the 5' end of each strand is at a fixed position on the genome with respect to restriction endonuclease sites (47, 634, 888), these results suggest that a free 3' end on the short

strand is available for elongation and thereby serves as a primer in the endogenous polymerase reaction (Figure 10). If DNA synthesis occurs only on the short strand and the sole function of the long strand is to serve as a template, then substitution of one or more deoxynucleoside triphosphates by an analog capable of being incorporated into DNA should result solely in its being found in the short strand. When bromo-deoxyuridine triphosphate (Br-dUTP) was used in place of thymidine triphosphate (TTP) in the polymerase reaction, and the isolated DNA denatured and analyzed by equilibrium centrifugation in CsCl density gradients, the bands of heavy DNA and light DNA failed to reassociate with themselves but readily reassociated when mixed together or upon addition of denatured Dane particle DNA (42). Incorporation of radioactive precursors for increasing lengths of time, followed by analysis of the DNA on alkaline sucrose gradients, resulted in radioactivity being associated with the sedimenting, partially single-stranded DNA species, but not with any shorter strands, suggesting that the precursors were incorporated by formation of a covalent bond with the 3' terminus of the short strand and that in any given molecule incorporation proceeded from a single initiation site (638). Hybridization of Dane particle derived DNA with strand specific HBV DNA probes and binding of T4 gene 32 protein specifically to single-stranded regions in the viral genome confirmed the presence of a gap region in the HBV genome (889). Greater than 99% of Dane particle derived DNA molecules were partially double stranded; less than 1% were partially single stranded; and no supercoiled molecules were detected (889). Observing the extent of single-stranded character in individual Dane particle DNA molecules using the gene 32 protein and electron microscopy, virtually all molecules were single stranded in a region spanning 650-700 nucleotides downstream from the S gene in the long strand to approximately the 5' fixed position of the short strand (889). The 3' end of the short strand in most molecules was found in the region of the genome encompassed by the S-gene on the long strand. The fact that the gap in HBV DNA possesses a fixed minimum length in a single region of the genome, combined with the potential for secondary structure in the form of hairpin loops and perfectly base paired stems within this region, suggests that the gap might provide a level of genetic control of expression encoded by the short (S)

strand within the gap region until after the region has been synthesized by the endogenous polymerase. Alternatively, the gap may be an important physical feature required for packaging the genome in core particles during morphogenesis (889).

In addition to finding DNA associated with serum derived core particles (46), several studies have demonstrated a similar DNA molecule in core particles isolated from the nuclei of infected human liver (223, 631a, 636). An early study has described the presence of a DNA molecule derived from liver cores and possessing a molecular weight of 2.3×10^6 daltons (639). Although this species was 40% larger than DNA isolated from serum cores prior to the DNAp reaction, its size is comparable to that of Dane particle DNA following an extensive polymerase reaction. DNA apparently exists in the plasma of some infected patients, since it could be detected by hybridization (640, 641). In one study, the $Cot_{\frac{1}{2}}$ value of plasma derived HBV related sequences was substantially higher than that expected for the known size and complexity of Dane particle DNA (641). Although the average length of the plasma DNA by electron microscopy was 2-3 μm corresponding to a molecular weight of $4-5 \times 10^6$ daltons, it is not known whether these larger molecules are HBV dimers joined at their sticky ends, HBV DNA covalently attached to host sequences, or some combination of the two (641). Nick translated, HBV DNA probe was used to assay the number and size of fragments generated by HaeIII digestion of like DNA in a Southern blot (47). The results showed a complexity of approximately 3900 nucleotide base pairs. Similar results were obtained using HaeIII following the endogenous DNA polymerase reaction and detection of bands by ethidium bromide staining (607). Use of ^{32}P in the endogenous DNAp reaction, followed by the HaeIII digestion, gel electrophoresis, and autoradiography revealed an additional two fragments not detected by ethidium bromide staining. Adding the sizes of these additional two fragments to the others detected by staining yielded a total of 4910 nucleotide base pairs (607). Again, these results suggest a complexity greater than the 3200 nucleotide base pairs determined for core particle DNA in other assays. It is possible that this complexity is due to the presence of a limited degree of sequence heterogeneity within different Dane particle derived DNA molecules from a single source. Indeed, DNA sequence variations have been documented in seven

different locations in six individual clones isolated from the serum Dane particles of a single patient (282). In cloning experiments using Dane particles from an adr positive serum HBV DNA from the serum was resistant to EcoRI digestion, yet a completely sequenced clone derived from that serum demonstrated an EcoRI site (900). Other recombinant plasmids from the same serum also showed some differences in restriction patterns and one insert was digested with HindIII, a site not present in any other cloned isolate (900). In contrast, comparative restriction endonuclease mapping of polymerase repaired Dane particle DNA and cloned HBV DNA from a single source yielded identical results with a panel of enzymes, suggesting the presence of a major molecular species in a single infection (888).

Restriction endonuclease analysis.

Restriction endonuclease mapping of Dane particle derived HBV DNA better defined the source(s) of complexity mentioned above. ^{32}P-DNA labeled by the endogenous DNA polymerase reaction followed by HaeIII digestion yielded a pair of fragments present in less than stoichiometric amounts, suggesting incomplete cleavage at these sites in the genome. Upon extensive DNAp reaction, these bands were detected in nearly equimolar amounts to the other bands resulting from restriction endonuclease digestion (164, 607, Figure 11). Further, an incomplete polymerase reaction, followed by digestion with HaeIII or HincII, often yielded doublets of some bands by gel electrophoresis (607). Completion of the polymerase reaction in some but not all DNA molecules accounted for this, and underscores the importance of working with fully double-stranded molecules when comparisons of complexity measurements are made by restriction endonuclease analysis. Treatment of cloned HBV DNA with nuclease S1 and XhoI, EcoRI or BamHI also resulted in the production of minor bands, possibly due to incomplete S1 digestion, real heterogeneity in the DNA being cleaved, or a combination of both (642). Since electron microscopic examination of polymerase repaired molecules shows that only about 10% are fully double stranded (642), AMV reverse transcriptase was used to make the repaired Dane particle DNA fully double stranded, and various isolates so treated were compared by restriction endonuclease analysis (164). HBV DNA from subtype adw2 made fully double stranded showed no heterogeneity upon analysis of restriction fragments, suggesting

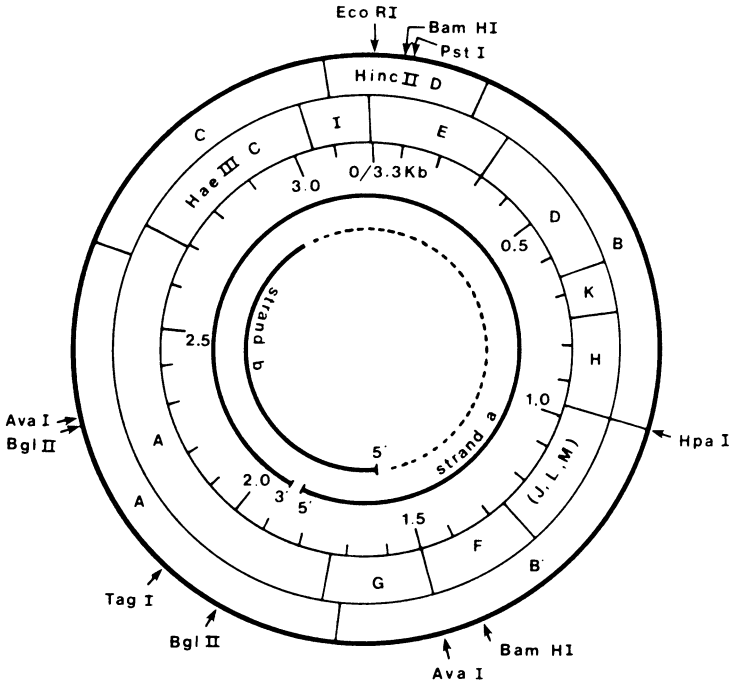


Figure 11-A. Map of HBV DNA (HBsAg subtype adw$_2$) (3.3 kb) including cleavage sites for several restriction endonucleases and the locations of the nick in strand a, the 5' end of strand b, and the single-stranded region of the DNA. The 3' end of strand b in different molecules occurs at different sites within the region shown by the dotted line. The single-stranded region lies between the 3' and 5' ends of strand b and thus is of different length in different molecules. [Reprinted with permission from Robinson, W. S. in the Annals of the New York Academy of Sciences (Genetic Variation of Viruses) 354:371-378. Copyright 1980 by the New York Academy of Sciences (ref. 161)].

that a large proportion of DNA derived from a population of serum Dane particles from a single source contained no differences in sequences at the points of enzyme cleavage. Similarly, no heterogeneity was observed among 20 clones of HBV DNA derived from another single source (165). These conclusions have been independently confirmed (888). Identical analysis of isolates from other patients having the same subtype showed occasional differences, while comparison of the restriction maps from patients having different subtypes of HBsAg have resulted in similar but

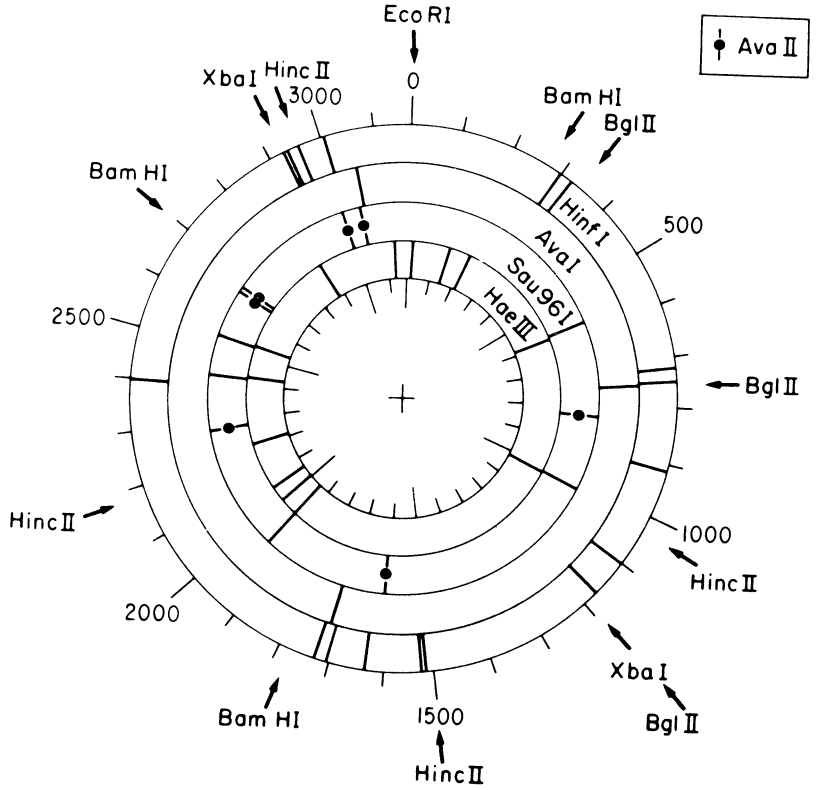


Figure 11-B. Composite restriction endonuclease map of cloned DNA from HBV of HBsAg subtype ayw. The EcoRI cleavage site was chosen as the zero position for the map. [Copyright 1980 by Alan R. Liss, Inc. (ref. 162)].

not identical maps (47, 162, 164, 642, Figure 11). Regular differences have been noted in the restriction fragment patterns of virus DNAs derived from different subtypes. In one study, major differences were observed in the analysis of an ayw clone in comparison to an adw clone (162). However, whether these differences are subtype related or correspond to one or more sites elsewhere in the HBV genome was not determined (164). The sequence analyses of some of these clones point to differences in the HBsAg gene region among different isolates (358), but many nucleic acid sequence differences have also been found in other regions of the genome (155-157, 536a). Clearly, nucleic acid

sequencing of isolated HBsAg gene regions from different isolates will be needed to properly address the question.

Genome isomers.

The results of both restriction endonuclease mapping and sequence analysis suggest that isolates from different patients possess differences in their nucleic acid sequences. Whether these differences are due to point mutations, deletions, inversions or other nucleic acid rearrangements is not known, but it is possible that at least some of the nucleic acid heterogeneity in HBV could reflect the production of defective-interfering genomes. These differences may also, in part, give rise to silent mutations, antigenic variants (subtypes) of HBsAg, or important variants of other HBV gene products. If defective-interfering particles exist, then the state (linear, circular, etc.) or size of the genome packaged into some particles should be distinguishable from DNA in fully infectious particles. In most studies, DNA isolated from Dane particle cores is circular, although some is linear, and no superhelical forms are found, suggesting that the predominant form of HBV is that of nicked or open circles (42, 889). Agarose gel electrophoresis of Dane particle derived DNA usually results in a band 3.2 kilobases (kb) in length, representing the fraction of fully double-stranded linear molecules and a smear of faster migrating material representing partially double-stranded DNA species (105). Analysis of Dane particle derived DNA from the serum of chronically infected patients or chimpanzees by blot hybridization using a ^{32}p-labeled HBV DNA probe resulted in diffuse hybridization between 2.0 and 3.25 kb (643, 644). A full-length supercoiled genome would migrate as a 2.3 kb band and would not be apparent in the smear. The possible presence of a supercoiled HBV DNA molecule, an isomer to the 3.25 kb linear species, was assayed for by using single-stranded nucleases S1 and Bal 31, which slowly nick and eventually linearize supercoiled DNA in a two-stage reaction. In the first stage, either enzyme nicks a susceptible single-stranded backbone forming part of a hairpin loop; while in the second stage, cleavage occurs in the strand opposite the site of the first nick (643). The product of the first cleavage was detected migrating at 4 kb, in a region of the gel devoid of other HBV material, while continued degradation converted virtually all of the hidden 2.3 kb supercoiled DNA to

the 4 kb intermediate and finally to the linear 3.25 kb isomer (643, 644). However, independent observations in HBeAg positive carrier chimpanzees failed to detect a 3.2 kb DNA band from serum Dane particles (646), and similar experiments with HBeAg positive human carriers yielded the same results (607, 646). In these experiments, hybridization to material derived from serum pellets resulted in a smear from 2-2.8 kb, corresponding to the partially double-stranded DNA species. Although these apparent discrepancies do not have a clear explanation, the detection of full-length, double-stranded HBV DNA species in serum may depend upon the extent of viral replication in the liver. For example, in a group of human carriers characterized by very high serum concentrations of HBsAg who were also positive for serum e antigen, full-length and partially double-stranded linear DNA were detected in their sera (653, 654). Carriers who had lower titers of serum HBsAg and were e antigen negative (or anti-HBe positive) had little or no HBV DNA in their sera. Further, results from in situ cytohybridization have demonstrated a positive correlation between the presence of intracytoplasmic HBV DNA and that of morphologically damaged hepatocytes, suggesting that in infected liver tissue foci where gene expression and replication are taking place, hepatocytes may become damaged and release HBV DNA (655, 656, 890).

Percutaneous liver biopsy specimens derived from chronically infected chimpanzees also contained full-length linear and nicked, circular HBV DNA at 3.2 and 4 kb, respectively (643-645). No integrated DNA was detected in these chronic carriers, indicating that chronicity was being maintained by a different mechanism. Independent observations in HBeAg positive carrier chimpanzees revealed a prominent nonintegrated DNA band at 3.2 kb, representing the fully double-stranded linear species, a family of partially double-stranded molecules in a smear 2-2.8 kb in size, and another smear of material 1.6 kb and smaller, possibly representing replicative intermediates (646). The presence of free viral DNA in persistently infected cells is not unique to HBV, since free forms have also been found in epidermal cells persistently infected with or transformed by bovine (647-650) or rabbit (651, 652) papilloma viruses, suggesting that integration is not necessary for either of these processes. Other papovaviruses, including SV40 and

polyoma (657, 660), as well as a number of herpesviruses (661-663), also have been shown to give rise to persistently infected cells in tissue culture. Their mechanism of persistence seems to center around the generation of defective interfering particles characterized by deletions in viral DNA, incorporation of host DNA sequences into the viral genome, duplications of a portion of the viral genome--usually the origin of replication--and polymerization of portions of the viral genome into lengths similar in size to the unaltered virus genome (664). The presence of a small amount of supercoiled HBV DNA in comparison to the full-length or partially double-stranded linear forms, is consistent with the supercoiled form representing an important replicative intermediate and/or the infectious form of the genome, while the dominant linear molecules may be ineffective, in that they possess one or more of the characteristics of defective interfering (DI) genomes. In most viral systems studied, DI particles are present in concentrations several logs above that of infectious particles (665, 666) and in the case of HBV, might effectively prevent establishment of a replicating system in tissue culture. The heterogeneity in the number and size of fragments generated in restriction endonuclease cleavage of HBV DNA from different isolates, the heterogeneity in the nucleic acid sequence in and around the surface antigen gene encoding region (177), and the differences in the total length of the HBV genome derived from complete sequence analysis of several independent isolates (155, 156), demonstrate sequence variability which is one characteristic of defective genomes. Certainly, the production of spherical and long filamentous 22 nm diameter HBsAg particles, as well as DNAp negative core particles, are likely to be antigenically identical to infectious virions, suggesting that one of the consequences of chronic HBV infection is the production of multiple types of DI particles.

HBV IN HEPATOCELLULAR CARCINOMA AND THE CHRONIC CARRIER STATE

One of the outcomes of persistent viral infection is carcinogenesis, often characterized by viral genome or genome fragments integrated into cellular DNA at one or more points (667). The strong association of HBV with primary hepatocellular carcinoma (PHC) (89, 90, 94, 95, 536, 537, 668), combined with the apparent findings of greater than unit

length HBV DNA association with Dane particles (607, 641), suggest that these longer species may be derived from integrated virus DNA which carried additional host material with it upon excision (639). The presence and state of HBV DNA in tumor tissues was subsequently examined by accelerated annealing of a ^{32}P-labeled HBV DNA probe in solution and by agarose gel electrophoresis (669, Table 15). Two tumors from different patients had detectable viral DNA at levels of approximately 1-2 genomes per cell, a third tumor from another patient resulted in levels less than 0.1 genome per cell, while a fourth isolate from another source contained no detectable viral DNA. Although the cellular DNA in the autopsy tumors was extensively degraded, preventing assessment of any integrated sequences, full-length HBV DNA was detected in some cases, suggesting its protection inside Dane particle cores. The absence of HBV sequences in a tumor from an HBsAg negative patient who was anti-HBs and anti-HBc positive suggests that viral DNA need not be present in every tumor cell to maintain the transformed state (669). Among chronically infected chimpanzees (643-645, see above) and a subset of chronically infected woodchucks (683), nonintegrated viral DNA species have been similarly detected in the absence of any integrated sequences. In contrast, an early study has shown the presence of HBV DNA in rapidly sedimenting cellular DNA in patients with chronic active hepatitis (605), suggesting the presence of integrated viral DNA in at least some human chronic carriers.

HBV DNA in human hepatoma cell lines.

Studies with the human hepatoma cell line PLC/PRF/5, which secretes small spherical surface antigen particles indistinguishable from serum derived HBsAg (113, 141-146), show that extracted cell DNA accelerates the reassociation of ^{32}P-labeled HBV DNA (114, Table 15). Quantitation of this reassociation resulted in the finding of four copies of HBV DNA per haploid cellular genome. Reassociation kinetics of all individual ^{32}P-labeled HincII and HaeIII restriction endonuclease fragments were also accelerated, indicating the presence of most if not all of the viral nucleic acid sequences in the cell line. Southern blot analysis of HindIII digested PLC DNA (HindIII does not cleave most known isolates of HBV DNA) resulted in the detection of three fragments larger in size than HBV DNA, suggesting three unique integration sites (114).

Table 15. Presence of HBV DNA in liver tissue, serum and cell lines in comparison with other markers of HBV infection.

Study	Number and origin of sample	Diagnosis	HBV DNA in liver: unintegrated	integrated
108,676	2, Senegal	PHC	-	+
	2, Senegal	PHC	-	-
	1, Senegal	PHC	-	-
	1, Germany	PHC	-	-
	1, Germany	PHC	+	+
	1, France	cirrhosis	+	+
	1, Germany 1, France	cirrhosis	-	-
	1, Germany	cirrhosis	-	+
	1, Germany	cirrhosis	-	-
	1, Senegal	angiosarcoma	-	-
		PHC	-	+
112, 678	3, Ivory Coast	PHC	-	+
	5, Ivory Coast	PHC	-	+
	1, Ivory Coast	PHC	-	+
	1, biopsy, Caucasian	PHC	-	+
	1, biopsy, Caucasian	PHC	-	+
	1, biopsy, Caucasian	PHC	-	+
	1, biopsy, black	PHC, CAH + cirrhosis	+	+ ?
	3, biopsy, Caucasian	cirrhosis	-	+
	4, biopsy, Caucasian	cirrhosis	-	+
	3, biopsy, Caucasian	CAH + cirrhosis	-	+
	1, biopsy, Caucasian	cirrhosis	-	+
	3, biopsy, Caucasian	cirrhosis	-	+

Table 15. (Cont.)

Study	Number and origin of sample	Serum: HBsAg	anti-HBs	anti-HBc	anti-HBe	HBeAg	HBV DNA
108,676	2, Senegal	+					
	2, Senegal	-					
	1, Senegal	+					
	1, Germany						
	1, Germany	+					
	1, France	+					
	1, Germany	-					
	1, France						
	1, Germany	+					
	1, Germany	+					
	1, Senegal	+					
		+					
112, 678	3, Ivory Coast	+	-	+		-	
	5, Ivory Coast	+	-	+		-	
	1, Ivory Coast	-	-	+		-	
	1, biopsy, Caucasian	+	-	+		-	
	1, biopsy, Caucasian	-	-	-		-	
	1, biopsy, Caucasian	-	+	-		-	
	1, biopsy, black	+	-	+		+	
	3, biopsy, Caucasian	+	-	+		-	
	4, biopsy, Caucasian	-	-	+			
	3, biopsy, Caucasian	-	+	+			
	1, biopsy, Caucasian	-	+	-			
	3, biopsy, Caucasian	-	-	-			

Table 15. (Cont.)

Study	Number and origin of sample	Diagnosis	HBV DNA in liver: unintegrated	integrated
	3, biopsy, Caucasian	CPH or CAH	+	?
	1, biopsy, black	acute hepatitis	-	+
	1, autopsy, Caucasian	fulminant	-	+
106,111,	3, Ivory Coast	PHC	?	+
105	1, biopsy	mild inflammation	+	-
	3, biopsy	CPH	+	-
	11, biopsy	CAH	+	-
	2, biopsy	CAH & PHC	+	-
	1, biopsy	mild inflammation	+	+
	3, biopsy	mild inflammation	-	+
	3, biopsy	CPH	-	+
	1, biopsy	CAH	+	+
	7, biopsy	CAH	-	+
107,109, 677	2, S. Africa	PHC + cirrhosis	-	+
	2, S. Africa	PHC	-	+
	1, S. Africa	PHC	-	-
	1, S. Africa	PHC	-	-
	1, S. Africa	PHC	-	-
	1, S. Africa	PHC	-	-
	1, S. Africa	PHC, cirrhosis	-	+
	1, S. Africa	PHC	-	+
	1, S. Africa	PHC	-	+
	1, S. Africa	PHC	-	+
	1, S. Africa	PHC, cirrhosis	-	+
669	3, autopsy	PHC	+	-
	1, autopsy	PHC	-	-

Table 15. (Cont.)

Study	Number and origin of sample	Serum: HBsAg	anti-HBs	anti-HBc	anti-HBe	HBeAg	HBV DNA
	3, biopsy, Caucasian	+	-	+		+	
	1, biopsy, black	-	+	+			
	1, autopsy, Caucasian	+	-	+		-	
106,111,	3, Ivory Coast	+	-	+		-	
105	1, biopsy	+			-	+	+
	3, biopsy	+			-	+	+
	11, biopsy	+			-	+	+
	2, biopsy	+			-	+	+
	1, biopsy	+			+	-	-
	3, biopsy	+			+	-	-
	3, biopsy	+			-	-	-
	1, biopsy	+			-	-	+
	7, biopsy	+			-	-	-
107,109, 677	2, S. Africa	+	-	+	-	+	
	2, S. Africa	+	-	+	+	-	
	1, S. Africa	-	+	+	-	+	
	1, S. Africa	-	+	-	+	-	
	1, S. Africa	-	+	+	-	-	
	1, S. Africa	-	+	+	-	-	
	1, S. Africa	+	-	+	-	+	
	1, S. Africa	+	-	+	+	-	
	1, S. Africa	+	-	+	+	-	
	1, S. Africa	-	+	-		-	
	1, S. Africa	+					
669	3, autopsy	+					
	1, autopsy	-	+	+			

Table 15. (Cont.)

Study	Cell line	Origin	HBV DNA in cell line: unintegrated	integrated
114	PLC/PRF/5	PHC	-	+
106	PLC/PRF/5	PHC	-	+
115	PLC/PRF/5	PHC	-	+
116	PLC/PRF/5	PHC	-	+
108	PLC/PRF/5	PHC	-	+
	Mahlavu	PHC	-	-
	Hep 3B	PHC	-	+
	BEL lines	PHC	-	-
110,679	PLC/PRF/5	PHC	-	+
	Hep 3B 217	PHC	-	+
	Hep 3B 14	PHC	-	+
	Hep 3B F1	PHC	-	+

^{32}P-labeled RNA also bound to all restriction endonuclease fragments of viral DNA. While this suggests that all of the viral DNA is present and transcribed, it is not clear whether full-length genomes or fragments, alone or in tandem, are at a particular integrated site. Indeed, the restriction endonuclease cleavage pattern of HBV DNA fragments from the PLC cell line share only a few similarities with similarly analyzed DNA from Dane particles (114). Independent observations confirmed that HBV ^{32}P-DNA probe reannealing could be accelerated by PLC DNA and that the DNA isolated from other human hepatoma cell lines derived from HBV infected patients did likewise (110). Quantitation of genome equivalents in each cell line yielded different results: Hep 3B 217 and Hep 3B 14, derived from the same patient, contained approximately two and one copies, respectively, while the PLC/PRF/5 line was again shown to contain approximately four. Independent observations using the PLC cell line resulted in the finding of six integrated HBV DNA copies: four complete and two partial (115). Further analysis by Southern blot hybridization using HindIII resulted in a different pattern of fragments for each of the cell lines, none of which corresponded to fully or partially double-stranded episomal HBV DNA. Even though the annealing of all restriction endonuclease fragments tested was accelerated by the DNA from these cell lines, indicating that virtually all the genome fragments were present, the organization of these integrated forms at multiple and independent sites in the cellular genome remained to be clarified (110). As

Table 15. (Cont.)

Study	Cell line	Cell line characteristics: HBsAg	HBcAg	HBeAg	DNAp	HBV RNA
114	PLC/PRF/5	+				+
106	PLC/PRF/5	+				
115	PLC/PRF/5	+				+
116	PLC/PRF/5	+				+
108	PLC/PRF/5	+				
	Mahlavu	-				
	Hep 3B	+				
	BEL lines	-				
110,679	PLC/PRF/5	+				
	Hep 3B 217	+				
	Hep 3B 14	-				
	Hep 3B F1	+				

mentioned above, cleavage of cellular DNA with restriction endonucleases capable of cutting HBV DNA into identifiable fragments yielded few of the expected fragments, indicating that the arrangement of integrated DNA was probably considerably different from that in Dane particles (110). Other studies confirm the presence of integrated HBV DNA in the PLC/PRF/5 human hepatoma cell line (106, 114-116). However, Southern blots of HindIII digested PLC DNA using the entire HBV DNA as probe yielded bands 6.5 kb, 5 kb and 3.5 kb in one study (114); 6.65 kb, 5.5 kb, 4.5 kb and 3.6 kb in another study (116); 24 kb, 17 kb, 12 kb, 6.5 kb and 4.2 kb in others (106, 111); and a group of six bands between 4.2 and 30 kb elsewhere (115). Independent analysis of HindIII fragments derived from PLC/PRF/5 DNA by Southern blotting resulted in the finding of six HBV specific bands at 25.1 kb, 22.1 kb, 17.4 kb, 12.4 kb, 6.3 kb and 4.4 kb (108). Although these results are consistent with others reported above (106), the apparent discrepancy between these results and those of most other studies may reflect clonal differences in the origin of the PLC cell line in each case. In contrast to the PLC cell line, Hep 3B contained two HindIII generated bands at 24.2 kb and 12.1 kb which were detected by an HBV probe (108). The sizes of the HBV specific bands in Hep 3B were different in two independent studies using HindIII fragments analyzed by Southern blot hybridization (108, 110), again suggesting clonal differences, although other explanations were not excluded. Among these cell lines, differences were also noted in the number and

size of bands detected when other restriction endonucleases were employed. Among four HBsAg negative human hepatoma cell lines tested for the presence of HBV sequences, all were negative. These results indicate a close relationship between the presence of integrated HBV DNA and surface antigen gene expression (108). All of the HBsAg positive hepatoma cell lines, additionally, share the characteristic of not possessing any full-length or partially double-stranded unintegrated viral DNA, as described in the carrier chimpanzees and in some human carriers. Combined with the lack of core, e and DNA polymerase activity, the lack of free virus DNA species in these HBsAg producing cell lines suggests that they are not productive for mature virus particles and that the expression of HBsAg occurs independently from these other gene products.

Recent experiments have shown HBV DNA integrated at seven distinct sites from PLC/PRF/5 cells grown in culture or in nude mice (891). HindIII digestion of culture or tumor derived DNA, followed by gel electrophoresis and Southern blotting with an HBV DNA probe, resulted in bands at 29.5 kb, 24 kb, 16.2 kb, 11 kb, 6 kb, 4.2 kb and 2.1 kb, suggesting stable integration during tumor formation. EcoRI digestion yielded 11 bands by Southern blot analysis, including a faint band at 3.2 kb and a prominent band at 2.8 kb. This result suggests that some of the integrated HBV DNA is present in head-to-tail tandem arrangements involving predominantly shorter-than-genome-length DNA. The absence of core gene expression in this and other like hepatoma cell lines through integration of an HBV genome fragment may be responsible for the escape of such infected cells from immune elimination. Such cells might then grow to become neoplastic nodules (891, 896, 897).

Analysis of HBV DNA in other human hepatoma cell lines yielded similar results. For example, HindIII digested DNA analyzed by Southern blotting from HBV associated huH-1, KG-55-T and huH-2 suggested the presence of multiple integration sites and subgenomic integrated fragments in these cell lines (892). Further characterization of these bands using S-gene and core-gene probes demonstrated partial or complete S-gene in all sequences reacting with the full-length genome probe, but only a subset of bands reacting with the full-length genome probe also reacted with the core-gene probe. The failure to detect a HindIII

fragment with HBcAg sequences alone suggests that sequences in or near the core gene may have been deleted at or after the integration event; leaving mostly HBsAg sequences. Analysis of HBsAg sequences from these lines with various restriction endonucleases suggests the likelihood of deletions and/or rearrangements in this part of the genome as well. Cloning of integrated HBV sequences from the PLC/PRF/5 cell line and from a primary tumor also showed viral genome rearrangement and host-virus junctions mapping to within the core or x gene regions (893). Indeed, the only region of HBV DNA conserved among these clones was the pre S/S reading frame. Although the flanking host DNA sequences in each clone were different and a number of retroviral oncogenes (v-abl, v-fps, v-ras, v-myc, v-myb and v-src) tested were not present, the structure of these clones indicates that integration may occur at or near the nicked region of the HBV genome (893). Similar results have also been observed in some patients with hepatocellular carcinoma (895). Analysis of three HBV containing clones independently derived from the PLC/PRF/5 cell lines, however, suggests that integration of viral DNA occurs within the single stranded gap region of the unintegrated genome (894). Two of these clones, being 10.5 kb and 10.7 kb in size, contained contiguous HBV sequences which were interrupted at a HindIII site within the integrated viral DNA. Although the HBV DNA in these clones shared most of their restriction endonuclease fragments and were integrated into host DNA within the X region of the genome, the flanking host sequences from each clone were completely different from each other. A 6 kb clone, bound on either side by cellular sequences, also yielded many bands in restriction endonuclease analysis which were characteristic of cloned HBV DNA. The HBV DNA of this clone, like the larger ones above, was integrated within the gap or X region of the viral genome. In all clones, the surface antigen gene sequences were present, and these were shown to be functional in the two larger clones by cotransfection of HSV thymidine kinase negative (tk^-) mouse L cells. That the 6 kb clone contained only about 2.6 kb of viral sequences and failed to express HBsAg in a cotransfection assay suggests that a portion of the viral genome could be deleted upon integration. Combined with the loss of cellular sequences near the point of virus integration and the absence of LTR-like structures, it is possible that the single-stranded or gap structure of HBV DNA molecules plays an important role

in the mechanism of integration (894). In such a model for integration, it is proposed that cellular DNA polymerases switch to the single-stranded gap in the HBV genome during the course of cellular replication. Following recombination between the long strand of HBV DNA and cellular DNA, the remaining short strand is filled in an ligated to the other strand of host DNA (894). Although other points of the genome could be used for integration, the single-stranded gap region would be preferred.

HBV DNA in tumorous and nontumorous tissues.

The finding of integrated HBV DNA in HBsAg producing cell lines derived from HBV infected patients possessing primary hepatocellular carcinoma suggests that tumor tissue obtained from biopsy or autopsy may contain integrated sequences as well (Figure 12, Table 15). The lack of HBV DNA integration in chronic chimpanzee carriers, combined with an absence of increased incidence of HBV associated PHC, also suggests that the integration event is important in the progression toward PHC (896, 897). Southern blot analysis of seven PHC isolates and five cirrhosis tissue samples, each from different patients, revealed the presence of HBV DNA in three of the tumors and two of the cirrhotic tissue samples (108). One hepatoma patient, seropositive for HBsAg, whose cellular DNA was cleaved with HindIII and fragments analyzed by Southern blotting, yielded HBV DNA containing fragments larger in size than genome length, suggesting integration. The same sample also revealed a band at the position of full-length free viral DNA whether or not HindIII was used prior to electrophoresis, indicating the presence of free virus DNA within cells or adjacent to hepatoma tissue (108, 896, 897). Other studies have also documented the presence of both free and integrated HBV DNA in the liver tissue of patients with PHC (895, 898). The absence of larger bands at gel positions characteristic of free full-length viral DNA multimers suggests that the larger bands detected were not aggregates of these nonintegrated species and that they most likely represent integrated virus genome(s), genome fragments or both (108). In hepatoma samples derived from other patients seropositive for HBsAg only integrated HBV DNA sequences were observed, while HBsAg seronegative hepatoma patients lacked any evidence of integrated or free DNA in their tumor tissue samples. Interestingly, similar patterns of HBV DNA were observed in cases of cirrhosis in the

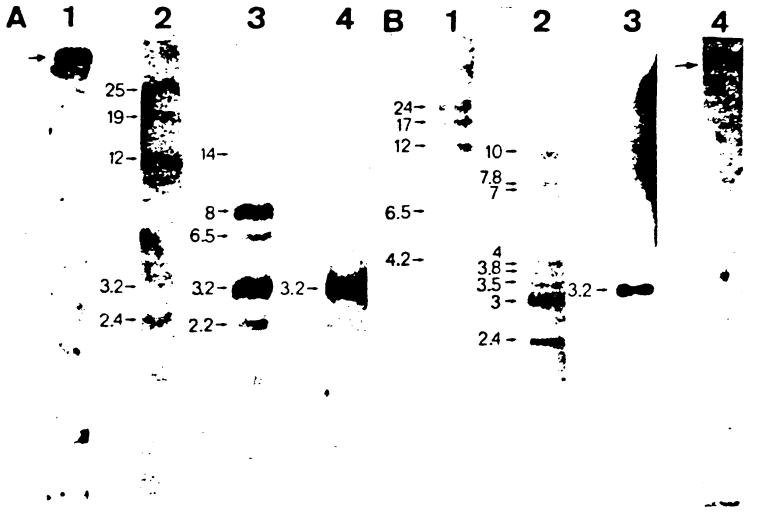


Figure 12. State of HBV DNA in hepatocellular carcinoma. (A) Tumor tissue. Lane 1: 30 µg of nondigested cellular DNA; lane 2: 80 µg of cellular DNA digested with HindIII; lane 3: 80 µg of cellular DNA digested with EcoRI; lane 4: 30 µg of cellular DNA from normal liver cells mixed with 10 pg of cloned HBV DNA. (B) Cell line PLC/PRF/5. Lane 1: 20 µg of cellular DNA digested with HindIII; lane 2: 20 µg of cellular DNA digested with EcoRI; lane 3: 10 pg of cloned HBV DNA; lane 4: 20 µg of nondigested cellular DNA. Cloned HBV DNA digested with BamHI and λplac5cl857S7 DNA digested with HindIII were used as molecular weight markers. [Reprinted from Brechot, C., et al in Progress in Medical Virology 27:99-102. Copyright 1981 by S. Karger AG, Basel (ref. 111)].

absence of PHC. For example, among patients seropositive for HBsAg, one had detectable integrated and unintegrated sequences, while others had unintegrated sequences only; and in a number of HBsAg seronegative patients, no evidence of HBV DNA sequences were found (108). Apparently, the integrated state of HBV DNA is not always associated with PHC. Some nontumorous cirrhotic tissues from surface antigen seropositive patients contain integrated sequences, and some cases of HBsAg seronegative PHC associated with HBV infection (as determined by the presence of anti-HBc, anti-HBs or both in many such patients) lack detectable integrated and free HBV DNA. The possibility of random integration of the virus genome with respect to host sequences would result in the detection of no integrated viral sequences by Southern blot hybridization. However, the

reproducible detection of discrete high molecular weight bands in nontumor or tumor DNA by Southern blotting suggests that they originate when integration at a particular site results in clonal expansion (896, 897).

The presence and state of HBV DNA in hepatocytes was independently studied using surgical biopsy or autopsy materials from individuals with no liver disease, acute hepatitis B, chronic hepatitis B without PHC, and from individuals with HBV associated PHC (112, Table 15). Nearly all of the samples from HBsAg seropositive or seronegative PHC or cirrhosis patients contained integrated HBV DNA by Southern blot hybridization, in contrast to other work (108). Further, one patient with HBsAg seronegative acute hepatitis and another with HBsAg seropositive fulminant hepatitis both contained HBV sequences which were apparently integrated (112). No free forms, suggestive of viral DNA replication, were present in either of these patients. In cases where free full-length and partially double-stranded DNA was found, independent of integration status, serum HBeAg was also detected, suggesting a direct relationship between the presence of e antigen and that of free, replicating forms of HBV DNA in the liver (111, 112). In two patients with hepatocellular carcinoma who were seronegative for HBeAg, the restriction enzyme patterns of the tumorous and nontumorous tissue samples from each patient were different. This was also seen in some HBeAg positive PHC patients (107). Levels of HBsAg and HBcAg gene expression in nontumorous tissues were high, as detected by immunofluorescent staining (671, 672), while in tumorous tissue, little surface antigen and virtually no core antigen was observed in infected cells (673-675). These results suggest that genetic alterations in the integrated viral nucleic acid, including deletion, duplication, translocation, or changes involving DNA methylation may play a role in changing the restriction enzyme sensitivities of integrated and viral DNA which is also paralleled by a distinctive change in both quantitative and qualitative HBV gene expression. Recent experiments with the PLC/PRF/5 cell line, in which only HBsAg is detectable, demonstrated little if any methylation in the surface antigen gene and considerable methylation in the core antigen gene. Since neither of these gene regions were methylated in virion DNA or in infected liver tissue, it

is possible that methylation represents one of the ways HBV gene expression is shut down in PHC in comparison to productively infected tissue (899). Treatment of PLC/PRF/5 cells with 5-azacytidine, which results in demethylation, yielded apparent core gene expression (798) which has not been confirmed (792). These results, however, do not exclude the possibility that independent integration events give rise to the different patterns of both restriction enzyme fragments and gene expression in PHC and neighboring cirrhotic tissue. The appearance of PHC in areas of long-term cirrhosis, combined with the finding of integrated sequences in both cirrhosis and PHC, are consistent with a shifting in the structural and/or functional makeup of the integrated virus sequences near or at the onset of PHC. However, the finding in one patient with early hepatocellular carcinoma of no differences in the restriction endonuclease pattern of tumorous tissue in comparison to nontumorous tissue suggests that nucleic acid rearrangements are not required to occur in order to bring about the appearance of PHC in cirrhotic liver tissue, although these rearrangements may be one in a number of factors leading to the development of PHC. Similar observations have been reported elsewhere (107).

HindIII digestion of PHC DNA derived from three different patients seropositive for HBsAg and anti-HBc, followed by Southern blotting and detection using a radiolabeled HBV DNA probe, resulted in the detection of fragments at 25 kb, 19 kb and 12 kb (111). Since their sizes were larger than genome length and they were not present prior to HindIII digestion, it is likely that they represent integrated HBV DNA species. Similar sized fragments were detected in the PLC/PRF/5 hepatoma cell line in at least one other study (106), suggesting that integration occurs at a very limited number of sites in cellular DNA or that chance integration at a single site resulted in proliferation of the infected clone (895). It is not clear from these studies whether the detected bands represent multiple sites of integration in a monoclonal tumor or different single integration sites from different clones of cells in a polyclonal tumor (111). It is possible that initial integration is random, resulting in a diffuse or nonspecific hybridization pattern, while chronic carriage would permit expansion of clones with altered growth characteristics (896, 897). In a larger related study by the

same investigators, the state of viral DNA, as detected by Southern blot hybridization of appropriately cleaved liver cell DNA, was correlated with serological and histological status (105, Table 15). HindIII DNA patterns derived from needle biopsy samples from 17 patients seropositive for HBsAg and HBeAg resulted in a strong band at 3.2 kb and a smear below it, suggesting the presence of large quantities of free, full-length and partially double-stranded viral DNA. Undigested material showed similar patterns. Although the histological status of these patients ranged from the appearance of slight inflammatory changes to CPH, CAH and PHC, only a single patient with CAH yielded a band larger than full-length DNA, possibly due to integration. It should be stressed that the detection of a 3.2 kb band and the conclusion that it is free, full-length viral DNA is no way proven by Southern blot analysis, since randomized linear sequences, common in defective interfering genomes of other viruses, could yield similar results. Only further restriction mapping of these genome length species could properly identify them. Further, the detection of high molecular weight DNA containing HBV sequences following HindIII digestion assumes that integrated HBV DNA maintains its resistance to this enzyme. Clearly, results from the hepatoma cell lines discussed above yield few fragments by restriction enodnuclease analysis that comigrate with those fragments generated from Dane particle DNA, suggesting considerable rearrangement of nucleic acid sequences in these tumor cell lines. Further, HindIII sites have been detected in clones of integrated HBV DNA in the viral nucleic acid (894) and in one clone of viral DNA (900). Despite these limitations, the results of Southern blotting are consistent with the presence of free and integrated viral DNA. HindIII DNA patterns derived from needle biopsy of 15 other patients who were seropositive for HBsAg, seronegative for HBeAg, and presented varied histological pictures ranging from mild inflammatory changes to CPH and CAH resulted in the detection of HBV DNA sequences in association with high molecular weight bands and none at 3.2 kb. Combined with the finding that HBeAg positive patients possess detectable 3.2 kb HBV DNA in serum and that HBeAg negative patients do not, these results suggest the existence of two types of HBV carriers. HBeAg positive carriers show high levels of viral DNA replication by virtue of 3.2 kb long sequences being detected in both

serum and liver. HBeAg negative carriers do not possess detectable free viral DNA in either serum or liver but instead possess HBV sequences which may be integrated (105).

The presence of HBV sequences associated with high molecular weight liver cell DNA derived from PHC patients seropositive for HBsAg has been independently confirmed (107, 109, 893, 894, Table 15). Interestingly, PHC patients who were anti-HBs positive and HBsAg negative failed to demonstrate the presence of HBV DNA in association with high molecular weight cellular DNA, suggesting that an earlier immune response against surface antigen in some way alters the nature of persistent infection (109). Alternatively, these antibodies may have reactivities toward human polymerized serum albumin, as described above. Among tumors where HBV DNA appeared to be integrated, the number and size of bands in each tumor detected by Southern blot hybridization was unique; yet one or more of the detectable bands in each tumor comigrated with a subset of bands in the other tumors. Further, all of the HBV DNA containing bands generated from HindIII plus EcoRI cleavage of PLC/PRF/5 DNA were found, in part, in similar analyses of tumor DNAs. While these results suggest that a limited number of common integration sites are present, comigration of DNA bands of a given sequence length is not a strong measure of identity, making any conclusions drawn from this data somewhat speculative (109).

If one mechanism of HBV persistence in chronic carriers and PHC patients involves integration into the host genome, then the time after infection in which this event takes place would mark an important step in pathogenesis toward PHC. In one study, carriers who were HBsAg seropositive and had chronic hepatitis for up to two years possessed HBV DNA in their livers in nonintegrated form. In contrast, in carriers possessing circulating HBsAg for more than eight years, only integrated DNA was detected by Southern blot hybridization (107). While these results suggest that integration of HBV DNA occurs during the course of chronic infection prior to the onset of PHC, other results cited above in which apparent integration was detected in cases of acute and fulminant hepatitis (112), as well as the inconsistent finding of integrated sequences in HBsAg negative, anti-HBs positive PHC patients (107-109, 112), again suggest that the timing and establishment of integration

into specific host genome sites might not be required for the development of PHC (896, 897).

The development of PHC in woodchucks chronically infected with WHV has recently provided a model to further study HBV associated PHC in man (121, 154, 681). Chronically infected woodchucks have been found to possess two states of persistent infection similar to those observed in HBeAg seropositive and negative human carriers (105, 121). Some woodchucks having long-term WHV infection were seropositive for WHsAg, possess both surface and core antigens in the liver, and demonstrate only integrated viral DNA by Southern blot hybridization (121). In contrast, the human hepatoma cell lines and liver tissue samples possessing solely integrated viral DNA by blot hybridization have demonstrated little evidence of core antigen gene expression (113, 141-146, Table 16), suggesting that the regulation of core antigen expression in these closely related viruses in their respective hosts is markedly different. The other group of chronically infected woodchucks were also WHsAg positive in both their sera and livers, possessed liver associated WHcAg and demonstrated large amounts of free viral DNA in liver and, to a lesser extent, in serum (121). By analogy to man, the latter group of woodchucks had large quantities of free, full-length and partially double-stranded molecules of viral DNA, reflecting high levels of DNA replication. The finding of free, full-length viral DNA in serum suggests the presence of Dane-like particles in these woodchucks similar to those present in HBeAg seropositive chronic human carriers. The detection of apparently integrated WHV DNA sequences and large quantities of free viral DNA in one carrier suggests that integration occurs during the course of chronic infection and that an integrated state could persist some time prior to the appearance of PHC, as in man.

The demonstration of two carrier states in both woodchuck and man provides unique opportunities for reassessment of the types of responses observed following ara-A and/or interferon treatment in human chronic hepatitis. In practice, treatment of human HBV carriers with these drugs results in a variety of responses which may be partially dependent upon the state of viral nucleic acid in infected hepatocytes. Some patients under treatment cleared HBsAg, HBeAg and viral DNAp activity from serum; others likewise cleared HBeAg and viral DNAp

Table 16. Phase relationships of the various open reading frames in HBV DNA

Comparison of the translation of the long strand of two HBV genomes (from 358). The amino acid sequences deduced from the long strand in three reading frames were compared for the two complete nucleotide sequences (156, 536a) after excluding the additional sequences. The amino acid differences were scored for significant changes, such as nonpolar to basic. The long strand was divided into 4 segments consisting of the known genes S and C, and two intervening segments.[a]

Nucleotide numbers in segment[a]	reading frame region		number designation of open reading frame	% amino acid difference
2451 to 154	p[b] -	phase 1	6	16.6
	pre-S --	phase 2	7	11.7
	-	phase 3		22.0
155 to 833	p	phase 1	6	4.9
	s[c]	phase 2	7	2.2
	-	phase 3		6.6
834 to 1900	p	phase 1	6	3.0
	-	phase 2		10.7
	x	phase 3	5	3.9
1901 to 2450	-	phase 1		13.1
	c[d]	phase 2	8	1.1
	-	phase 3		13.1

[a]Nucleotide base pairs are numbered clockwise from the unique EcoRI site in HBV DNA as shown in Figure 13.

[b]p = open reading frame which may encode the HBV DNA polymerase.

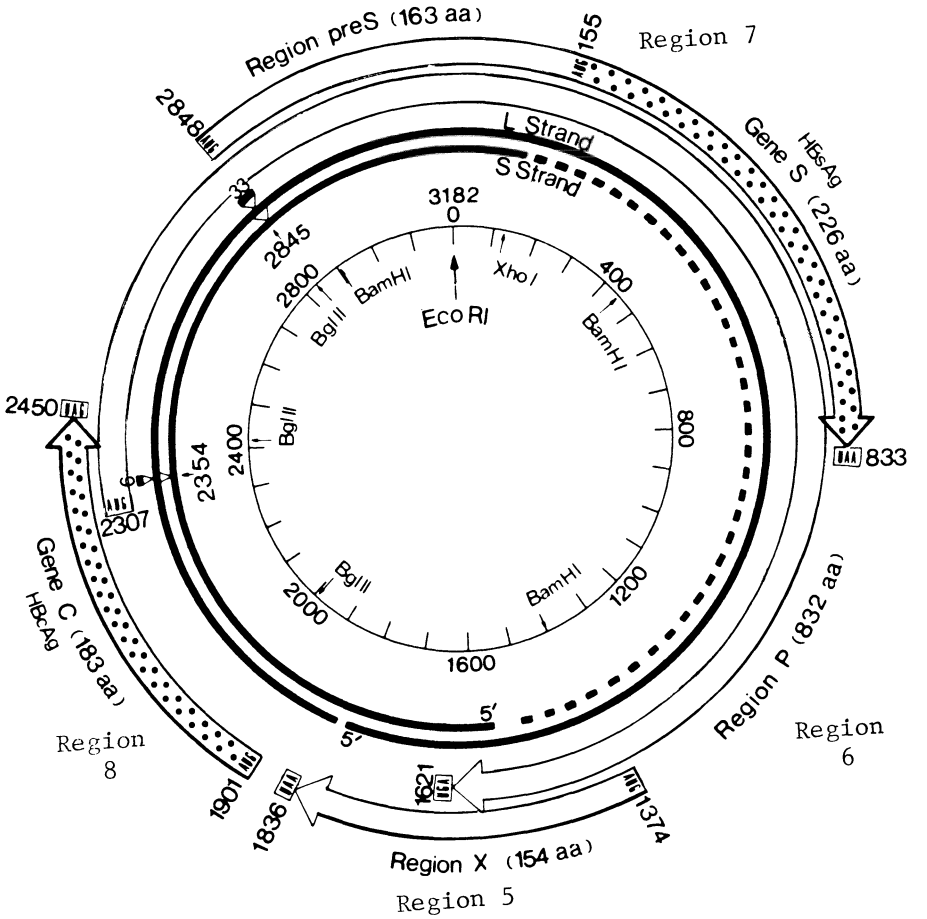
[c]s = open reading frame encoding the major surface antigen polypeptide.

[d]c = open reading frame encoding the major core antigen polypeptide.

activity but remain seropositive for HBsAg; while other carriers showed only a transient decrease in one or more of these serum markers during the period of anti-viral treatment (623-625). Since these drugs interfere with viral replication, it would be expected that the subset of carriers receiving greatest benefit from such therapy would be those showing abundant free forms of HBV DNA and no evidence of integrated sequences in their hepatocytes (684). Permanent elimination of HBsAg, HBeAg and DNAp from serum may be a consequence of the successful elimination of all replicating HBV DNA in the liver. In drug responses where HBsAg production persists at a lower level than observed prior to therapy, it is possible that the loss of replicating and full-length free viral DNA species directly results in the loss of core gene expression and core particle synthesis in the hepatocyte. If this were true, then only free, full-length HBV DNA would possess all of the information and the proper configuration for core antigen synthesis. The recent finding of replicative intermediates in the "immature" core particles of DHBV (188, 189), suggests that an intimate relationship exists among intact "immature" core particles and nucleic acid replication. If nucleic acid replication is dependent upon the proper expression and assembly of "immature" core particles, or if the assembly of core particles depends upon the existence of replicating nucleoprotein complexes, then direct inhibition of nucleic acid synthesis or virus core gene expression could effectively abort viral replication. The fact that surface antigen expression persists in some carriers may be due to the presence of integrated viral DNA at one or more sites behaving much as it has been described in the human hepatoma cell lines. The appearance of HBsAg in the supernatants of human hepatoma cell lines containing integrated HBV sequences and its frequent appearance in the sera of PHC patients (especially in Africa and Asia) stand in sharp contrast to the repeatedly unsuccessful attempts in finding other HBV associated gene products from these sources. It is not known whether integration regularly results in some alteration in the genetic material required for core gene expression. In one study using the PLC/PRF/5 human hepatoma cell line and specific endonuclease restriction fragments of HBV DNA as probes, it was shown that the probable orientation of the HBV genome in the cell line was such that the virus-host junctions were at the sticky

ends of the virus DNA (115, 893-895). Whether the organization of viral genes between these sticky ends is colinear with those in Dane particle DNA remains to be determined, especially since different numbers and sizes of fragments in restricted PLC DNA compared to Dane particle DNA have been reported (106, 108, 110, 111, 114-116). As discussed, recent work has documented both rearranged genome fragments and colinear inserts. If one or more of the core associated polypeptides is partially encoded for by two open reading frames, namely c and x (Figure 13), separated by the nick in the long (minus) strand of DNA, as suggested by tryptic peptide mapping studies (56), then the orientation of these reading frames to each other, which would be different in supercoiled, nicked-circular and linear integrated DNA, may have a bearing upon whether the proper transcript could be synthesized. In addition to proper juxtaposition of structural gene segments, the presence and fidelity of core gene promoter sequences may differ with DNA conformation or state. If one common site of HBV DNA integration is at its sticky ends, then core encoding information would be separated to either end of the integrated site, effectively splitting the functional gene. Where intact genome copies are inserted into host DNA, the surface antigen gene will always be found intact near the middle of the integrated HBV sequence. Since the promoter for HBsAg gene expression has recently been defined (168, 680) and its location is far from the nick in the long strand (see below), it is likely that its integrity will be maintained following integration of HBV DNA at its sticky ends, suggesting that the genetic elements necessary for expression of HBsAg are present and that expression would be likely under these circumstances.

The finding of very high frequencies of hepatocellular carcinoma in WHV chronically infected woodchucks held in captivity in comparison to uninfected controls (121, 154, 681, 685) and the successful transplantation of such PHC tissues into nude mice (901) have prompted studies directed toward better defining the relationship between viral and host DNA in chronically infected and hepatoma tissue cells. Southern blot analysis of PHC DNA digested by single restriction endonucleases and detected by a WHV DNA probe showed high molecular weight bands consistent with viral DNA integration, and low molecular weight forms consistent with the presence of full-length and partially double-stranded



forms of the viral genome (123). The appearance of DNA in only free or integrated form, characteristic of the different groups of woodchuck carriers described above (121), was not observed in most tumors analyzed. The finding of free viral DNA in quantities averaging about 2000 genome equivalents per cell, which often obscured detection of low copy integrated sequences, resulted in similar experiments being performed on isolated liver nuclei, where much less viral DNA was present. DNA extracted from an enriched nuclear preparation contained a major band at 2.5 kb. Since this band gave rise to fragments characteristic of

Figure 13. Physical structure and proposed genetic organization of HBV genome. The 5' end of the long (L) strand is base-paired with the 5' end of the short (S) strand. Dashed line corresponds to the variable single-stranded region. Certain restriction sites indicated by arrows correspond to the physical map of the HBV/ayw genome analyzed by (157). The unique EcoRI site is used as the point of origin in the physical map, and the unique XhoI site is indicated in the conventionally clockwise orientation of numbering the nucleotides from 0 to 3182. The two sequences of 6 and 33 nucleotides at positions 2354 and 2845 are those present in the HBV/adw clone analyzed by (155). The broad arrows surrounding the genome correspond to the four large, open regions of the L strand, conserved in the three published sequences. These four potential coding regions are called regions X, P, S (divided into pre-S and gene S) and C; or regions 5-8, respectively. The number of amino acids in the brackets corresponds to the length of the hypothetical polypeptide. The two regions corresponding to the defined genes S and C are sippled. [Reprinted with permission from Tiollais, P., et al. in Science 213:406-411. Copyright 1981 by the American Association for the Advancement of Science (ref. 358)].

cloned WHV DNA upon digestion with restriction endonucleases and its migration was very similar to the 2.3 kb supercoiled HBV DNA described above (643, 644), these results support the presence of such a species in tumor nodules (123). Independently, both integrated and supercoiled WHV DNA forms have been detected in hepatoma and adjacent nontumor tissue in several chronic carrier woodchucks (902). If supercoiled WHV DNA is a template for replication and densitometric analysis of the Southern blots suggest approximately 50 supercoiled molecules per cell, then substantial replication may be taking place in hepatoma tissue (123). Whether the DNA replication is vegetative or includes maturation is not clear from these studies, although the data presented are more consistent with the former possibility (123). The detection of high molecular weight bands containing WHV DNA is consistent with integration occurring at multiple sites in the genome. The finding of different digestion patterns of integrated viral DNAs from different tumor nodules suggests that either they represent metastases of undetected cells in the primary tumor, independently occurring primary tumors, or a combination of both. Although the second interpretation is favored by histological criteria, the presence of nontumorous tissue within or adjacent to the analyzed nodules, may partially account for these results.

The relationship between integration and transformation was further studied by examining the state of viral DNA in nontumorous WHV

chronically infected liver (687). A library of clones containing nuclear DNA derived from a chronically infected woodchuck liver was constructed using lambda phage charon 30 as a vector. Hepatocellular carcinoma was not detected in the liver tissue from which the library was made. Two classes of recombinants containing WHV DNA sequences were found, both in low copy number. a) Two clones contained viral sequences integrated into the host cell DNA, demonstrating that integration is not unique to carcinoma cells and that it occurs during the course of persistent WHV infection (687). Although integrated HBV DNA has also been found in nontumorous liver tissue from chronically infected patients, Southern blot analysis suggests multiple sites of HBV DNA integration (105, 108, 112, 676, 678), while similar analysis of chronically infected woodchuck liver tissue has not resulted in the finding of discrete high molecular weight bands, suggesting that random integration did not result in clonal proliferation (687). Independent observations, however, have resulted in the finding of several distinct high molecular weight bands by Southern blot hybridization in a subset of chronically infected woodchucks (121). Perhaps integration of selected sites within the host genome (near a cellular oncogene) results in expansion of these hepatocyte clones sometime during chronic infection and may be responsible for the apparent discrepancies in the results of Southern blot analysis. b) Three other clones comprised a second class of recombinants, possessing greater than unit length viral DNA and undetectable host DNA sequences. Further characterization of these apparently unintegrated, long, "novel form" clones of WHV DNA by partial or complete digestion with various restriction endonucleases resulted in the finding of inverted, deleted and duplicated portions of the WHV genome (Figure 14). The presence of these variably long (7-10.5 kb) complex forms were confirmed and strengthened by heteroduplex mapping experiments. Although the origin of these "novel forms" is not clear, they are similar in complexity to cloned integrated viral sequences derived from acutely infected, chronically infected, and hepatoma tissue in woodchucks. The presence of these "novel forms" at about one copy per cell in comparison to at least 50 copies per cell of full-length and partially double-stranded WHV DNA suggests that these forms do not play an important role in replication. Although they may

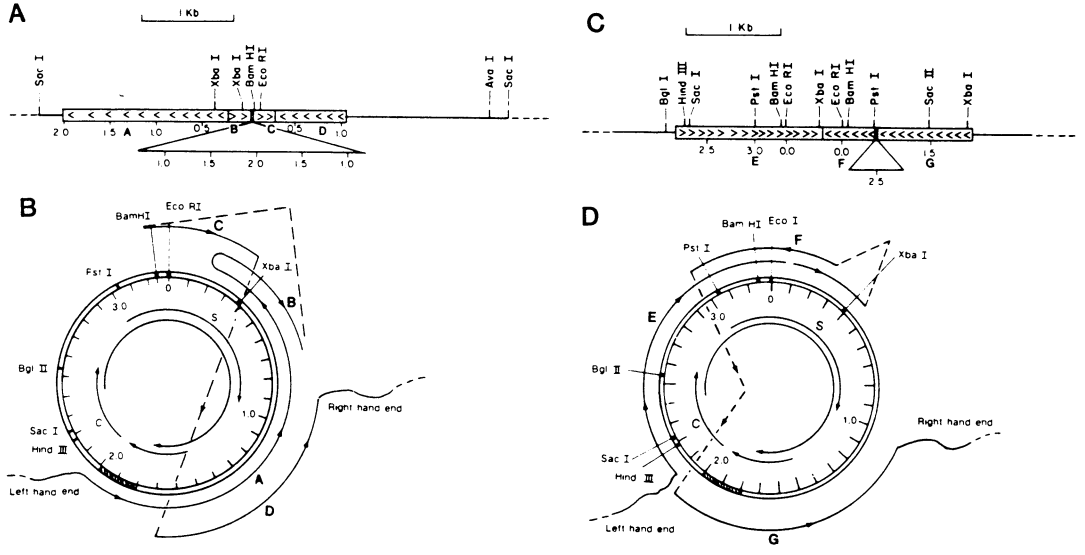

A
1 Kb
Sor I
Xba I
Xba I
Bam HI
Eco RI
Ava I
Sac I
2.0
1.5
A
1.0
0.5
B
C
0.5
D
1.0
1.0
1.5
2.0
1.5
1.0
B
BamHI
Eco RI
C
Pst I
Xba I
B
0
3.0
S
Bgl II
1.0
C
Sac I
Hind III
2.0
Right hand end
Left hand end
A
D
C
1 Kb
Bgl I
Hind III
Sac I
Pst I
Bam HI
Eco RI
Xba I
Eco RI
Bam HI
Pst I
Sac II
Xba I
2.5
3.0
E
0.0
0.0
F
1.5
G
2.5
D
Bam HI
Eco I
F
Pst I
Xba I
E
0
3.0
S
Bgl II
C
1.0
Right hand end
Sac I
Hind III
2.0
Left hand end
G

FIGURE 14

Figure 14. Integration pattern of WHV DNA in chronically infected woodchuck livers. (A) Linear map of the CW309 integration site showing restriction sites and the extent and orientation of viral DNA sequences. Viral sequences are represented by the box, with map coordinates given in kilobases originating at the EcoRI site, nonviral sequences by the thin line. Arrowheads in the box represent the 5' to 3' direction of the viral plus strand. The deletion is shown by the triangle, whose base is equivalent to the length of the deleted viral sequences. (B) Map of the viral sequences in CW309, relative to the circular map of WHV. Arrowheads point in the direction equivalent to left to right in (A) above. The map of the virus is based on the sequence obtained in ref. 159, and the scale is given in kilobases clockwise from the EcoRI site, with the 5' to 3' direction of the plus strand also proceeding clockwise. The open reading frames inferred in ref. 159 from the sequence are shown in the middle, identified as follows: S, surface antigen (WHsAg) polypeptide; C, core antigen (WHcAg) polypeptide. The hatched segment denotes the cohesive end region of the virion DNA. (C) Linear map of the HW99-0 integration site, represented as in (A). (D) Circular map of the HW99-0 viral sequences, represented as in (B). The letters A-G identify the colinear segments of viral sequences. [Reprinted with permission from Ogston, C. W., et al. in Cell 29:385-394. Copyright 1982 by M.I.T. (ref. 688)].

possibly arise by the excision of integrated sequences or as a consequence of defective DNA replication, the role of these "novel forms" in infected cells is yet to be determined.

Detailed characterization of the integrated WHV DNA sequences in two different hepatocellular carcinomas demonstrating integration at a single site yielded long, complex forms similar to the apparently unintegrated "novel forms" discussed above (688). It is not known from these results whether the clones studied have anything to do with the induction and/or maintenance of the transformed state. However, it is possible that highly rearranged viral DNA is especially liable to become integrated or that integration of intact viral genomic DNA destabilizes the affected chromosome, resulting in extensive rearrangements until a stable form is again achieved (688). Highly rearranged integrated forms are also characteristic of other DNA tumor viruses, including SV40 (689-691) and adenovirus 2 (692), and it is possible that, like these viruses, HBV and WHV encode a virus polypeptide responsible for induction and/or maintenance of the transformed phenotype. If this were so, then integration would not be required for transformation, and the complex integrated forms and apparently unintegrated "novel forms" would both be capable of transforming capability. Indeed, both clones of integrated

complex forms contained, intact, the short open reading frame, X (688). Many of the apparently unintegrated "novel forms" also contain intact copies of most or all of the X gene (687), suggesting that information encoded for by this region of the genome may be involved in transformation. Sera derived from some patients with HBV associated hepatocellular carcinoma contain antibodies which reacted by anticomplement immunofluorescence with one or more proteins in the nuclei of cultured human hepatoma cells (903, 904). Only sera from HBV associated PHC were reactive and only HBV associated hepatoma cell lines (PLC/PRF/5 and Hep 3B) were positive, suggesting that the reactive component was specific to HBV. Since the presence of such a nuclear antigen did not correlate with HBsAg production or anti-HBc reactivity, the latter being negative in these cell lines, it is possible that the new antigen system specifically associated with PHC could be related to the X gene product of HBV if it is a virus gene product at all (903, 904). Alternatively, the virus may act in accordance with the typical promoter-insertion model (693-697). In this case, HBV or WHV DNA would integrate near at least one cellular oncogene and promote expression of the oncogene which would result in the transformed phenotype. Both mouse mammary tumor virus (698) and avian leukosis viruses (695-697) have been found to promote the expression of cellular oncogenes at least partially responsible for the transformed phenotype. It is interesting in this context that both clones of integrated WHV DNA possess the single region of the virus genome which is not in an open reading frame (688). This region of the genome is also present in the apparently unintegrated "novel forms" of WHV DNA (687). If this region acts as a promoter in the virus, then it might direct the expression of a nearby cellular oncogene when the genome is integrated into the appropriate host site(s). If HBV and WHV acted by promoter-insertion, then different complex forms with different numbers of deletions and rearrangements would be present, so long as the promoter remained intact. Using EcoRI digested HBV DNA as a template in an *in vitro* transcription system derived from HeLa cell extracts, core antigen gene sequences were transcribed from a promoter region located just prior to the proposed translation initiation site of the major HBcAg polypeptide (797). Using similar fragments in the same transcription system, another study showed that one

of two promoter sites for RNA polymerase II in HBV DNA mapped to the sequences preceding the HBcAg gene (905). In vitro, however, the putative core gene promoter appeared to be weak (905), and it is equally possible that the sequences including the surface antigen gene promoter play a role in establishing and/or maintaining the transformed state. As noted above, whether HBV DNA integration is colinear or accompanied by extensive rearrangements, the pre S/S gene regions have often been found present (892-894). The expression of HBsAg upon transfection of cells with clones encompassing HBV DNA integrated into host cell DNA suggests that this transcriptional unit is intact and functional. However, what may be important in terms of carcinogenesis is not whether HBsAg is expressed, but whether its promoter is active. The finding of surface antigen gene sequences integrated at multiple sites in a variety of hepatoma cell lines suggests that they behave like multi-copy mobile genetic elements (892, 906-908). The presence of S-gene sequences within a HindIII fragment, the restriction endonuclease site presumably in host DNA, is also consistent with this hypothesis (892). The discovery of a polyadenylated viral transcript in PLC/PRF/5 cells containing both HBsAg and cellular sequences in covalent linkage (896, 897) provides further evidence consistent with the movement of one or more pseudogenes through an mRNA intermediate to various locations in the genome. The ability of the HBsAg promoter to express human fibroblast interferon from a recombinant molecule demonstrates that a cellular gene could be expressed from this promoter (909). Further, comparable levels of interferon were produced from the S-gene promoter as well as the SV40 early promoter. The additional observation that HBsAg is produced in comparable quantities by both promoters suggests that expression under the S-gene promoter is constitutive as are early products expressed under the control of the SV40 early promoter. Since in nonpermissive cells containing integrated viral DNA, early gene products are made in large amounts compared to late gene products (910), the S-gene promoter as a mobile genetic element may substantially alter the expression of one or more host oncogenes. Although this has not been demonstrated, a sequential increase in c-myc followed by c-ras^H oncogenes occurs transiently during liver regeneration (911). Certainly, random integration of HBV DNA or DNA fragments into the cellular genome, followed by

activation of one or more oncogenes, suggests that integration may be one step of many leading to PHC. Alternatively, integration may result in the inactivation of a gene or genes which inhibit cell division, permitting uncontrolled continued proliferation of the affected clone. Further characterization of the relationship between virus and host genetic material will help to clarify this relationship and further define the role of the virus in PHC.

Karyotyping of human hepatoma cells.

Recently, karyotyping of the human hepatoma cell lines PLC/PRF/5, Hep 3B and Hep 2B from different patients with HBV associated hepatocellular carcinoma [PLC (113) and Hep 3B (141, 146) lines] or from a patient with hepatoblastoma [Hep 2B (141, 146)] has demonstrated the presence of multiple genetic rearrangements (699). In addition to polysomy of some chromosomes, chromosome 1 was involved in multiple translocations. Among other solid tumors, chromosome 1 has been frequently affected in lung (705), testicular (701), rectal (708), cervical (700, 704), bladder (707), breast (706) and ovarian carcinomas (702, 703). In the PLC/PRF/5 cell line, multiple deletions and pentasomy of a single region were observed in four of the six chromosomes 1, while in Hep 3B some of the multiple copies of chromosome 1 demonstrated regions translocated from chromosome 11; and in Hep 2B, translocation was observed between some of the normal chromosomes 1 and chromosome 21. Polysomy of chromosomes 2, 6, 9, 10, 15 and 17 was observed in most karyotype analyses. Translocations have also been consistently documented between chromosomes 7 and 11, between 10 and 13, and between 14 and 21 in the PLC/PRF/5 cell line. Chromosome 15 in the Hep 3B line contained additional genetic material, while polysomy of chromosome regions were observed in chromosomes 6, 10 and 17. Chromosome 15 in the Hep 2B cell line also contained additional genetic information. Most other changes seen in karyotype analysis of these cell lines involve deletions of various chromosomes. If HBV or WHV DNA integration into the host genome is partially responsible for PHC by the promoter-insertion model, and integration involves both rearrangement of the viral DNA into long, complex forms (688) and destabilization of chromosomal DNA in the affected chromosome(s), then multiple rearrangements of host DNA could result until new stable forms are

achieved. Since the results of Southern blotting of hepatoma DNA for integrated HBV DNA species yield different numbers and sizes of fragments in different tumors, it is not likely that HBV integrates at preferred sites of the human genome. Integration is probably random, and the surviving clones having integrated sequences possess different proliferative capacities, dependent upon whether HBV DNA integration alters the growth characteristics of each cell undergoing an integration event. The complex karyotypes of the hepatoma cell lines may be a reflection of cell variant selection from a primary tumor upon long-term cell culture but might also result, in part, from integration of the viral genome. Recent demonstration of carcinogenesis associated with DNA rearrangements in which a cellular oncogene is transposed to the influence of a cellular or viral promoter (693-696, 709-711) may play a role in HBV or WHV associated hepatocellular carcinoma. Other molecular mechanisms, however, cannot be excluded.

NUCLEIC ACID SEQUENCING OF DANE PARTICLE DNA AND EXPRESSION OF HBV GENES

Nature of open reading frames.

The cloning of Dane particle associated DNA has provided material for detailed physical and chemical characterization of the HBV genome, including complete nucleotide sequence analysis. Restriction endonuclease mapping of various cloned HBV DNA isolates has provided suitable overlapping fragments for 5'-terminal labeling (712) and sequencing by the chemical degradation (712) or dideoxynucleotide (713, 714) methods. In two reports where the entire HBV genome was sequenced, it was found to be 3182 nucleotides (156) or 3221 nucleotides (536a) in length. When the additional sequences of 6 and 33 nucleotides in the larger genome were deleted by computer analysis, substantially better alignment of the sequenced DNAs was possible (358), although the two analyses still differed in 9.5% of their nucleotide base pairs. Independent sequencing of adw and adr isolates resulted in HBV DNA inserts 3.2 kb and 3.188 kb respectively (900). The overall sequence divergence between adr and ayw was 11.2%, 10% between adw and ayw, and 9.8% between adw and adr. Among two adw isolates, however, the overall divergence was only 1.6% (155, 900). Although no mutational hot spots were noted among these sequenced isolates, several contained apparent deletions. These deletions were found in adw and ayw isolates, but

not adr, in the beginning of the pre-S region (900). Another deletion approximately 100 bp before the core gene, near the region of the nick in the long strand, was observed with adr, but not with adw or ayw (900). Complete sequence analysis of the plus DNA strand--that is, the short strand which acts as a primer for and can be specifically labeled by the endogenous DNA polymerase activity--reveals four regions capable of encoding polypeptides of at least 100 amino acids (Figure 13). In HBV DNA, open regions 1-4 could potentially encode polypeptides 170, 109, 149 and 115 amino acids in length respectively. If splicing were to occur, larger polypeptides could be produced from any combination of these small regions (156, 158, 159). However, their presence, size and position on the short strand are not conserved in WHV DNA (358). Comparison of the genome sequences of HBV and WHV DNA demonstrate that the position of region 1 is conserved among these closely related viruses (158, 159). Region 1 sequences, however, were unrelated in these viruses, suggesting that the short strand probably does not encode any polypeptides. It is completely absent from the nucleotide sequence of the DHBV genome (912). Complete nucleotide sequence analysis of the minus DNA strand--that is, the long strand which acts as a template for the endogenous DNA polymerase activity--also reveals four open reading frames, 5-8, which potentially encode polypeptides 166, 851, 432 and 219 amino acids in length respectively. Each of the open regions was defined as beginning with an ATG codon, signaling the start of translation, and ending with a TAA codon, signaling the termination of translation (156). Unlike the short strand, in which regions 1 and 3 as well as 2 and 4 existed as pairs, the open reading frames on the long strand extended over most of the genome and overlapped with each other in different reading frames (Figure 13, Table 16). Each of the open reading frames on the long strand of HBV DNA (156, 536a) is conserved in WHV DNA (158, 159), DHBV DNA (912) and GSHV DNA (913), suggesting that these larger regions possess important encoding functions. Although comparison of HBV and WHV DNA demonstrated 62-70% sequence homology, this homology was considerably lower between regions 6 and 8, corresponding to a portion of region 5 (the X gene) near the nick in the long strand (159). This low region of homology is also present upon comparison of GSHV (913) and DHBV (912) DNA sequences to HBV.

Although the significance of these differences remains to be studied, a short region of noncoding DNA between regions 5 and 8 is highly conserved in the genome of these mammalian hepatitis viruses (159, 913). In DHBV, the structure of the genome is different in this region because regions 5 and 8 are fused (912). However, a hairpin loop at the beginning of the 5/8 region in DHBV DNA exists which is similar in structure but different in sequence than the analogous hairpin between regions 5 and 8 in the mammalian viruses (912). Combined with the location of these sequences near the nick in the long strand and their palindromic structure which suggests the existence of stable hairpin loop, it is possible that these sequences might play an important role in the initiation of virus DNA replication (159).

Surface antigen gene structure and expression.

The availability of limited N- and C-terminal amino acid sequence data of HBsAg p22-25 and p26-29, combined with the nucleic acid sequence data, resulted in exact localization of the region in the HBV genome responsible for encoding these polypeptides (155-157, 177, 358, 536a, 900). A unique sequence of 57 nucleotides in the HBV genome corresponding to the N-terminal 19 amino acid residues of either HBsAg p22-25 or p26-29 was located at the third ATG initiation codon in open region 7 on the long strand of DNA. The single carboxy terminal dipeptide of p22-25 and p26-29 (264) has also been located on the viral DNA in phase with the amino terminal sequence at the end of the open region 7 just prior to the TAA codon signaling termination of translation (157). Assuming no intervening sequences, this S-gene would be 678 nucleotides in length and encode a polypeptide 226 amino acids long and approximately 25,400 daltons in size. This prediction is consistent with the finding of a major HBsAg associated polypeptide on SDS-gels in the same size range (Table 4), with the number and size of aminoethylated tryptic peptides resolved following cleavage of HBsAg p22-25 (155), and by the close agreement in amino acid composition between p22-25 and that deduced from the appropriate nucleotide sequence [155, 157 (Table 5)]. In addition to this data, any intervening sequences in the S-gene would have to be small (< 200 nucleotides) and keep the amino and carboxy terminus of p22-25 in phase, suggesting that intervening sequences probably do not exist in the S-gene. Comparison of the S-gene

sequences published to date, however, still demonstrate several differences (Figure 3). The largest differences among subtypes have been found in the central hydrophilic region spanning residues 113-143, where there are six changes between adr and adw, eight changes between ayw and adw and seven changes between ayw and adr (900) which are related to subtype differences. Quantitative amino acid analysis data obtained from protein hydrolysis or deduced from nucleic acid sequences each predict high contents of proline, tryptophan, aromatic amino acids (phe, tyr, trp), and hydrophobic amino acids [val, leu, ile; Table 5]. That these amino acids constitute greater than 50% of the composition of surface antigen polypeptide implies that surface antigen polypeptide possesses large hydrophobic regions probably locked into a tight overall globular configuration by the many disulfide bonds of HBsAg. The amino acid composition deduced from nucleic acid sequence data of WHV DNA also contains a high percentage of aromatic and hydrophobic amino acids (160), and both S-gene sequences contain considerable amounts of cysteine in their central regions (157, 160). The nucleic acid sequence of HBV S-gene predicts three regions of beta sheets (residues 1-31, 75-109 and 157-226), which include the amino and carboxy terminal domains that are highly conserved among the sequences of the different HBV DNA clones (177, 358). Although the deduced sequences of surface antigen polypeptide show little helical structure, two hydrophilic regions (residues 32-74 and 110-156) defined by beta turns and separated by a hydrophobic region make up the middle of the polypeptide. Many of the differences in sequence corresponding to changes in amino acids are located in the large hydrophilic domain (residues 110-156) and probably contribute to the antigenic heterogeneity of HBsAg.

In addition to the ATG codon defining the N-terminus of HBsAg p22-25, region 7 is characterized by two other upstream ATG codons without intervening TAA triplets (156, 167, 177, 358, 536a, 912, 913). Sequence analysis of the WHV DNA genome demonstrates an analogous genetic organization and suggests that if the major WHsAg associated polypeptide is encoded for starting at the third ATG of the open region, then a polypeptide 222 amino acids long, slightly smaller than its HBsAg analog, would be made (159). A similar relationship would be concluded from examination of the GSHV DNA sequences (913). Indeed, SDS-PAGE analysis

of HBsAg, WHsAg and GSHsAg yielded results consistent with this relationship (180, 181). The existence of this pre-S gene region in all four viruses suggests that it may encode one or more polypeptides larger than the major surface antigen component. Analogous situations have been documented with p33 and p100 of adenovirus 2 (717) and with VP2 and VP3 of SV40 (718, 719). The present data does not exclude the possibility that HBsAg p22-25 is a product cleaved from a larger precursor polypeptide initiated at any of the upstream ATG codons of region 7. Deduced amino acid sequences from the pre-S region of HBV DNA clones demonstrated larger differences than were present in the S-gene regions. Comparison of the nucleotide sequences of two HBV DNA clones further showed that one carried an additional 33 nucleotides near the first ATG in the region (358, 536a). Other clones carry similar deletions (900). The pre-S gene region of WHV, GSHV and DHBV genomes showed little homology with the analogous region in HBV DNA, suggesting no conservation during evolution. Interestingly, the deduced amino acid sequence of pre-S from some of these viruses contains a large amount of proline and no cysteine residues, suggesting the presence of a random coil with little or no secondary structure (159, 912, 913). Although it has been suggested that all of the components larger than the major surface antigen polypeptide of HBV are aggregates of the latter (206), two dimensional tryptic peptide mapping of such polypeptides associated with HBsAg, GSHsAg, WHsAg and DHBsAg (768) yielded a number of larger components containing more spots than their respective major polypeptides. That p32 and p43 of HBsAg shared all of their spots with p22-25 and that p43 shared a subset of spots present in p32 but not p22-25 suggests that pre-S may be translated. Consistent with these results is the finding of like-sized polypeptides by SDS-PAGE, possibly initiated at the first ATG (p43) or second ATG (p32) and terminating at the end of region 7. Further evidence proving the existence of these discrete surface antigen associated polypeptides has already been discussed in the context of their ability to bind polymerized serum albumin as isolated components or as part of intact HBsAg particles. The finding of an RNA transcript encompassing the entire pre-S and S-gene regions of HBV DNA following transfection of mouse L cells and the finding of a TATA-like promoter sequence preceding the first ATG codon of region 7

are also consistent with the expression of pre-S sequences (168, 680). Although the function of these sequences is not well defined, they are not required for the assembly of 22 nm spherical HBsAg, since isolated p22-25 and p26-29 have been shown to reconstitute such particles (350). A possible function of such amino acid sequences may be to provide a matrix important in the maturation and physical stability of Dane particles or, as discussed above, serve as a viral receptor for infectivity.

The regulation of HBsAg gene expression has been studied from a variety of different perspectives with the hope that a recombinant HBV vaccine could be made in the future. HBV nucleotide sequence in the pre-S gene region reveals a palindromic sequence near the unique EcoRI site which may form a hairpin loop in linear or supercoiled viral DNA (156, 643, 644). Although no role has been assigned to such a region, it is possible that the presence of this hairpin loop may regulate HBsAg expression. HBsAg expression also seems to be regulated in the hepatoma cell line, Hep 3B (146). HBsAg accumulation was not noted at low cell densities in culture supernatant, while albumin was produced and secreted. HBsAg production increased dramatically as cultures became confluent, much beyond the quantities calculated based upon lower cell densities, while albumin levels increased in accordance with an increase in cell numbers. These results suggest that HBsAg production was under cell cycle control and that the greatest production occurred during G_o or resting phase. Interestingly, HBsAg production also seems to correlate with the number of integrated copies of HBV DNA in a number of hepatoma cell lines (110), suggesting that greater HBsAg production was a consequence of more copies of integrated HBV DNA.

Numerous studies using cloned HBV DNA have attempted to better define the sequences responsible for the one or more antigenic determinants of HBsAg and the portions of the viral genome responsible for the regulation of surface antigen expression (Table 17). In one study, Dane particles isolated from a patient demonstrating a complex serotype, adyw, were used to create a family of recombinants in E. coli HB101 from several large restriction endonuclease fragments fused to the beta-lactamase signal sequence of pBR322 (178). Bacterial colonies grown on Millipore filters were lysed with phage lambda, and HBsAg was detected by binding to antibody adsorbed to polyvinyl disks followed by binding

Table 17. Systems expression one or more HBV gene products.

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
167,169	pTH 194	752 bp	Pst 1	S-gene less 5' 10 bp; 85 bp beyond 3' S-gene	pBR322
	pTH 195 λHBV1-1a	318 bp	Pst 1	S-gene less 5' 10 bp; N-terminal amino acids 11-120	pBR322, λ
	pTH 313	403 bp	Pst 1	S-gene less 5' 328 bp; C-terminal amino acids 120-226	pBR322
	pBHsAg 57	760 bp	Xho/Hpa II	intact S-gene, plus some 5' and 3' HBV sequences	pBR322
	pTH 201	318 bp	Pst 1	S-gene less 5' 10 bp; N-terminal amino acids 11-120	pBR322
174	pXba HBs-1	980 bp	Hha 1, Xba, EcoRI	S-gene less 5' 102 bp; plus downstream sequences past C-terminus	λplac5-1UV5
179	pSA4A	744 bp	HincII	S-gene less 5' 79 bp; plus downstream sequences past C-terminus	ptrpL1
171, 721	SVHBV	1350 bp	BamHI	S-gene plus flanking sequences	pBR322-SV40

Table 17 (Cont.)

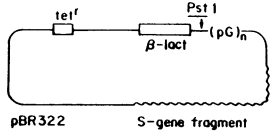
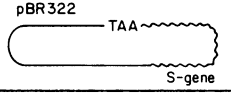
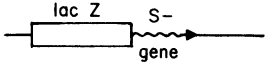
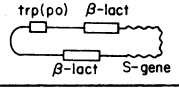
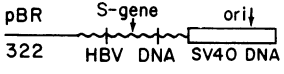
Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
β-lactamase gene (leader)	E. coli HB101	fused polypeptide; HBsAg(+)		
β-lactamase gene (leader)	E. coli HB101	fused polypeptide; HBsAg(+)	H_2N–β-lact–$(gly)_n$–HBs–COOH	
β-lactamase gene (leader)	E. coli HB101	fused polypeptide; HBsAg(+)		
not β-lactamase site	E. coli HB101	soluble polypeptide? HBsAg(+)	p22-25?	
β-lactamase gene (> leader)	E. coli HB101	fused polypeptide; HBsAg(-)	H_2N–β-lact–$(gly)_n$–HBs–COOH	Similar to pTH 195
β-galactosidase gene (lacZ)	E. coli C600 rec BC rk^-, mk^-	fused polypeptide; HBsAg(+)	H_2N–β-gal (1005 aa)–HBs–C-term. (192 aa)–COOH	
β-lactamase gene	E. coli HB101	fused polypeptide; HBsAg(+)	H_2N–β-lact (182 aa)–HBs–β-lact–COOH	
late SV40 genes	E. coli HB101; monkey kidney cells	22 nm spherical HBsAg	Fig. 1	

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
168, 170 173, 909, 927	pCP10	genome dimer	EcoRI	S-gene, pre-S and further upstream sequences intact in dimer	pBR322
722	pHBV 130-4	up to genome tetramers	BstEll	All open reading frames intact in the tandem tetramer	pBR322
724	pHBS-16	835 bp	Taq 1, Hpa 1	S-gene intact, but lacks nearly all pre-S	pBR322 derivative
726	pMSVHBs4 pMSVHBs9	2800 bp 5600 bp	BglII	HBs4 has 1 copy of S-gene; HBs9 has 2 tandem copies	pBR322-MSV
727	pTHBV-1	6364 bp	EcoRI	tandem genome dimer	pBR322
728	pAO1-HBV	3182 bp	EcoRI	pre-S region interrupted at EcoRI site in clone	pAO1
	pAO1-HBV	3182 bp	EcoRI	HBV DNA EcoRI cleaved and recircularized	pAO1

Table 17 (Cont.)

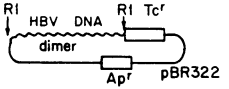
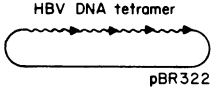
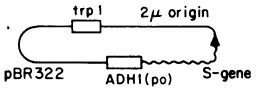
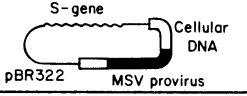
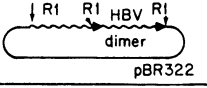
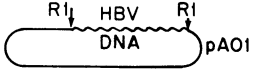
Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
EcoRI site of pBR322	E. coli DP50; with pAG0, mouse LMtk$^-$ cells	22 nm spherical and tubular HBsAg	Fig. 1	
pBR322 Bst EII	E. coli HB101; with pFG5, tk$^-$ rat2 cells	22 nm spherical HBsAg; HBcAg and HBeAg(+)	Fig. 1	
Downstream ADH1 promoter	yeast cells (XV610-8C)	22 nm spherical HBsAg; not secreted	Fig. 1	
Downstream from cloned MSV provirus genome	E. coli HB101, NIH 3T3	22 nm spherical HBsAg	Fig. 1	
EcoRI site of pBR322	NIH 3T3	22 nm spherical HBsAg	Fig. 1	
EcoRI site	NIH 3T3	no expression	-	
EcoRI site	NIH 3T3	22 nm spherical HBsAg	Fig. 1	Recircularized HBV DNA

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
	pKKHBs34	2800 bp	BglII	Has pre-S and S-gene regions intact	pKK92cZ
	pBRHBV9	∿ 1300 bp	BamHl	Pre-S region interrupted at BamHl site in clone	pBR322
175	pHBV-1	3200 bp	EcoRI	HeLa cells transfected with recircularized ligated HBV DNA	pBR322
168	pCP9	3182 bp	EcoRI	Pre-S region interrupted at EcoRI site in clone	pAGO
	pAC1	2337 bp	BglII	Pre-S region plus S-gene intact	pAGO
	pAC2	∿ 2700 bp	BglII	Pre-S region plus S-gene plus some upstream sequences intact	pAGO
	pAC3	3182 bp	BglII	Pre-S, S-gene, and more upstream sequences intact	pAGO
	pANC1	3182 bp	BamHl	Downstream of S-gene not intact	pAGO

Table 17 (Cont.)

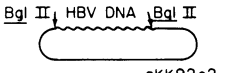
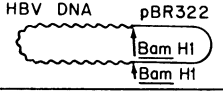
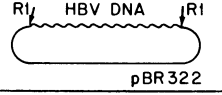
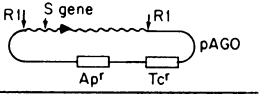
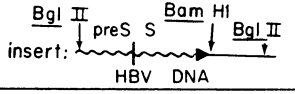
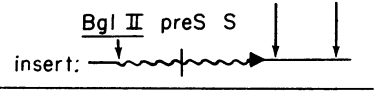
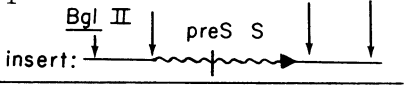
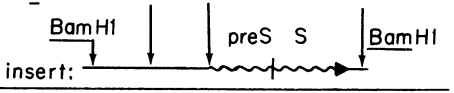
Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
BglII	NIH 3T3	22 nm spherical HBsAg	Fig. 1	
BamHI	NIH 3T3	no expression	-	
EcoRI site	E. coli x 1776, HeLa cells	22 nm spherical + tubular HBsAg, Dane like particles	Fig. 1	
EcoRI site	mouse LMtk$^-$ cells	no expression	-	
BamH1 site	mouse LMtk$^-$ cells	no expression	-	
BamH1 site	mouse LMtk$^-$ cells	22 nm spherical HBsAg	Fig. 1	
BamH1 site	mouse LMtk$^-$ cells	22 nm spherical HBsAg	Fig. 1	
BamH1 site	mouse LMtk$^-$ cells	no expression	-	

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
167, 177, 178	pHBV-64 pHBV-66 pHBV-138 pHBV-139 pHBV-110	varies (1900-2400 bp)	BamHl, Kpnl	many recombinants contain stop, restart sequence in HBV DNA at start of core gene	pBR322
179	pCA246	1005 bp	Hhal	two ATG codons near start of core gene in recombinant	ptrpL1
176	pKH81	1037 bp	BamHl	low amount of core production may be due to stop, start codons in core gene	pKH80
172, 737 738, 799	pHBV-R1-1 to pHBV-R1-11, pH80	varies	Pstl	beta-lactamase gene and UV5 promoter 5' to core sequences	pBR322 derivative pEX150
915	pTRPSS-6	809 bp	Sau3A-HpaI	entire S gene.	pTRP801
	pTRPSS-39	744 bp	HincII-HpaI	S gene minus N-terminal 20^{+} amino acids	pTRP801
	pTRPSS-50	712 bp	XbaI-HpaI	S gene minus N-terminal 30^{+} amino acids	pTRP801
917, 918	pSVHBSA	1179 bp	EcoRI-BamHl	entire S gene + some pre-S to EcoRI site	pSVR
919	pSH6, pSH18	2800 bp	BglII	entire genome except core	pSV010

Table 17 (Cont.)

Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
PstI in β-lactamase	E. coli HB101	fused polypeptide; HBcAg(+)	H_2N—β lact.—$(gly)_n$—HBc—COOH	tet^r β-lact (pG)$_n$ pBR322
ClaI site	E. coli HB101	HBcAg polypeptides p19 & p22	core antigen polypeptide complexes	trp (po) core gene ptrpL1
BclI of pKH80	E. coli K-12 strain HB101; B. subtilis MI119	free core polypeptide	major core polypeptide	Km^r Em^r core Em^r pBD9 pBR322 HBV DNA partial
EcoRI or HindIII	E. coli DS410 (minicells) E. coli HB101	intact core particles	core antigen like particles	Pst I core Pst I HBV DNA pBR 322
ClaI ClaI ClaI	E. coli K-12 strains C600 and P678-54	(SS-6) low level HBsAg p22-24 polypeptides. (SS-39 and 50) higher level p22-24 polypeptides.	HBsAg p24 in lysate N-terminal truncated HBsAg p24	trp(po) Tc^r Cla I S-gene Ap^r or Fragment
in place of SV40 VP1	monkey CV-1 cells	22 nm spherical HBsAg	Fig. 1	SV40 pBR322 ori R1 Ap^r S-gene BamH1
BglII	E. coli HB101, COS (CV-1) cells	22 nm spherical HBsAg	Fig. 1	HBV DNA SV40 ori pBR322

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
920	p311E, p311L,	1986 bp	EcoRI, BglII	insert from EcoRI site in pre-S, through S-gene, plus downstream sequences	pML (pBR322)
921	LSV-HBsAg	1273 bp	TaqI, BamHI	S-gene.	pLSV
	LSV-HBpresAg	2181 bp	AvaI, BamHI	pre-S + S gene	pLSV
927	pAG85	2330 bp	BglII	S-gene + flanking bp; pAG85 near TK promoter (p2)	pAGO
	pAG83	2330 bp	BglII	S-gene + flanking bp; pAG83 near HSV p3 promoter	pAGO
	pAG59	2361 bp	RsaI, HincII	pre-S + S gene.	pAG60
	pAG61	1662 bp	XbaI, ClaI	pre-S/S gene + some downstream sequences.	pAG60
	pAG66	2587 bp	XbaI, TaqI	pre-S/S gene + 400 bp downstream more than pAG61.	pAG60

Table 17 (Cont.)

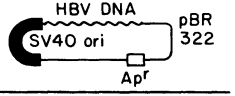
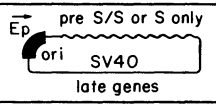
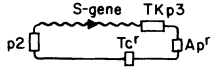
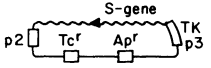
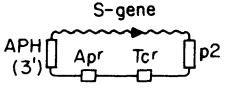
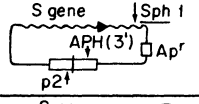
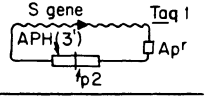
Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
EcoRI, BglII	E. coli, COS (CV-1) cells	22 nm spherical HBsAg	Fig. 1	
BclI, HindIII	COS (CV-1) cells	22 nm spherical HBsAg	Fig. 1	
BglII	vero cells	22 nm spherical HBsAg	Fig. 1	
BglII	vero cells	22 nm spherical HBsAg	Fig. 1	
SphI	vero cells	22 nm spherical HBsAg	Fig. 1	
SphI	vero cells	no HBsAg	Fig. 1	
TaqI	vero cells	22 nm spherical HBsAg	Fig. 1	

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning site	other characteristics	cloning vehicle
916	pAH203	3200 bp	XhoI	whole HBV genome.	pAM82
	pAH301	1300 bp	XhoI, BamHI	HBsAg gene.	
928	pYeHBsd	870 bp	EcoRI, HindIII from pHS94	S-gene + 3' untranslated region	YEp13
930	pMZ6V4	2743 bp	BglII	S-promoter, pre-S/ S gene sequences.	pML2
931	pBPVHBsL/1, pBPVHBsR/8	2300 bp	BglII	S promoter, pre-S/ S gene sequences.	$pBPV_{T69}$
934	pHBs2, pHBs4	1350 bp	BamHI	S-gene only; expression under early promoter.	vaccinia virus
	pHBs1, pHBs3	1350 bp	BamHI	S-gene bp in opposing orientation to promoter.	vaccinia virus
	pHBs5	1350 bp	BamHI	S-gene controlled by TK promoter.	vaccinia virus

Table 17 (Cont.)

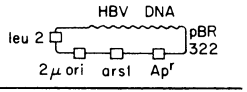
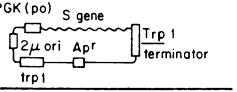
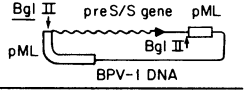
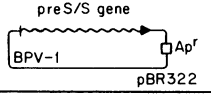
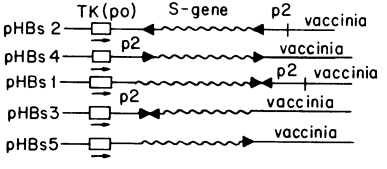
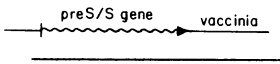
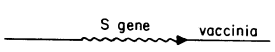
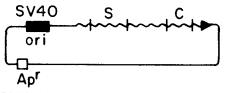
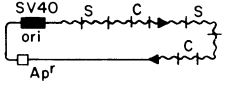
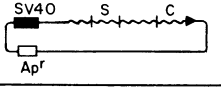
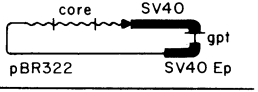
Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
XhoI	yeast AH22	22 nm spherical HBsAg	Fig. 1	
HindIII, BamHI	yeast strains, 20 B-12, XV610-8C and GM3C-2	p25 HBsAg + 22 nm spherical HBsAg	Fig. 1	
BamHI	E. coli HB101, C127 cells	22 nm spherical HBsAg	Fig. 1	
BamHI BamHI	E. coli HB101, NIH 3T3	22 nm spherical HBsAg	Fig. 1	
BamHI	CV-1 cells	22 nm spherical HBsAg	Fig. 1	
BamHI	CV-1 cells	no HBsAg produced		
BamHI	CV-1 cells	no HBsAg produced		

Table 17 (Cont.)

Study	fragment designation	HBV insert: size/ length	HBV cleavage/ cloning sitee	other characteristics	cloning vehicle
935	vP11	2300 bp	BglII	pre-S/S-gene intact.	pDP120-vaccinia
	vP59	1100 bp	HhaI	S-gene only.	pDP120-vaccinia
940	pSH2.1	3200 bp	EcoRI	single copy HBV DNA insert.	pSV08
	pSHH2.1	6400 bp	EcoRI	HBV DNA dimer.	pSV08
	pSH2.6	3584 bp	HindIII-HaeII	fragment of pSHH2.1.	pSV08
798	pKYC-200	1850 bp	BamHI	core + flanking sequences	pSV2 neo, pSV2 gpt

Table 17 (Cont.)

Insertion site (in plasmid)	propagation	expression	structure of product	structure of recombinant
BamHI of HindIII fragment of vaccinia	CV-1 cells	little HBsAg	Fig. 1	
		22 nm spherical HBsAg		
EcoRI	COS cells	22 nm spherical HBsAg	Fig. 1	
EcoRI	COS cells	low HBsAg	Fig. 1	
HindIII + HaeII	COS cells	low HBsAg	Fig. 1	
BamHI	NCI H292 (HuT 292)	core by RIA and immunofluorescence	not characterized	

of radioactive antibody to the solid phase complex. HBV fragments in positive clones were cleaved to smaller sizes and recloned in pBR322 (167, 169). Although RIA was again used for HBsAg detection, the ability of cell lysates from these recombinants to generate detectable anti-HBs in rabbits was found to be a more reliable assay. Recombinant pTH194, fused to the beta-lactamase signal sequence and containing all except the first 10 nucleotide bases of the S-gene, yielded anti-a and anti-y. pTH195 and lambda HBV 1-1a recombinants, containing approximately the 5' 50% of the S-gene fused to the beta-lactamase signal sequence, yielded anti-a and anti-d. pTH313, containing approximately the 3' 50% of the S-gene in a similar construction, yielded anti-a only. Recombinants including larger portions of the beta-lactamase sequences fused to HBsAg sequences, yielded fused products which did not give rise to antibody (e.g. pTH 201). Interestingly, only one of several recombinants containing the entire S-gene joined to another site in pBR322 yielded bacterial extracts resulting in the production of anti-HBs. Although polypeptides made under these conditions contained little extraneous sequences, their ability to compete against native HBsAg was low, and the anti-HBs raised in vivo was usually of low titer, suggesting that either lack of HBsAg glycosylation in E. coli or the state of physical aggregation in bacterial cells, or both, accounted for these results. Insertion of the HhaI plus XbaI generated fragment of S-gene, which includes all except the 5' 102 base pairs encoding the major HBsAg polypeptide, into a derivative of bacteriophage lambda (λplac5-1UV5) near the 3' end of lacZ resulted in the production of a 138,000 dalton beta-galactosidase-HBsAg fusion polypeptide with antigenic determinants of both HBsAg and beta-galactosidase (174). Although the molecule could be precipitated by anti-HBs, suggesting that native conformation was present in a detectable percentage of fusion products, immunogenicity was not tested. In an attempt to increase the production of HBsAg as a fused product in a prokaryotic system, the HincII fragment of S-gene, including all sequences except the 5' 66 base pairs, was fused to the beta-lactamase gene in a pBR322 plasmid containing the promoter-operator sequences of the bacterial trp operon (179). Relatively large quantities of overproduced beta-lactamase-HBsAg fusion protein was detected at 41,000 daltons on

SDS-polyacrylamide gels (179). Unlike the beta-galactosidase-HBsAg fusion product which represented approximately 0.05% of the total bacterial cell protein (174), the beta-lactamase-HBsAg fusion product represented about 8.5% of the total protein (179). The latter was immunoprecipitated with anti-HBs, and precipitation was totally inhibited by addition of cold HBsAg, although again immunogenicity was not assayed. Although the production of fused polypeptides helped to protect HBsAg sequences from being degraded by bacterial enzyme systems, the physical state of these fused products is unlike that of p22-25 and p26-29 in 22 nm spherical HBsAg particles, suggesting that their immunogenicity, like that of denatured HBsAg polypeptides, will probably be low--precluding use of these products in their present form for vaccine production.

Among the problems encountered studying HBsAg (or other gene) expression in E. coli are the possible detrimental effects of protein production upon bacterial cell growth, proteolytic degradation of the fused or free polypeptide, and/or suboptimal expression of the gene in a new environment (174, 179, 914, 915). A family of expression vectors was constructed from pTM778, a plasmid containing a tryptophan operon; pBR322 sequences; and HBsAg adw gene sequences derived from pHBV 933, a plasmid containing an EcoRI HBV DNA insert (915). Recombinants containing the complete S-gene (pTRPSS-6) or fragments lacking 60-100 bp from the 5' end of the S-gene (pTRPSS-39 and pTRPSS-50) corresponding roughly to the amino terminal hydrophobic domain of HBsAg p22-25 were assayed for HBsAg expression under trp operon control in E. coli (915). Among positive recombinants, the full-length S-gene inset (pTRPSS-6) partially inhibited colony growth, while the colonies containing the truncated S-gene constructs grew normally, suggesting that the N-terminal hydrophobic domain may embed in the bacterial membrane and in some way inhibit growth. With the truncated constructs, the expression levels in minicells from culture broth ranged 20-45 ng/ml (915), which is comparable to that recently observed under the control of a yeast dehydrogenase I promoter (916), suggesting that direct expression of HBsAg could be achieved in bacteria at levels comparable to some eucaryotic systems. In another attempt to circumvent these limitations, a two plasmid system capable of rapidly producing relatively

large quantities of a foreign protein in free or fused form, has recently been described (914). In this system, the gene of interest is expressed under the control of the E. coli lacZ regulatory region of a plasmid (pJJS1011) which exhibits runaway replication at 43°C but not at 30°C. The other plasmid (pJJS1002) contains a temperature sensitive lac repressor gene which regulates foreign gene expression on the runaway plasmid at low temperatures (914). In this fashion, synthesis of relatively large quantities of some HBV gene products could be made for use in further understanding its molecular biology (914).

The use of recombinant DNA technology to express HBsAg in mammalian cells has resulted in the production of both antigenic and immunogenic small spherical HBsAg particles. A 1350 base pair BamHI fragment of HBV DNA (approximately 40% of the genome), including a portion of the pre-S region, the entire S-gene, and some downstream sequences, was cloned into a pBR322-SV40 plasmid in the region of the SV40 late genes (171, 721). The presence of pBR322 sequences permitted propagation in E. coli, and the presence of SV40 sequences permitted expression upon lysis in African green monkey kidney cells. Approximately 45% of the monkey kidney cells infected with the SVHBV recombinant were positive for cytoplasmic HBsAg after 72 hours, and up to 1 µg of surface antigen was detected in the culture media (171). Partial characterization of the released material showed that it shared morphology, density and sedimentation characteristics with small spherical HBsAg. These results have since been independently confirmed (917, 918). Immunoprecipitation, followed by SDS-PAGE, demonstrated the presence of p22-25, p26-29 and, on some gels, p49.

The strict packaging requirements of SV40 DNA recombinants into mature particles and the very limited time in which molecular events could be studied restrict the usefulness of SV40 lytic vectors. Therefore, a number of investigations have analyzed HBsAg expression using nonlytic pBR322-SV40 recombinants in which HBV sequences have been inserted into the early region of SV40 (918-922) and the defective recombinants amplified and expressed transiently in SV40 transformed CV-1 cells (COS cells) which produce T antigen (923-925). Small spherical HBsAg particles were synthesized and secreted by COS cells transfected with S-gene containing plasmids pHBs311-E and pHBs311-L

which differ only by the orientation of the SV40 origin of replication with respect to early and late promoters, respectively, transcribing the S-gene (918). When COS cells were transfected with either of two plasmids, pSH6 or pSH18, which contained in opposite orientations the large 2.1 kb fragment of HBV DNA with all sequences except the core gene, HBsAg was made and secreted (919). Such particles possessed a pair of major polypeptides at 25,000 and 29,000 daltons that were immunoprecipitable with anti-HBs. Approximately 40-50 μg HBsAg/10^7 cells was produced, which is 10-15 fold more material than that reported in earlier studies (113, 171, 173, 175). Insertion of the S-gene into a plasmid where expression was controlled by a 311 bp fragment of the SV40 origin of replication or by a larger 342 bp fragment containing the SV40 72 bp repeat enhancer sequences resulted in HBsAg detection in COS cells by indirect immunoflourescence, RIA or isotopic labeling in most cases (920). Recombinants designated p342E, p342L and p311L, directing HBsAg synthesis from early or late promoter sequences within opposite orientations of the SV40 origin of replication, were positive for HBsAg expression. The failure of some recombinants to produce surface antigen, including pML-HBs which lacks SV40 sequences, and p311E, which lacks an intact 72 bp repeat, suggests that SV40 sequences are required for HBsAg expression but that the SV40 72 bp repeat is not required. The mapping of the 5' terminus of the mRNA producing HBsAg to within the SV40 late promoter, combined with the fact that deletion mutants of the 72 bp repeat enhancer sequences in p342E still expresses HBsAg at levels comparable to intact p342E, suggest that transcription begins within the SV40 origin and that heterologous enhancer elements may be present in the HBV DNA fragment (920). The latter point is supported by the finding that the intact 72 bp repeat region seems to be essential for both early and late SV40 gene expression (926). In order to assess the role of pre-S sequences in surface antigen production, SV40-HBV recombinants were made so that HBV sequences spanning the S-gene (in LSV-HBsAg) or the entire pre-S/S region (in LSV-HBpresAg) were placed under the control of the early SV40 promoter which included the 72 bp repeat sequences (921). COS cells transfected with these recombinants demonstrated similar levels of plasmid DNA replication, yet LSB-HBsAg expressed about four-fold more HBsAg specific RNA than did LSV-HBpresAg. Further,

LSV-HBsAg produced and secreted up to 800 ng of HBsAg/10^6 cells while LSV-HBpresAg yielded 40-50 fold less HBsAg under identical experimental conditions (921). In comparison to the PLC/PRF/5 cell line, LSV-HBsAg produced about 10-fold more HBsAg, suggesting efficient expression under the SV40 early promoter. To account for these differences, the long pre-S antigen leader could attentuate both the SV40 early promoter and the S-gene promoter in LSV-HBpresAg and/or the presence of RNA processing signals within the pre-S region could result in an increased rate of transcript turnover (921). Clearly, the relatively high levels of surface antigen RNA and protein production by these recombinant molecules in COS cells will continue to yield greater understanding as to the nature of HBsAg expression.

An alternative nonlytic system for the expression of surface antigen has been developed using the bovine papilloma virus (BPV) DNA replicon in mouse cells (930-932). In one study, BPV-1 DNA was separated from the pre-S/S gene and presumptive S promoter by pBR322 (pML) sequences so that the recombinant plasmid could be replicated in prokaryotic and eucaryotic cells (930). Transfection of C127 mouse cells with the plasmid pMZ6V4 possessing these characteristics resulted in clones which secreted 50-200 ng HBsAg/10^6 cells/24 hours. These levels are comparable to those reported from integrated HBV DNA (173) or under control of the SV40 late promoter (171). Small spherical particles similar to serum derived HBsAg were found, and SDS-gel analysis of ^{125}I labeled immunoprecipitates demonstrated the presence of the major nonglycosylated and glycosylated components. Although the viral nucleic acid remained episomal, restriction enzyme analysis of the DNA from these clones compared to that of the input plasmid suggests substantial rearrangement of sequences (930). Since comparable levels of HBsAg expression were demonstrated independent of the BPV-1 DNA orientation in the plasmid, it is likely that HBsAg expression was under control of its own promoter and that the RNAs produced may be similar to <u>in vivo</u> transcripts (930). Using a similar recombinant, independent workers transfected NIH 3T3 cells with a plasmid containing the pre-S/S gene regions, the 69% transforming fragment of BPV-1 DNA and pBR322 sequences (931). A peak of HBsAg production was observed five to ten days post-transfection, then dropped to intermediate levels and rose again with the appearance of

foci two weeks later. By manipulating culture conditions of individual clones, HBsAg accumulation was linear with time and reached 39 $\mu g/10^7$ cells/week with one clone. Again, this was independent of the respective HBV-BPV DNA orientations in the transfecting plasmid. HBsAg was secreted as small spherical particles similar to those obtained from infected sera, was immunogenic in guinea pigs, contained polypeptides of molecular weights 24,000, 28,000, 34,000 and 37,000 daltons, and was produced at rates approximately 20-fold higher than observed in the PLC/PRF/5 cell line (931).

Mutant mouse L cells, deficient in thymidine kinase ($LMtk^-$), were used to study surface antigen gene expression by cotransformation of cultures with EcoRI HBV DNA ligated to pBR322 (pCP10) and with plasmid pAGO carrying the cloned herpes simplex virus tk gene (170, 173). Since the site of HBsAg transcription initiation has not yet been precisely mapped and since the unique EcoRI site of HBV DNA is within the pre-S region about 150 bp upstream from the S-gene, pCP10 was constructed as a dimer of HBV DNA in a tandem head-to-tail arrangement. Twenty days after cotransformation and HAT selection, HBsAg, but not other virus markers, was detected in infected cells and in culture media. Particles resembling small spherical HBsAg were produced by one clone of cells at about 150 ng antigen/ml/day at saturation density. This is comparable to rate of HBsAg production in the PLC/PRF/5 hepatoma cell line, which is about 250 ng antigen/ml/day under the same conditions (113, 720). Attempted cotransformation of $LMtk^-$ cells with a plasmid containing one EcoRI HBV DNA fragment did not result in HBsAg production, suggesting that the integrity of viral sequences prior to the S-gene was necessary for expression. Analysis of the state of HBV DNA in HBsAg producing mouse L cells showed that it was integrated at multiple sites in the mouse genome. Although the structure of the integrated sequences has not yet been determined, these cells share this property with all of the HBV associated human hepatoma cell lines studied. The constitutive expression of HBsAg in pCP10 infected mouse cells was not observed when other cell lines were tested (927). For example, Vero cells cotransfected with pCP10 and pAG60, the latter vector containing the dominant selective marker aminoglycoside 3'-phosphotransferase (APH(3')), resulted in cells which produced HBsAg transiently between 10 and 30 days after

transfection. To exclude the role of other HBV DNA sequences or gene products in affecting HBsAg expression, fragments of pCP10 containing the S-gene and variable flanking sequences were inserted into pAG60 or tk positive pAGO. Transfection of Vero cells resulted in the same later transient expression as that observed with pCP10, although the levels of expression lower and the duration shorter. Expression of these plasmids in other cell lines of human or rabbit origin also differed from that observed with mouse cells, suggesting that expression is cell type dependent. Further, transient expression seems to occur whether the HBsAg coding region is under the control of the herpes virus tk promoter or its own promoter, suggesting that the S-gene promoter is not responsible for such an expression pattern. Repression of stable HBsAg synthesis was not due to a trans-acting substance, since Vero cells transfected with a recombinant in which transient expression had already passed could be transfected again with the same recombinant, resulting in another round of transient expression. In addition, mouse cells transfected with the same recombinant(s) stably produced HBsAg, and this characteristic could be maintained even after fusion with Vero cells. Finally, the transient nature of HBsAg expression may be related to the fact that integrated copies of the recombinant plasmids had highly rearranged S-gene sequences which may have occurred following the initial integration event (927).

Independent studies using pBR322 recombinant plasmids containing up to four HBV genome copies in tandem head-to-tail configurations were used to cotransform a tk^- rat 2 fibroblastic cell line or mouse $LMtk^-$ cells with herpes simplex thymidine kinase gene as a selectable marker (722). A plasmid containing four tandem, head-to-tail HBV genome copies (pHBV130-4) cotransformed rat 2 cells and resulted in a stable clone which produced and secreted small, spherical HBsAg particles. Like the Hep 3B (146) and PLC/PRF/5 (723) hepatoma cell lines, Rat 2/130.4/TK4 secreted little HBsAg at low cell densities and much higher quantities than expected at high cell densities. Mouse $LMtk^-$ cells cotransfected with the same recombinant plasmid also yielded a number of clones synthesizing HBsAg particles (722).

In addition to mammalian cells, HBsAg particles have recently been produced in yeast (724). Here, an 835 bp genome fragment, isolated

following double digestion of HBV DNA with Taq 1 and Hpa 1, contained an intact S-gene and some flanking sequences. This fragment was ligated next to the promoter for yeast alcohol dehydrogenase (ADHI) in a pBR322 fragment also containing the yeast 2μ replication origin and the trp 1 gene for selection in yeast cells. Roughly 2-5 μg of surface antigen per 200 ml of yeast cell culture was detected only in cell extracts from mid-log phase cultures. Although no surface antigen was secreted, the extracted particles shared most of their properties with HBsAg produced by the PLC/PRF/5 cell line. SDS-PAGE showed a single immunoprecipitable band from these yeast cells which comigrated with the major nonglycosylated component of HBsAg. That the yeast material was as antigenic and immunogenic as authentic HBsAg, even though the major glycosylated component was absent on SDS gels, suggested that glycosylation is not required for either formation of particulate HBsAg nor for immunogenicity (724). Immunization of mice, grivet monkeys and chimpanzees with a single 40 μg dose of alum adsorbed recombinant HBsAg resulted in high anti-HBs titers which were maintained at least one year (929). Four vaccinated chimpanzees challenged with infectious human plasma were each protected from HBV infection, while four unvaccinated chimps remained susceptible, indicating that sufficient neutralizing determinants on HBsAg p25 were present for protection to be conferred (929).

The possibility of using yeast generated HBsAg as a recombinant vaccine has provided the impetus for further study with different recombinant molecules. Using a shuttle vector containing pBR322 sequences necessary for amplification in E. coli and yeast DNA sequences which permit HBsAg expression under the control of the repressible acid phosphatase promoter, moderate quantities of HBsAg were detected following lysis of the recombinant containing yeast (916). Approximately 2-3 μg of HBsAg was produced per ml of yeast lysate, corresponding to 400,000-450,000 molecules of HBsAg p25 synthesized by each cell. The HBsAg produced was similar to small spherical particles derived from serum, was precipitated by anti-HBs and was immunogenic in guinea pigs (916). Similarly, expression of the S-gene under control of the highly active yeast 3-phosphoglycerate kinase (PGK) promoter produced HBsAg p25 approximately 1-2% of the total cellular protein (928). About 2-5%

of this p25 was aggregated in the form of 22 nm small spherical HBsAg particles. Yeast transfected with the recombinant pYeHBsd in which the S-gene was sandwiched between the PGK promoter and the yeast trp1 gene terminator generated a unique RNA species. Radiolabeling of the cells with ^{35}S-methionine or cysteine followed by gel electrophoresis yielded a band analogous to HBsAg p25, but no major glycosylated component at p29 (928). Such a band was immunoprecipitated with anti-HBs raised against native particles. The low amounts of small spherical HBsAg produced by the recombinant yeast were immunogenic in mice and yielded anti-HBs titers equal to or exceeding those of native particles without adjuvant, although their yield and presence of yeast proteins outline problems for future use.

The BglII fragment of HBV DNA encompassing the entire genome except the major core antigen polypeptide encoding sequences was cloned into a pBR322 vector containing the integrated proviral form of Maloney mouse sarcoma virus (MSV). Transformation of mouse NIH 3T3 fibroblasts by such recombinants resulted in the establishment of several stable cell lines producing 22 nm spherical HBsAg particles (726). Cell line J1, derived from cells transfected with the recombinant plasmid pMSVHBs4 containing a single BglII HBV DNA insert, secreted 20 times more HBsAg particles than the PLC/PRF/5 line under identical maintenance conditions. Another cell line, J2, derived from cells transfected with the recombinant plasmid pMSVHBs9 containing two copies of the BglII HBV DNA in tandem, produced 3-4 times the amount of HBsAg than the PLC/PRF/5 line. Although expression of HBsAg in J1 was similar to that observed using BPV DNA as a vector (726) or upon cotransfection of the HBsAg gene with the dehydrofolate reductase gene (727), high levels of expression in the pBR322-MSV system may be due to the presence of the retroviral LTR sequences (635), while in the other situations increased expression has been attributable to amplification of the S-gene sequences (726, 727). Analysis of the secreted HBsAg particles by PAGE showed the major pair of polypeptides at 24,000 and 28,000 daltons, as well as two higher molecular weight components at 34,000 and 37,000 daltons (726). The particles were also shown to be immunogenic in guinea pigs, resulting in anti-HBs titers greater than 512 after 6 weeks.

The apparent requirement for a selectable marker in cells cotransfected with a plasmid containing S-gene has been eliminated by using recircularized HBV DNA isolated from recombinant plasmids (175, 728, 933). EcoRI cleaved HBV DNA cloned as a single copy in pBR322 (pHBV-1) was cut by the same enzyme into full-length molecules and recircularized by T4 ligase (175). When this material was used to transfect HeLa or mouse L cells, cytopathic changes were observed and both intra- and extracellular HBsAg was detected (175, 933). pHBV-1 or the EcoRI digested products of the clone did not result in surface antigen gene expression in HeLa cells, suggesting that HBV DNA could act as an infective agent only when it is in the form of a 3200 bp double-stranded circle (175). HBV DNA cloned at its unique EcoRI site into a plasmid vector pA01 resulted in no detectable HBsAg expression following transfection of NIH 3T3 mouse fibroblasts (728). However, when the same HBV DNA was excised with EcoRI and recircularized prior to transfection, HBsAg particles were detected in culture media within two days (728). The requirement of an intact pre-S gene region for surface antigen production was confirmed using another recombinant containing the large BglII fragment of HBV DNA (728). The failure to see HBsAg expression following transfection of NIH 3T3 cells with the S-gene containing BamHI fragment of HBV DNA in pBR322 suggests that the surface antigen promoter resides somewhere in the BglII-BamHI fragment near the beginning of the pre-S region or in sequences upstream from it.

The use of vaccinia virus as a eucaryotic expression vector has prompted development of live vaccines expressing HBsAg (934, 935). In this system, HBsAg encoding sequences are inserted in place of the vaccinia tk gene, and expression is controlled by the tk promoter or another early promoter so that translation would be initiated from within the insert (934). In a family of such constructs, pHBs2 and pHBs4 contain S-gene sequences under the control of an early promoter translocated to the tk region of the genome (Table 17). Wild type vaccinia virus infected CV-1 cells transfected with these recombinants resulted in easily detectable HBsAg in the cell extract (1.66-1.86 μg HBsAg/10^7 cells/24 hrs.) and culture medium (3.4 μg HBsAg/10^7 cells/24 hrs.). pHBs1 and pHBs3, having S-gene sequences in opposite orientation with respect to the translated early promoter so that the latter occurs after the S-gene, generate barely

detectable levels of HBsAg. In pHBs5, where the S-gene is under control of the tk promoter, small quantities of HBsAg were found in the culture medium (0.16 μg/10^7 cells/24 hrs.). HBsAg produced was in the form of particles with a density of 1.2 g/ml, showed characteristic major polypeptide bands at 23,000 and 25,400 daltons which were immunoprecipitable with anti-HBs, and was highly immunogenic in rabbits following intradermal inoculation (934). Similarly, recombinants were made from a tk^- vaccinia variant, in which a HindIII fragment from the F portion of the vaccinia genome was combined with pDP120, a pBR322 derivative and either S-gene (vP59) or pre-S/S-gene (vP11) sequences (935). vP59 in transfected CV-1 cells produced 1.5-2.0 μg HBsAg/10^7 cells/24 hrs. under vaccinia control, most of which was secreted. These amounts were several thousand times greater than cells similarly transfected with vP11. Again, the biophysical properties of such HBsAg particles produced were indistinguishable from serum derived particles and they were highly immunogenic in guinea pigs (935). The low cost, ease of administration and high antibody titers associated with such recombinant vaccinia virus vaccines suggest that they would be useful not only against HBV, but against a variety of other infectious agents (936). However, since a large proportion of the world population has already been vaccinated with vaccinia to eradicate smallpox, revaccination would probably lead to limited recombinant growth and expression, meaning that HBsAg production and the anti-HBs response would probably be low, and the effectiveness of such a vaccine questionable. Only further work will be able to discern whether another vector will be more suitable.

Analysis of the nucleic acid sequence of HBV DNA in the segment upstream from the first methionine initiation codon of the pre-S region (156, 177, 536a, 900) reveals a TATA-like sequence 72 bp 5' from this point (159, 168). It has been postulated that the sequence TATAAATA or a variant thereof, like the Pribnow box sequences which bind E. coli RNA polymerase (729), serves as a site near animal virus and eucaryotic genes for RNA polymerase binding (730, 731). Further, this proposed binding site seems to occur just prior to the site of transcription initiation (729-731), suggesting that transcription of the S-gene and pre-S region might initiate near this point in the genome. Independent sequencing of HBV DNA, however, demonstrates a TATA-like box 184 bp 5'

to the initiator methionine codon of the S-gene in the pre-S region, suggesting, instead, that transcription may initiate near this site (155). The nucleic acid regions responsible for initiation of HBsAg transcription and promotion of surface antigen expression were further localized by studying HBsAg production in mouse LM cells transfected with a series of recombinant plasmids containing the S-gene and increasing amounts of contiguous pre-S and upstream sequences (168). Plasmid pCP10, containing a head-to-tail tandem genome dimer in which the S-gene, pre-S region and upstream sequences were intact, resulted in HBsAg production. Plasmid pCP9, containing a single copy of the S-gene and pre-S region sequences up to the EcoRI site, was negative for HBsAg production. The plasmid pAC1, containing an intact S-gene and pre-S region and terminating at the BglII site near the start of the pre-S region, was also negative for HBsAg, even though the whole of genome region 7 was intact. Construction of pAC2, which included the genetic formation in pAC1 plus a number of upstream sequences 5' to the pre-S region and ending at the next BglII site, was HBsAg positive (Figure 11). The inclusion of further upstream sequences (to the next BglII site) in pAC3 also yielded HBsAg. Although this localizes the promoter region for surface antigen in the approximately 400 bp BglII fragment just 5' to the pre-S region, the lack of HBsAg expression from the plasmid pANC1 lacking some sequences downstream from the S-gene that the positive recombinants retained suggests that they are also important for surface antigen mRNA biogenesis (168). The possibility of the S-gene promoter being at the TATA box 72 bp upstream from the beginning of the pre-S region, as suggested above, has been called into question by the finding that the 2.4 kb surface antigen associated RNA made in mouse or rat cells transfected with a plasmid containing a head-to-tail tetramer of HBV DNA contains hybridizing sequences extending upstream from this point (942). Either the nature of the cells transfected or the recombinants used account for these apparent differences, or it is possible that S-gene promotion occurs from variant TATA-like sequences located at 103 bp or 158 bp upstream from the start of the pre-S region (942). Precise mapping of RNA species from infected liver tissue will be needed to discern both the RNA initiation site(s) and the most probable promoter sequences responsible for S-gene expression.

Characterization of the polyadenylated mRNAs transcribed from HBV DNA or DNA fragments following transfection of mouse LMtk$^-$ cells with recombinant plasmids showed a major species at 2.3 kb (168). Using RNA from cells containing pCP10, additional but weaker HBV specific bands were detected at 4.3 kb and 0.9 kb. A major band at 2.3 kb was also detected in extracts from other HBsAg producing recombinants and from the human hepatoma cell line PLC/PRF/5 but not from other recombinants which failed to produce surface antigen in mouse LMtk$^-$ cells. Mapping of this large mRNA to the virus genome with various restriction endonuclease fragments of HBV DNA as probes showed that transcription initiation was located just upstream from the pre-S region within the BglII fragment containing the TATA box and the promoter for surface antigen gene expression. Further analysis showed that transcription continued through the pre-S region, the S-gene and for about another 1900 bp after the S-gene ending somewhere in the region of the nick in the long minus strand of DNA. No transcripts corresponding to core antigen gene were detected. Interestingly, the lack of HBsAg production by cells transfected with pANC1, which, by construction, does not have sequences which would encode the 3' end of such a transcript, could be due to the absence of polyadenylation of a shorter transcript.

A number of other investigations have been directed toward better characterizing the nature of transcription in the hepatoma cell line PLC/PRF/5. HBV DNA aliquots digested with different restriction endonucleases, separated by agarose gel electrophoresis and transferred to nitrocellulose were detected by ^{32}P-labeled PLC/PRF/5 RNA (114). The detection of all restriction fragments by the labeled RNA suggests that all of the HBV DNA sequences are transcribed in the cell line. Independent analysis of the HBV specific RNA's produced by the same cell line resulted in a major species detected at approximately 2.0 kb with an AluI fragment of the HBV genome, which includes all of the S-gene sequences, a portion of pre-S (300 bp) and 700 bp beyond the termination of the S-gene (115). Somewhat different results were obtained using poly A$^+$ and poly A$^-$ fractions of RNA from the same cell line (116). Cytoplasmic poly A$^+$ RNA separated into individual species by gel electrophoresis, blotted onto diazobenzyloxymethyl (DBM)-cellulose paper, and detected by a ^{32}P-labeled HBV DNA probe revealed two bands

at 3.05 kb and 2.5 kb. Total nuclear RNA (poly A^-) showed an RNA species at 4.7 kb in addition to those observed in the total cytoplasmic fraction. Although the differences among these studies need to be clarified, the transcription of cellular sequences along with viral sequences could produce molecules of different lengths, some of which may not be functional in translation assays (116). Alternatively, cellular sequences expressed under the direction of the HBsAg promoter may play a central role in maintaining the transformed state. The nearly consistent finding of no core gene expression in a wide variety of recombinants as well as with recircularized DNA suggests that the regulation of surface and core gene expression is different. Indeed, many asymptomatic carriers and some with CPH have demonstrable HBsAg in their sera and cytoplasm of hepatocytes with little evidence of any other virus gene expression (348, 725, Figure 2). The type II response of HBV positive patients, in which ara-A and/or interferon treatment resulted in the disappearance of Dane particles but the persistence of circulating HBsAg, also suggests that virus replication and HBsAg synthesis are independent processes (623, 624, 631). That antiviral chemotherapy may be effective against replicating virions but not against HBsAg production from integrated HBV DNA is consistent with the characteristics of all the human hepatoma cell lines studied to date which only produce HBsAg and only contain integrated viral DNA.

As mentioned above, COS cells transfected with SV40-HBV recombinants could also be used for studying the biogenesis of HBsAg. For example, the RNA species generated from LSV-HBsAg or LSV-HBpresAg, plasmids containing the S-gene or pre-S/S-gene sequences, respectively, were characterized by S1 mapping and primer extension (921). Although all of the RNA species from LSV-HBsAg and some from LSV-HBpresAg initiated within the SV40 early promoter, a number of transcripts from LSV-HBpresAg apparently initiated from within the pre-S region. Further, one RNA from LSV-HBpresAg mapped to the putative HBV pre-S cap site approximately 25 bp downstream from the RNA polymerase binding site (TATA box). Together, these results suggest that splicing from the S-gene promoter region may generate a variety of mRNAs which encode HBsAg polypeptides containing pre-S sequences (921). In another study, S-gene expression was examined using a series of plasmids with progressive deletions in the 3' sequences

flanking the gene within the small BglII fragment spanning the region around the nick in the long strand (922). The full length SV40-HBV plasmid, pDLR1, containing the HBV DNA fragment from the EcoRI site within the pre-S region to the BglII site at the beginning of the core gene, gave rise to a predominant mRNA species of about 2 kb and a minor species of 1.1 kb. The larger RNA probably initiated within the SV40 late promoter and terminated at the end of the HBV sequences in the construct. Hybridization using probes from different portions of the HBV DNA insert showed that all of the probes were reactive with the 2 kb mRNA but that a probe spanning the carboxy terminal region of the polypeptide p25 and adjacent nontranslated sequences did not react with the 1.1 kb species, suggesting that the smaller RNA was a spliced version of the larger transcript. Construction of a cDNA library from these mRNA species and nucleotide sequencing of some of the clones demonstrated that a splice of 846 bp beginning near the middle of the S-gene and ending just before the X gene was the nature of the difference between the two sized RNAs. The 3' end of these cDNA clones contained a nucleotide stretch resembling the polyadenylation consensus sequence, located just prior to the core gene on HBV DNA (922). When a series of plasmids with increasing deletions in this region were tested for RNA production, only recombinants lacking the poly A consensus sequences were negative for RNA production, suggesting that both processing and polyadenylation require the presence of these sequences (922).

The splicing of one or more transcripts from the pre-S/S-gene of HBV DNA as a mechanism by which a family of distinct but highly related HBsAg associated polypeptides is generated has been suggested by the results of several more studies. Expression of interferon in mouse L cells under the control of the putative S-gene promoter yielded similar sized transcripts whether intervening pre-S sequences were present or not, suggesting that splicing of pre-S sequences occurs when they were present in the recombinant (909). In C127 mouse cells transformed with pMZ6V4, a recombinant containing BPV-1 DNA and HBV sequences spanning the putative S-gene promoter, pre-S and S-genes, S1 analysis of the RNA species most likely encoding the HBsAg major nonglycosylated polypeptide mapped the 5' end at position 3160, approximately 40 bp from the EcoRI site, suggesting that this position could act as a splice acceptor site

(930). Alternatively, this site could be the actual 5' end of the transcript (937). The fact that the same RNA was generated independent of the BPV-1 DNA orientation with respect to the HBV sequences further implies that transcription is under control of the S-gene promoter (930). Analysis of surface antigen specific RNA from infected chimpanzee liver by S1 protection experiments also showed that the 5' end of the major species mapped just upstream from the EcoRI site in the pre-S region (937). Treatment of surface antigen RNA with exonuclease VII yielded the same result, suggesting that a loop representing a spliced region was not present in the transcript. Identification of the 5' terminus 185 bp upstream from the beginning of the S-gene places it in a region of HBV DNA possessing significant homology with a region in SV40 DNA unrelated to the TATA-like sequence but important in promotion of SV40 late gene expression (937, 938). About 60 bp upstream from this site is another stretch of HBV sequences sharing significant homology with a portion of the SV40 replication origin which is also important in promotion of late SV40 gene expression (937, 939, 940), suggesting that promotion and/or regulation of HBsAg p25 expression may be at least under partial control of these genetic elements. S1 mapping of the 3' end of this transcript demonstrated termination 21 bp beyond the start of the core gene; a result similar to that reported elsewhere (168, 922, 942). The constitutive nature of HBsAg expression (909), whether controlled by its own promoter or by the genetic elements described here, combined with polyadenylation of one or more transcripts within the core gene (937), may effectively suppress expression of X, core and polymerase genes since they, too, overlap the region of DNA encoding this S-gene associated transcript. Whether the mRNA encoding the major nonglycosylated polypeptide of HBsAg is spliced or not, and whether it is controlled by the putative S-gene promoter or the SV40-like sequences within the pre-S region, will only be resolved by more detailed characterization of the transcripts including their ability to translate one or more HBsAg polypeptides.

Another approach in studying S-gene transcription is *in vitro* transcription using different HBV DNA templates. A shuttle vector containing S-gene sequences downstream from its own promoter or from the SV40 late promoter (940) produced an RNA species initiating 185 bp upstream from

the S-gene. Using templates with different cleavage points upstream from the translation initiation codon of HBsAg p25, it was shown that a cleavage 30 bp upstream from the start of p25 abolished transcription, while a cleavage 250 bp upstream had no effect, suggesting that all of the promoting sequences for the S-gene were within 250 bp upstream (937). The full length 3.2 kb EcoRI generated HBV genome and the large BamHl fragment (1.85 kb) lacking the S-gene were also used as templates for in vitro transcription (905). Both templates initiated transcription just preceding the pre-S sequences, which was sensitive to α-amanitin, indicating that the transcripts were made by RNA polymerase II. The S-gene promoter synthesized quantities of RNA similar to that made by a strong promoter controlling the adenovirus major late transcript, again suggesting the high levels of S-gene promoter activity (905). Sl analysis of the in vitro transcript yielded the same result as that observed in vivo, in which COS cells expressing S-gene sequences from an SV40-HBV recombinant were characterized, suggesting that the site of initiation was the same in both cases. Although the nature of S-gene expression has been partially illuminated by recombinants or free genome fragments both in vivo and in vitro, the relationship of these transcripts and controls to what occurs in natural infection remains to be further elucidated. Animal models naturally or experimentally infected with HBV-like viruses would be very useful in this context.

Core antigen and e antigen gene structure and expression

Based upon the size of the major HBcAg polypeptide on SDS-gels (Table 9), and assuming that little or no splicing occurs within an open reading frame of the HBV genome, it is possible that region 8 may encode such a polypeptide (156, 177, 358). This was proven in recombinants carrying intact region 8 and expressing HBcAg determinants (Table 17). Region 8 is conserved in WHV DNA, encoding a polypeptide 188 amino acids long, while the analogous region in HBV encodes a major component 183 amino acids in length (159). Region 8 is also conserved in GSHV DNA (913) and is found as a fusion product with region 5 (the X gene) in DHBV DNA (912). The deduced amino acid sequence of the major core component in HBV, GSHV and WHV shows that they share considerable homology and that they have a carboxy terminal protamine-like structure, the latter of which is also shared with DHBV (912). Within the

90 bp preceding the presumed start codon for core polypeptide in mammalian viral genomes are a number of other ATG codons (159, 913), making assignment of the actual initiation codon difficult in the absence of protein sequencing data. A recent attempt to determine the amino terminal sequences of the major HBcAg polypeptide has not been successful (716), suggesting that the amino terminus may be blocked. In both HBV and WHV, the core encoding region is in a different reading frame than the surface antigen gene sequences (159, Table 16). Further, most of the core encoding sequences do not substantially overlap open regions in other reading frames, which may explain the relatively large number of silent mutations noted in this region upon comparison of WHV and HBV DNA sequences. In one HBV isolate, an open region from the nick in the long strand extends approximately 75 bp, terminates, and is followed three nucleotides later by a methionine initiation codon thought to be the N-terminus of the major core polypeptide (167, 177). The study of core antigen (HBcAg) expression under conditions where the beta-lactamase gene of pBR322 was fused to fragments of HBV DNA extending from near the nick in the long strand to the pre-S or S-gene regions resulted in fused polypeptides in which reactivity was dependent upon successful read-through of the in-phase TAA codon just prior to the presumed initiation of the core antigen gene. Since these recombinants were expressed in bacteria, it is likely that the fused products were generated through the translational restart phenomenon operative in other bacterial protein systems (739). Whether translational restart occurs naturally in HBV infection is speculative. However, such occasional restart may give rise to large, unique polypeptides having core antigen and possibly other reactivities. Further characterization of core association polypeptides will undoubtedly reveal whether this is the case.

HBcAg expression was studied in a plasmid constructed from the *trp* operon promoter-operator region of *E. coli* (179). The recombinant contained HBcAg sequences with two initiation codons capable of encoding polypeptides 183 or 212 amino acids in length, depending upon the actual site of initiation. HBcAg produced by one recombinant made up 3.2% of the newly synthesized protein which was increased to 10% upon addition of the inducer 3-β-indolylacrylic acid. HBcAg material was detected by

RIA and immunoprecipitated by anti-HBc. Analysis of immunoprecipitates by SDS-PAGE resulted in major bands at 19,000 and 22,000 daltons, suggesting that both initiation sites were used and/or that the 19,000 dalton component resulted from breakdown of the larger polypeptide. The presence of a number of minor components in the SDS-gel profiles of immunoprecipitated HBcAg polypeptides, combined with the finding that the HBcAg produced in E. coli sedimented at greater than 100S, is consistent with the conclusion that the core reactive polypeptides are present in a tightly associated complex. Although the presence of nucleic acid in these complexes was not ascertained, and such complexes may be due to simple polypeptide aggregation, it is possible that nucleoprotein complexes derived from the core polypeptides bound to bacterial nucleic acid may account for these observations.

As discussed, HBcAg has been successfully expressed in E. coli as a fused polypeptide having additional sequences derived from β-galactosidase (178). HBcAg expression was dependent upon successful read-through or restart following an in-phase TAA termination codon. Higher levels of HBcAg expression were achieved in some recombinants when synthesis was controlled by the lac UV5 promoter instead of the normal lac promoter (737). Here, recombinants were constructed from sequences encoding the first eight amino acids of the β-galactosidase gene followed by HBV sequences containing variably sized deletions from near the nick in the long strand through the region encoding the first few amino acids of core polypeptide. Recombinants retaining the in-phase TAA codon just prior to HBcAg encoding sequences resulted in detectable core in solid phase RIA using serial dilutions of bacterial lysates having similar total protein concentrations. One recombinant, pR1-11, in which the deletion extended through the TAA codon and the presumed methionine initiation codon, yielded approximately 100 fold more HBcAg than most other recombinants in which the deletion did not include the in-phase TAA. Another recombinant, pR1-4, which was similar in construction to pR1-11 except that the deletion extended two more amino acids into the core antigen polypeptide encoding region, resulted in very low levels of detectable core. Variations in HBcAg synthesis between pR1-11 and pR1-4 may be due to differences in mRNA secondary structure, which may affect the efficiency of translation in each case, or due to differences in the efficiency of antigen-antibody binding as a consequence

of different amino terminal core structures generated by each recombinant. While similar considerations may explain the differences between the levels of antigen production in pR1-11 and the other expressing clones, the presence of an in-phase TAA codon in these other cases may also contribute (737). Further characterization of the core antigen produced by pR1-11 demonstrated that the material was aggregated in gel chromatography, that it was immunologically identical to liver derived HBcAg in immunodiffusion, and that it contained a polypeptide similar in size to the major core antigen component on SDS-gels (737). The major HBcAg polypeptide from both sources was reactive with anti-HBc and anti-HBe by Western blotting (799). Electron microscopic observations of such material demonstrate the presence of small particles similar in size and density to liver derived HBcAg (737, 799). These particles have also been shown to aggregate only after addition of anti-HBc containing sera, showing that they possess core determinants (738).

B. subtilis strain MI119 was transformed with a recombinant of pBR322 containing plasmid pBD9 into which the BamHI fragment of the HBV core antigen gene region was inserted within the erythromycin gene (176, Table 17). A nonfused core reactive product was detected in bacterial lysates. That this product made up only about 0.1% of the cellular protein is consistent with the stop/restart phenomenon operative in this case. Further, the expression of HBV genetic information in B. subtilis, a nonpathogenic organism, demonstrated the feasibility of such production for human consumption, since it is already used commercially for enzyme and antibiotic biosynthesis.

The biosynthesis of HBcAg and HBeAg in eucaryotic cells with HBV promoters and terminators seems to be dependent upon the configuration of the integrated HBV DNA (722). The plasmid pHBV130.4, containing four tandem head-to-tail copies of the HBV genome, was used to cotransfect tk^- rat 2 and mouse $LMtk^-$ cells. Cleavage of the DNA from transfected cells (rat 2/130.4/TK4) with KpnI or SstI, which do not cleave pHBV130.4, followed by Southern blot analysis, resulted in the appearance of multiple bands 15.5 kb to greater than 50 kb with KpnI and 7.8 kb to greater than 50 kb with SstI. The size and relative intensity of the fragments generated by each enzyme suggest that some of the

fragments may have one or more regions of cellular sequences. The presence of multiple integration events, possible intervening cellular sequences in the integrated HBV, and fragmentation of the HBV genome as a consequence of integration, as suggested by Southern blot hybridization, are consistent with the finding of complex forms of WHV DNA with these characteristics integrated into chronically infected woodchuck hepatocytes (688) and with the idea that early in transformation co-transferred sequences join prior to integration (732, 733). However, cleavage of rat 2/130.4/TK4 DNA with XhoI or PstI, which cleaves pHBV130.4 once, and with BamHI, which cleaves the recombinant twice, resulted in the finding of intact copies of integrated HBV DNA. Therefore, some integration events are characterized by the formation of complex forms of viral and cellular DNA, while others occur by insertion of intact viral genome copies. When rat 2 tk^- and mouse LMtk^- cells were cotransfected with pHBV130.4, a number of transformed clones were found to synthesize and secrete HBcAg or HBeAg. Synthesis of these HBV antigens did not always occur in the same clones producing HBsAg. Further, the amounts produced were different in each of the clones, and synthesis was not related to the number of intact, integrated HBV DNA sequences. However, expression of HBcAg and HBeAg may require the presence of multimeric HBV genome sequences if the mRNA encoding one or more of these products is greater than genome length. Transfection of cells with pHBV130.4 under this assumption would increase the possibility that the relevant greater than genome length segment of HBV DNA would become integrated (722). Evidence presented above that HBsAg could be synthesized from mRNA molecules less than genome length is consistent with the finding that smaller multimers or fragments of HBV DNA usually result in HBsAg production but not in the synthesis of HBcAg or HBeAg. Certainly, the existence of greater than genome length mRNA would not be unique to HBV, since such a situation has already been described for the polyoma late mRNAs (734, 735). In this light it is possible that even though the human hepatoma cell lines carrying multiple copies of HBV DNA all synthesize surface antigen, they do not possess the proper complement or configuration of integrated sequences for expression of the other viral gene products (722).

The establishment of mouse and rat cell lines stably producing HBsAg, HBcAg and/or HBeAg provides opportunities for studying the nature of the RNAs produced which may define some of the transcriptional units in HBV (942). Rat 2/130.4/TK4 cells, containing tandemly repeated HBV DNA integrated into the host genome and characterized by surface antigen, core and e antigen production, generated four polyadenylated RNAs which hybridized with full length HBV DNA probe in Northern analysis (942). These species were 1.054 kb, 2.435 kb, 3.968 kb and 4.425 kb long; the latter two transcripts being significantly longer-than-genome length. Using strand specific probes in M13 vectors, it was shown that each of these RNAs was encoded by the long or minus strand of the virus (942). With genome fragments as probes, the 2.435 kb RNA hybridized to all probes except that spanning the core region and downstream 3' sequences, suggesting that the RNA encoded S-gene polypeptide(s). The other subgenomic RNA, at 1.054 kb, hybridized to probes spanning the X gene region and flanking sequences between the core and surface antigen genes. The two RNAs greater-than-genome length hybridized with all probes, suggesting that they were encoded by sequences throughout the genome and that they may include some neighboring cellular sequences. The production of similarly sized greater-than-genome length transcripts by several cell lines producing core and e antigen reactivities, but not by lines that were core and e antigen negative, suggests that the transcripts were generated completely within HBV DNA sequences, since the integration site(s) in different cell lines would not be expected to be identical. Further, the production of such transcripts demonstrates that, at least under these circumstances, the RNA polymerase reads through a number of polyadenylation signals. Such events have been documented in the IgM μ chain synthesis during B cell development (943, 944) as well as during the adenovirus 2 (945) and polyoma (734, 735) life cycles. Alternatively, the large size of these transcripts may signify that they represent immature RNA species. Perhaps in differentiated hepatocytes where virus is replicated, these large transcripts are further processed, although in these mouse and rat fibroblasts translation of core and e antigen apparently take place from these species. If differential splicing is critical for HBV replication, and such splicing is a consequence of the state of cellular

differentiation, then only hepatocytes at the appropriate stage of differentiation will be able to replicate virus, while less differentiated hepatocytes will not. This is consistent with a recently proposed model in which there are liver cells susceptible to virus replication (S cells) and other cells resistant to virus replication (R cells) which differ from each other in that the latter is less differentiated than the former (948).

In an attempt to study the requirements of core gene expression, NIH 3T3 cells were cotransfected with a shuttle vector containing a head-to-tail tandem repeat of EcoRI HBV DNA and sheared genomic DNA from A29 hamster cells containing genes encoding a methotrexate-resistant dehydrofolate reductase (949). Methotrexate resistant cells positive for HBsAg by immunofluorescence were negative for core and e antigens. These cells contained multiple integrated copies of HBV DNA, secreted 22 nm HBsAg particles and soluble e antigen, as detected by appropriate radioimmunoassays (RIAs), but produced no detectable core antigen. The molecular basis for the latter observation was sought by characterization of the integrated DNA and the RNAs produced. Restriction endonuclease mapping of the integrated viral sequences showed the presence of one complete EcoRI copy of HBV DNA in tandem with another partial EcoRI copy joining cellular sequences within the C-gene region at multiple positions within the genome. HBV sequences were found to be unmethylated in the transfected cell lines, although at some integrated sites, neighboring cellular sequences were methylated. These results suggest that the lack of HBcAg production was not due to deletions in the core gene or methylation of the viral DNA. Using DNA probes from in and around the core gene, a 1.7 kb RNA band was consistently detected by Northern blot analysis, suggesting that one or more core polypeptides translated from such a transcript may be cleaved to e antigen reactive polypeptide(s) from these cells (949). As demonstrated with the integrated HBV DNA tandem repeat tetramer, e antigen expression here seems to be associated with an integrated block of viral DNA which is intact and greater than genome length organized in the form of a tandem repeat. However, the transcript associated with e antigen production was much smaller than genome length (949) in contrast to independent results describing greater-than-genome-length transcripts (942). The reason(s)

for this difference is not clear. Interestingly, the expression of e antigen by transfection of cells with pCP10 (a plasmid containing an HBV genome dimer in tandem) (927) or by recircularized, religated HBV DNA (933), further suggests that an intact genome copy is necessary for the core region to be expressed.

Mammalian tissue culture cells transfected with recircularized, religated HBV DNA (175, 933) or a recombinant molecule containing the core antigen gene sequences (798, 950) seem to demonstrate cytopathology following transfection, suggesting that core gene expression may have something to do with liver cell damage during viral replication. A human epidermoid carcinoma cell line transfected with the HBV core gene by protoplast fusion, followed by growth under one of two selectable markers, was tested for the plasmid DNA and for HBcAg (798). By Southern blotting, the plasmid was found in the selected cell clones. By adapting the commercially available HBeAg RIA, transient expression of core antigen in passage 2 following transfection of 5-azacytidine treated cells was observed. This correlated with cytopathology and cell detachment. Indirect immunofluorescence with human anti-HBc showed that more than 90% of 5-azacytidine treated cells expressed core in their cytoplasm. However, the dramatic increase in core expression following 5-azacytidine treatment of the PLC/PRF/5 cells has not been confirmed by independent efforts (792). The relatively low RIA P/N ratio, 3.5-3.8, and the lack of any other evidence demonstrating transcription or translation of the HBc gene sequences, weaken the proposed argument that core expression plays a primary role in cytopathology. In fact, a recent study with chronic HBV carriers has described an inverse relationship between virus replication and severity of liver disease, suggesting that the highest levels of HBcAg expression are accompanied by the least amount of cytopathology (951). Even under circumstances where replicating forms of HBV DNA have been demonstrated in the cytoplasm of liver cells showing histological evidence of cell damage, virtually all of the core antigen was found in the nucleus, not the cytoplasm (890). Further, in most forms of chronic hepatitis, expression of core antigen in the liver is at low levels (348). Immunosuppressed or immunodeficient patients having HBV infections most often show subclinical chronic infections without biochemical abnormalities and little or no inflammation even

though HBcAg could be found by fluorescence in 60-100% of liver cell nuclei (348). The natural history of chronic HBV infection suggests that markers of virus replication correlate with liver pathology and that seroconversion, usually accompanied by a disappearance of such markers, often results in histological improvement (823, 832). While this is consistent with the hypothesis that one or more HBV gene products possess cytopathic properties (823), it is possible that the host immune response to the virus, and not virus replication per se, is the important determinant in the severity of liver disease (951). The recent finding of HBcAg on the surface of infected hepatocytes replicating HBV (450) and that T cells reactive against HBcAg may target such cells (835), further suggests the importance of the immune system in liver damage.

Characterization of the possible transcripts involved in core antigen/e antigen gene expression has just started to elucidate some further requirements related to their expression. In vitro transcription of EcoRI linearized, full length HBV DNA, derived from a recombinant plasmid (pA01), resulted in the generation of a 1.7 kb RNA as determined by denaturing agarose gel electrophoresis (736, 797). In vitro transcription of individual BamHI fragments of HBV DNA demonstrated that a 1.9 kb segment, which included the core gene and flanking sequences, also produced the 1.7 kb RNA species. The sensitivity of transcription to α-amanitin suggests that the host cell (HeLa) RNA polymerase II recognized and successfully transcribed from a viral promoter probably somewhere upstream from the core gene (736, 797, 905). A possible core gene promoter was also evident in runoff transcription assays using different sized fragments within the 1.9 kb BamHI region as templates (905). The initiation sites of the RNA species generated each preceded the presumptive start of the major core antigen polypeptide. This mapped the core promoter to around nucleic acid residue 1689 ± 35 (Figure 13) within the cohesive ends of the genome that make up part of the X region. A TATA-like sequence approximately 30 bp upstream from this site could bind RNA polymerase II for core gene transcription (905). If this region serves as the core antigen gene promoter in vivo, then it would be on the opposite side of the nick in the long strand from all or most of the core antigen structural

gene, implying that the nicked structure of the genome may interrupt a transcriptional unit responsible for expression of at least the major core polypeptide. This, in turn, raises the possibility that supercoiled forms of HBV DNA, which have been found in infected liver, constitute the actual templates for viral transcription in vivo. The presence of one or more greater-than-genome-length RNA species associated with core gene expression is consistent with this conclusion and could easily be generated from a supercoiled template assuming read through of some polyadenylation signals in the genome.

The proposed polymerase gene of HBV DNA.

The genome of HBV contains a large open region spanning 75% of the viral DNA in the third reading frame with respect to surface antigen and core antigen genes (156, 159, 177, 358, Table 16, Figure 13). In one of the adw isolates, however, a stop codon was present approximately half way through this open reading frame (900). Among sequenced HBV DNA isolates, the greatest amount of divergence in amino acid sequence was found in the amino terminal region (358). WHV DNA also contains this large open region which shares little amino acid sequence homology with its human counterparts in the amino terminal region (159). Interestingly, the carboxy terminal portion of the major core polypeptide, which is encoded by the same region of the genome in a different reading frame, is well conserved. Substantial homology in both nucleic acid and polypeptide sequences between WHV and HBV occur for most of the remaining region 6 except for the area around the EcoRI site (159). Recent nucleic acid sequencing of the DHBV (912) and GSHV (913) genomes also demonstrate an analogous large open reading frame with similar characteristics. Region 6 in these viruses could encode a basic polypeptide approximately 90,000 daltons in size (358, 912, 913). Although there are several initiation codons within this region, a 90,000 dalton polypeptide would be in the same size range as other DNA polymerase enzymes; and several studies have suggested that the DNA polymerase of HBV and related viruses could be encoded by this open region in the respective viral genomes (156, 177, 358, 536a, 900, 912, 913). Alternatively, or in addition, appropriate splicing to the internal initiation codons could yield several polypeptides with various functions in the life cycle of these viruses. Examination of the sequences around

region 6 shows the presence of a TATA-box approximately 335 bp upstream from the first initiation codon (156), further suggesting the translation of this region. Examination of replicating nucleic acid-protein complexes from DHBV infected liver suggests that such replication takes place through an RNA intermediate by reverse transcription and that the associated polymerase would share some characteristics with other like enzymes (189). Indeed, comparison of the deduced amino acid sequences from several known reverse transcriptase enzymes with that of HBV and some of the related viruses show several regions of homology, including a number of invariant residues (912, 913, 952). Expression of HBV genes in the Rat 2/130.4/TK4 cell line demonstrate the presence of two greater-than-genome length RNA species (722, 942). While one of these may encode core and e antigen related polypeptides, the other might encode the polymerase product. Further characterization of these transcripts and those mRNA species produced in infected animals by *in vitro* translation may provide information as to the nature of any polypeptides having polymerase activity.

Open region X in HBV DNA.

HBV DNA region X (or region 8), located just upstream from the core polypeptide gene (region 5), could potentially encode a polypeptide of 154 amino acids. A similar open region has also been found in the sequenced genomes of WHV (159) and GSHV (913), further suggesting X encodes one or more polypeptides in these viruses. From the deduced amino acid sequences, WHV and GSHV X share 71% homology while GSHV and HBV X polypeptides share 33% (913). Interestingly, the X region in each of these virus genomes is interrupted by a nick in the long DNA strand, suggesting that its expression may be dependent upon the presence of a supercoiled molecule. In the DHBV genome, the core sequences (region 5) are fused to the majority of the sequences making up region 8 (the X region), leaving only a small portion of region 8 upstream from the nick in the long strand, suggesting a close association among these open reading frames (912). However, interpretation of this observation has been difficult since the "X" sequences of the fused reading frame in DHBV share little homology with the corresponding X regions of the other viruses. Relatedness in higher order protein structure and function may exist and await further work in structure-function correlations among core associated polypeptides.

It is noteworthy that the nucleic acid sequences preceding the X region lack a recognizable TATA-like sequence (905, 942) and that no X gene promoter has been defined by in vitro transcription assays (905). In this context, the expression of surface antigen sequences in COS cells, characterized by a 1.1 kb spliced RNA whose splice acceptor site was mapped to just upstream from the X region, suggests that this site may be important in the transcription of X gene sequences (922) during infection. Alternatively, the finding of a 1.1 kb RNA species in Rat 2/130.4/TK4 cells hybridizing to the X region and flanking sequences suggests that perhaps X polypeptide could be expressed by itself (not fused to the core gene) and that transcription initiation may occur in the absence of a TATA-like sequence (942), as described with adenovirus early region II and IVa2 mRNAs (953). It is possible that these apparent differences may be due to the nature of the recombinants and cell lines used. Characterization of the transcripts from infected tissues will be necessary to discern the mechanism(s) by which the various viral genes are expressed in vivo.

The finding that HBcAg associated p27.5 and p40 and GSHcAg associated p26 and p37.5 contain tryptic peptides which are shared with their respective major core associated polypeptide, and also contain peptides which are shared among themselves but not with their respective major core associated component, suggests that these minor high molecular weight polypeptides contain additional primary sequences partially (in p26 and p27.5) or totally (in p37.5 and p40) encoded for by the X region (56). More direct evidence demonstrating the association between X and core come from studies using antibodies made against two synthetic peptides derived from sequences in a hydrophilic region not far from the carboxy terminus of X (792). In these experiments, intact core particles from infected human, ground squirrel and woodchuck livers contained X reactive sites that copurified with each of them. Further, core polypeptides separated under reducing conditions in SDS-gels demonstrated reactivity with X antibodies in a Western blot, suggesting the presence of X sequences in core associated polypeptides. The same pattern of reactivity has also been shown with serum derived DHBV. Combined with limited manual sequencing data which fixes the N-terminus of the DHBcAg p37 component to the start codon in region 8 upstream

from the nick in the long strand (792), these results suggest that regions 5 and 8 make up a single transcriptional unit at least under these circumstances. Although the functional significance of these observations remains to be elucidated, if the expression of X as a free polypeptide or as a fusion product with core gene sequences is necessary for viral replication, then the presence of supercoiled viral DNA would be obligatory in the generation of molecules necessary for genome replication and/or core maturation. In the replication cycle, the X product might be the protein attached to the 5' terminus of the viral DNA (742, 913, 942) and thereby act as a primer. Whatever its role, the finding of X related sequences in core particles and virions will undoubtedly permit design of experiments focused toward elucidating the relevant structure-function correlates.

THE 5' TERMINAL PROTEIN AND POSSIBLE ORIGIN OF GENOME REPLICATION ASSOCIATED WITH HEPATITIS VIRUSES

An increasing number of single-stranded RNA and double-stranded DNA viruses possess a protein covalently attached to the 5' terminal base of their genomes (740, 741). Evidence from several systems studied in detail suggests that these proteins may be important in the initiation of replication and possibly in the encapsidation of the progeny genomes. The existence of such genome bound proteins has recently been described in HBV (742), GSHV (747) and DHBV (744), suggesting that these terminal polypeptides may play an important role in viral replication. Early evidence implying the presence of a covalent DNA-protein complex in HBV and GSHV resulted from the observations that the DNA released from SDS treated Dane or Dane-like particles was removed from aqueous solution by phenol extraction unless the DNA was first treated with pronase or proteinase K (48, 607, 742, 747). The resistance of such complexes to heating at 60°C in the presence of SDS and 2-ME is consistent with the nucleic acid and polypeptide moieties being covalently linked (742). For further characterization, Dane particle DNA made radioactive by the endogenous DNAp reaction and analyzed by rate zonal sedimentation in sucrose and 0.1% SDS demonstrated that the complex sedimented slightly faster (141S) than free (protease treated) DNA (140S) (742). The complex was shown to bind glass fiber filters at high ionic strength,

which is characteristic of these complexes but not of free DNA, as demonstrated in adenoviruses (743). Density equilibrium centrifugation of such complexes compared to free DNA resulted in density differences from 0.013-0.015 g/ml in CsCl/guanidine chloride gradients, while comparison of the complex density to that of free DNA and soluble polypeptides suggests that the complex consisted of about 10% protein (742). By agarose gel electrophoresis, the DNA-protein complex of each virus moved slightly slower than the corresponding free DNA. In DHBV, the complexes were not dissociated in gel electrophoresis by SDS or by strand denaturation at 90°C for 15 minutes in 0.1% SDS, suggesting they do not form during electrophoresis (744). Heating the HBV complex to 80°C, however, altered its mobility on agarose gels consistent with linearization, indicating that this conversion was not altered by the presence of the 5' protein. The resistance of the HBV genome termini to labeling with γ-^{32}P-ATP and polynucleotide kinase even after treatment of linearized DNA-protein complexes with alkaline phosphatase is consistent with the covalent binding of a protein to the terminal 5' base in the genome (742). The stability of the DHBV DNA-protein complex to 100 mM sodium hydroxide at 37°C for 1 hour suggests that the terminal linkage is different from that in adenoviruses (744, 746), the latter consisting of a phosphodiester bond between serine or threonine and the terminal nucleotide. Although some nucleic acid bound proteins are covalently attached by a phosphodiester bond to tyrosine (748) or phosphoamide bond to lysine (749), the nature of the bond in HBV and related viruses has not been characterized.

The strand to which the protein is covalently bound in HBV was assayed for by agarose gel electrophoresis of restriction endonuclease fragments generated from single digests using a panel of enzymes. Aliquots of digestion products, which were either not further treated or phenol extracted with or without prior proteinase K digestion, localized the position of the 5' protein to the long minus strand of DNA (742). In GSHV, the long strand appeared in agarose gels following phenol extraction only after proteinase K treatment, suggesting that the terminal protein is bound to the long strand here as well (747). In DHBV, virion DNA denatured after radiolabeling by the endogenous DNAp reaction, was chromatographed on benzoylated-naphtoylated-DEAE(BND)-cellulose (744). Since most of the radioactivity (present

only in the plus strand) was recovered under conditions where single-stranded DNA was eluted, it was likely that protein was bound to the minus DNA strand. This was confirmed by Southern blotting in the presence or absence of protease digestion using strand specific probes. The unique site of 5' protein attachment was ascertained in duck liver derived immature core particles by following the minus strand synthesis in such cores using ^{32}P-TTP incorporation in an endogenous reaction (744). By adding dideoxynucleotides to block extended minus strand synthesis and Actinomycin D to block plus strand synthesis, it was shown that minus strand fragments shorter than 30 bp already had covalently bound protein. The fact that sequencing of protease digested fragments was successful showed that the 5' end of the minus strand occurs at a unique site (744).

The short or plus strand of HBV DNA, which acts as a primer for the DNAp reaction, yielded no evidence of a terminally bound protein (742). Perhaps the resistance of the HBV DNA plus strand to label with polynucleotide kinase, combined with the apparent lack of a terminal protein by agarose gel electrophoresis, suggest that a 5' polypeptide, considerably smaller than its counterpart on the long strand, may exist. Alternatively, the 5' terminus of the short strand may be blocked in some other way.

Analysis of the HBV DNA forms in chronically infected chimpanzee liver by proteinase K digestion prior to phenol extraction and agarose gel electrophoresis showed that the 2.3 kb supercoiled and 4.0 kb nicked forms did not contain bound protein, while the 3.25 kb full-length linear and smear of partially double-stranded material contained the 5' protein (644). Analogous results were obtained upon analysis of viral DNA extracted from DHBV infected livers (744). Since the nicked and linear forms of HBV DNA differ from the supercoiled form by cleavage at one and two phosphodiester bonds respectively, it is possible that the 5' protein might act as a site-specific endonuclease which specifically cleaves HBV DNA from the supercoiled form (740-742). The gene A product of ØX174 has been shown to act as an endonuclease which generates a site-specific cleavage in one strand of the replicative intermediate and then becomes covalently bound to the terminus of the nicked strand (550). If this were so with HBV and like viruses, then the full-length linear and partially double-stranded DNA-protein complexes isolated

from Dane particles or the single-stranded minus DHBV DNA-protein complexes isolated from immature core particles may be replicative intermediates (643). In adenoviruses, the 5' covalently attached polypeptide is located at the origin of DNA replication (741). Sequence analysis of HBV and WHV cloned genomes suggests that a possible origin of replication exists in a single, conserved region of DNA which lies near the 5' terminus of the long strand (159). The finding of a protein covalently bound to the 5' terminus of the long strand, the presence of this attached protein in growing minus strands, and the proximity of this terminus to a possible origin of replication are consistent with the protein being responsible for initiation of DNA replication by acting as a site-specific endonuclease and a primer for minus strand synthesis. This role would be consistent with that of like protein in adenoviruses and in phage Ø29 in that the terminal protein in each case acts as a primer for DNA synthesis (740, 741, 752-755). An endonuclease activity associated with SV40 and polyoma chromatin isolated from infected cells has also been shown to cleave supercoiled to linear DNA at the origin of viral replication (750). Although the origin of the endonuclease activity was not determined, the finding of a terminal protein covalently linked to the 5' end of the linearized genome at this site (751), the conversion of supercoiled to linear DNA at the origin of replication, followed by covalent binding of a protein at the 5' terminus may be analogous to the events occurring in HBV.

The presumed origin of replication for HBV and related viruses shares a number of other characteristics consistent with the proposed function of this region. Analysis of nucleic acid sequences within the cohesive termini of these virus genomes reveals a hairpin loop flanked on either side by a 10-11 nucleotide long direct repeat (912, 913, 941). In HBV, GSHV and WHV the hairpin loop structure contains similar sequences and is near the presumed core promoter in HBV (905, 913). In DHBV the loop is located in the same region of the genome, although the sequence involved is different (912, 913). Despite these differences, this loop structure may serve to bind one or more enzymes important for the initiation of DNA replication. Sequences making up the direct repeats are located near the nick in the long and short strands in the

viral DNA, which are separated by 220-240 bp in HBV, GSHV and WHV, and by 46 bp in DHBV (913). Even though the function of these direct repeats remains to be elucidated, they may serve as recognition sequences for the site specific endonuclease activity possibly associated with the polypeptide or precursor which becomes covalently bound to the viral genome. Alternatively, if these conserved sequences are not crucial for recognition by one or more enzymes, then it is possible that the repeats are generated at each replication cycle from unique sequences, as are the short direct repeats present at both ends of retroviruses (635, 912, 913). Whether these sequences are important for replication, integration or other aspects in the life cycle of these viruses remains to be elucidated. In addition to the RNA polymerase II controlled core promoter, in vitro transcription experiments have revealed an RNA polymerase III dependent transcript generated from the short strand of HBV DNA within the same region of genome cohesive ends (905). Such a transcript was approximately 0.7 kb in length, initiating about 50 bp upstream from the core gene, and spanned sequences between the core and surface antigen genes (905). While the existence and function of such a transcript in natural infection remain to be discovered, if it does exist, these observations suggest that the promoter for this transcript and that of the core gene in opposing orientation on the other strand overlap and that their expression may be coordinately regulated (905). If such coordination exists within this region of HBV DNA, then under some conditions core and other individual HBV genes would be expressed, and under other conditions viral DNA replication would occur instead (905). Given the length of the transcript, 0.7 kb, also implies that such a transcript could only be expressed after the endogenous polymerase reaction repairs the short strand and the ends become ligated in a supercoiled molecule. Again, this suggests that the supercoiled form of viral DNA is central to the replication scheme of these viruses.

Finally, the successful production of typical acute hepatitis in chimpanzees infected HBV DNA derived from recombinant clones demonstrated that the virion proteins, including the 5' bound terminal polypeptide, were not required for infectivity (756). This differs markedly from the situation in adenoviruses and phage Ø29, which require the presence of the 5' protein for infectivity (740, 757).

The forms of HBV DNA used for chimpanzee infection included linear genome dimers constructed from single different restriction endonuclease sites and from a recircularized and ligated genome monomer. The absence of the nick and gap structure in these DNA species suggests that they play no role in infectivity, since the results clearly demonstrate that closed double-stranded circular HBV DNA is fully infectious (756).

REPLICATION SCHEME FOR HEPATITIS B AND RELATED VIRUSES

The preceding sections of this monograph indicate that hepatocyte membrane associated HBsAg, nuclear HBcAg and HBeAg, serum Dane particles, DNA polymerase activity and serum derived HBeAg forms correlate with virus replication. Examination of these markers in HBV infected carriers suggests the existence of two HBV replication patterns. One pattern (group 1) has been characterized by high concentrations of serum HBsAg, HBeAg and high DNA polymerase activity (640, 758, 759). These carriers also demonstrate high levels of genome length DNA in both serum and liver (653, 654). Both Dane particles and small spherical HBsAg from these individuals often have detectable receptor for polymerized albumin. The other pattern (group 2) has been characterized by lower concentrations of HBsAg and usually no HBeAg, DNA polymerase activity, or detectable HBV associated receptor for polymerized albumin. Group 2 carriers demonstrate genome length DNA in the liver but not in the serum. Other carriers, demonstrating very low serum HBsAg concentrations and having only integrated DNA, probably do not replicate HBV (653, 654). Analysis of the viral DNA forms in each group showed that groups 1 and 2 contained the nick and gap form and that group 1 additionally contained supercoiled molecules in both the liver and serum (653, 654). Biopsy specimens from carrier chimpanzees contained two species of apparently nonintegrated HBV DNA species corresponding closely to full-length linear molecules (3.4 bp) and relaxed supercoils having a nick in one strand (4.1 bp) (645). Independent observations with liver derived HBV DNA forms from carrier chimpanzees showed fully and partially double-stranded molecules at 3.2 kb and 2.0-2.8 kb, respectively, and another smear extending below 1.6 kb (646). Since the latter population of HBV associated sequences was

found in the liver but not the serum, it is likely that it represents replicative intermediates (646).

In studies with DHBV DNA derived from infected livers, full-length linear viral DNA was detected on Southern blots at approximately 3 kb (188). Another DNA species, corresponding to the supercoiled form, was detected at 1.94 kb. A smear of faster migrating material, possibly single-stranded DNA, was also observed. Since the rate sedimentation behavior of single-stranded DNA varies with salt concentration in sucrose gradients while the behavior of double-stranded DNA is relatively unchanged, such analysis carried out with DHBV DNA from infected liver demonstrated that the fastest migrating smear of material on agarose gels was extensively single stranded. These single-stranded molecules were not present in virion DNA. Electrophoresis of denatured cloned DHBV DNA resulted in a band comigrating with the slowest of the single-stranded species at the top of the smear, suggesting that the largest of these molecules was genome length. Using single-stranded DHBV DNA probes in Southern blot hybridization, it was shown that virtually all of the single strands were minus strand species and that the smear probably represented minus strands of increasing length, the largest being genome size (188). A doublet migrating slightly slower on agarose gels than the largest of the single-stranded species yielded results with strand specific probes consistent with there being full-length minus strand and small amounts of growing plus strand DNA. Further experiments with DHBV immature core particles isolated from infected liver were used to ascertain the nature of the template for minus strand synthesis (189). Soluble DHBV infected liver extracts were analyzed by velocity sedimentation on sucrose gradients, and the presence of replicating intermediates assayed for by DNA polymerase activity. Gel electrophoresis of the reaction products, which sedimented at about 100S, yielded the DNA species described above in DHBV infected liver. A major component comigrated with full-length single-stranded DNA, while the presence of several smaller single-stranded species suggests that preferred pauses occur during strand elongation. The resistance of the reaction products to DNase and RNase, as well as electron microscopic observations of the replicating complexes, suggest that both minus and plus strand synthesis occur

within immature core particles. This is in contrast to the activity found in virion cores, where only plus strand synthesis could be demonstrated.

Treatment of immature cores with actinomycin D, which inhibits DNA directed DNA synthesis, completely inhibited plus strand synthesis but did not alter minus strand synthesis. Agarose gel electrophoresis of the nucleic acid from immature cores treated with actinomycin D demonstrated only single-stranded minus DNA species. These experiments suggest that plus strand synthesis occurs on a DNA template while minus strand synthesis occurs on an RNA template. Treatment of the polymerase reaction products to S1 nuclease in the presence or absence of actinomycin D showed that the plus strand was largely resistant to S1 in either case, suggesting that it was hydrogen bonded to its template-minus strand DNA. In contrast, the S1 sensitivity of the minus strand increased upon addition of actinomycin D, suggesting that it was not bound to its template. Minus strand DNA in DNA-RNA hybrids migrated in agarose gel electrophoresis in a way which was not completely independent of the DNA-RNA ratio, suggesting that the length of the RNA template varies inversely with that of the growing minus strand. This not only offers an explanation for the S1 sensitivity of minus strand DNA but suggests the existence of an RNase H activity which progressively degrades the template soon after minus strand synthesis. Finally, the strand specificity of these products was confirmed by hyridization with strand specific probes and monitoring changes in electrophoretic mobility on agarose gels.

To further characterize the template associated with the minus strand, isopycnic gradients containing cesium sulfate were used. In the absence of actinomycin D, 28% of the polymerase product banded with RNA or was found at a density intermediate between RNA and DNA. In the presence of actinomycin D, similar analysis showed decreased amounts of product migrating at the density of DNA. In either case, heating prior to density equilibrium centrifugation resulted in product shifting to the DNA region of the gradient from the DNA-RNA hybrid, indicating that the DNA was hydrogen bonded to the RNA template. Minus strand DNA was observed in duplex DNA and DNA-RNA hybrid regions of the gradients, while plus strand was found localized only to the DNA region.

In a more direct test to ascertain whether RNA serves as a template for minus strand synthesis, the avian myeloblastosis virus reverse transcriptase was added to the putative template-primer complex isolated from immature core particles (189). Treatment of such complexes with actinomycin D selectively prevented appearance of partially of fully double-stranded species of DHBV DNA on agarose gels due to inhibition of plus strand synthesis. Prior treatment of the template-primer complex with RNase destroyed minus strand synthesis while not affecting plus strand synthesis and duplex RNA formation, demonstrating that the template is RNA in nature.

Somewhat similar structures have recently been found in cytoplasmic extracts of the human hepatoma cell line PLC/PRF/5 (147, 762, 763). Density equilibrium centrifugation of ^{35}S-cysteine labeled postmitochondrial pellets resulted in a peak of HBsAg positive material at density 1.2 (alpha particles) and another peak of HBsAg negative radiolabeled material at density 1.3 (beta particles). Both peaks were absent from HBsAg nonproducing hepatoma cell lines, suggesting that they contained virus related material. Beta particles treated with nonionic detergent were capable of incorporating tritiated thymidine in an endogenous DNA polymerase reaction which was inhibited by actinomycin D. This is consistent with the presence of DNA dependent plus strand synthesis in these particles. Further, hybridization of this material with radiolabeled DNA or RNA probes derived from cloned HBV DNA demonstrate that the sequences in beta particles are HBV specific (147, 762). Although the density and sedimentation characteristics of beta particles are similar to light cores, the presence of DNA polymerase and at least some HBV sequences suggest that these may represent defective or immature core particles (147). The sedimentation coefficient of beta particle DNA has been shown to be 11S, in contrast to HBV DNA, which is 15S (763). Further characterization of these particles as to their core and/or e antigen reactivities, as well as size of associated DNA species, will be necessary to understand what role, if any, these particles possess in the life cycle of HBV.

The unusual structure of Dane particle associated DNA described above appears inconsistent with semiconservative patterns of DNA replication. Combined with the finding of an RNA template for minus

strand synthesis, it is likely that the replication cycle of HBV and like viruses involves reverse transcription (Figure 15). In the proposed replication scheme, infection of hepatocytes with Dane or Dane-like particles results in uncoating of the viral genome. Exposure of these released nucleoprotein complexes to deoxynucleoside triphosphates may yield extensive plus strand synthesis. Those molecules having a small nick in each strand might then become covalently closed by virtue of the proposed topoisomerase function of the 5' protein or by a host enzyme. Early synthesis would include genome length RNA, a small number of core polypeptide related components, and DNA polymerase polypeptide, which would be required for minus strand synthesis in the replication complex formed later. Recently, polyadenylated plus strand RNA approximately genome length has been isolated from infected duck livers (766), but it is not known whether this represents the "pregenomic" RNA necessary as a template for minus strand synthesis, or a messenger encoding one or more polypeptides required for the replicative intermediate. Substantial polypeptide synthesis must occur early in the replication cycle, since pregenomic RNA, at the start of minus strand synthesis, is already packaged in an immature core structure. Although it is not known how minus strand synthesis is initiated, if the sequence of events in HBV is similar to that in adenoviruses (745, 746, 752-755, 761), then a free 5' protein or protein precursor may covalently bind to what will become the 5' nucleotide on the minus strand. This covalent complex would noncovalently associate itself with the terminus of the pregenomic RNA. The DNA polymerase and any other DNA binding protein required to complete the replication complex would then bind near the 5' protein. Polypeptides making up the premature core particle might then assemble around the nucleoprotein complex. The rest of the replication cycle would then occur within the maturing core particle. Under these circumstances, it is possible that the 5' protein is not only important in the initiation of DNA replication but may also be important in packaging the complex into cores.

Following initiation of minus strand synthesis, the composition of the replicative intermediate changes. Minus strand continues to grow to full genome length, while the RNA template is simultaneously degraded, probably by an RNase H activity. Plus strand synthesis

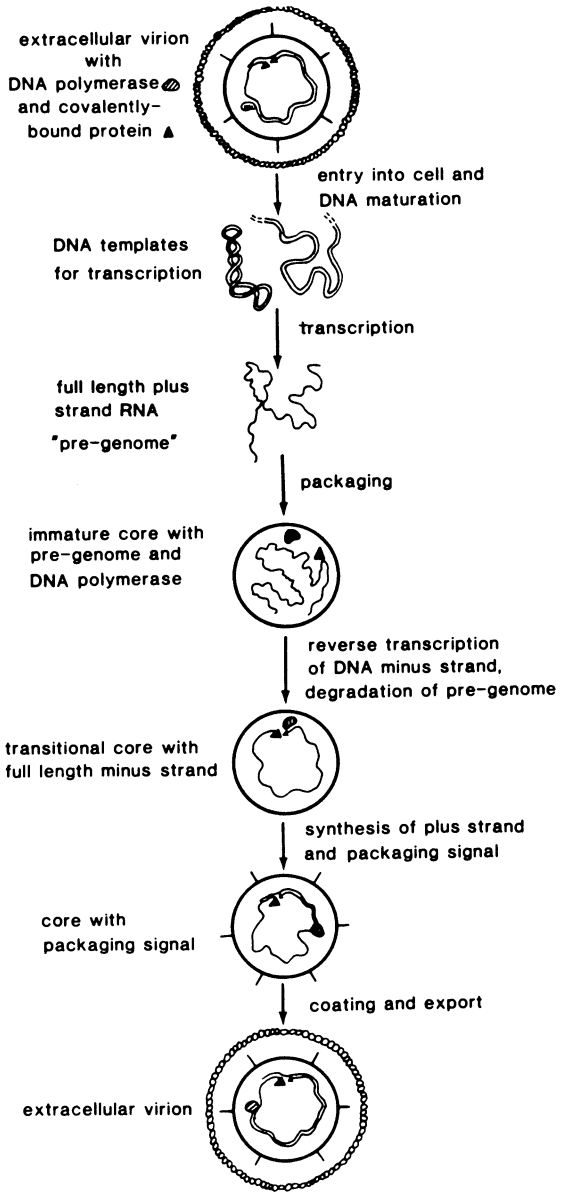


Figure 15. Proposed pathway for replication of the genome of hepatitis B-like viruses. [Reprinted with permission from Summers, J. and W. S. Mason in Cell 29:403-415. Copyright 1982 by M.I.T. (ref. 189)].

probably initiates at the 5' end of the minus strand, using the attached 5' protein as primer. As discussed, it is possible that the 5' protein includes sequences derived from the X gene. However, it is significant that the polymerase gene of retroviruses encodes at least three enzymes with polymerase, RNase H and DNA endonuclease activities, respectively, from a single transcriptional unit (954, 955). Further, the location of the DNA endonuclease activity seems to be in the carboxy terminal region of the polymerase gene product (955, 956). Since the polymerase gene of HBV and related viruses encompasses an open reading frame covering most of the genome, and since such an open reading frame contains a number of translation start signals which could potentially encode a family of polypeptides each with the same carboxy terminus, it is possible that the 5' protein may be a carboxy terminal cleavage product of the polymerase gene. Other members of polymerase gene transcriptional unit may have RNase H activity and/or polymerase activity analogous to that in retroviruses. Alternatively, if the 0.7 kb RNA polymerase III dependent transcript is made when the genome is supercoiled, it may affect mRNA processing, export or translation in a manner analogous to the way VA1 RNA in adenoviruses regulates translation of viral messengers by base pairing (941, 957). Alternatively, or in addition, this RNA might hybridize to the pregenomic RNA and help prime minus strand DNA synthesis. Since this RNA maps near the core antigen gene promoter, its synthesis may affect core gene or X plus core gene expression. If the core gene promoter, located at the "end" of the genome within the putative origin of replication, functions in a manner analogous to that of the retroviral promoter in the long terminal repeat, then it may play an important role in promoting the synthesis of the pregenomic RNA from linear or supercoiled DNA (905, 941). In oncogenesis, it may promote expression of one or more cellular genes in a promotion-insertion model. A further observation consistent with these facts is the order in which genes are arranged on the genome of HBV and related viruses compared to retroviruses. From the 5' end of the retroviral genomic RNA, the genes encoding the nucleocapsid proteins, followed by the viral polymerase and finally by the envelope antigen, parallel the situation in HBV if the nick in long strand prior to the core gene is interpreted as the "start" of the genome. Better definition of the

transcriptional units and their appearance after experimental infection of animals will help elucidate the sequence of events in viral replication.

The viral DNA forms described as replicative intermediates in the DHBV life cycle have also been found in analogous experiments with HBV (645, 646, 959, 960), GSHV (958) and WHV (123). However, important differences seem to exist as to the site of DNA replication in infected hepatocytes. Using HBV infected chimpanzee liver, replicating forms of DNA were found in predominantly nuclear and not cytoplasmic extracts (959). In contrast, using liver sections from patients having HBsAg positive CPH or hepatoma, in situ hybridization with a radioactive HBV DNA probe showed that most of the viral DNA was located in the cytoplasm (655, 656, 890). The intracytoplasmic distribution of HBV DNA was found diffuse in some samples, localized in others, or lining the cytoplasmic membrane. A small amount of hybridization was detected in the nuclei of infected cells, especially in hepatoma tissue, suggesting the sequences were integrated (960). Examination of liver sections for HBcAg by immunofluorescence demonstrated that HBcAg was present mostly in the nuclei of cells containing cytoplasmic HBV DNA (655, 890, 960), suggesting that the viral DNA was not encapsidated within core particles and that the replication cycle of HBV included a significant cytoplasmic phase (811). Other studies focusing upon core distribution in liver biopsies showed pure nuclear, mixed nuclear and cytoplasmic as well as diffuse cytoplasmic and/or submembranous patterns in patients with chronic hepatitis and circulating Dane particles (58, 452, 725), which probably reflect the entire spectrum of steps in the replication cycle of the virus. Taking HBV DNA into consideration, it was found that the large majority of cells positive for cytoplasmic core were also positive for cytoplasmic HBV DNA (960). However, among cells containing a large amount of replicating cytoplasmic HBV DNA, the largest fraction contained no detectable core, a smaller fraction demonstrated cytoplasmic core, and a relatively small number showed nuclear core (960). All of these cells were positive for HBsAg. Combined with the finding of low levels of replicating HBV DNA in anti-HBe positive patients (640, 961, 963), these results further suggest that HBV DNA could replicate free of intact core particles. This is in contrast to DHBV,

which appears to replicate in the cytoplasm exclusively within immature core particles. Although these differences may be virus and/or host specific, it is clear that accumulation of cytoplasmic HBsAg or nuclear HBcAg could occur in cells lacking significant amounts of HBV DNA, suggesting that the expression of these genes does not depend upon DNA replication and that their expression under these circumstances may be indicative of latent or abortive infection (960). Conversely, the presence of replicating viral DNA in the absence of core expression or expression only in the nucleus might indicate a state of vegatative reproduction. If the cytoplasmic phase of HBV DNA replication is central to the life cycle of the virus, then encapsidation of replicating DNA may occur at a later stage of replication with HBV than is the case with DHBV. That budding of 22nm spherical HBsAg and Dane particles takes place from the membranes of the endoplasmic reticulum in infected hepatocytes positive for membranous HBsAg (764), suggests that nucleic acid containing cores must be present in the cytoplasm. The presence of core particles in cytosols prior to budding (764) is consistent with these being core particles in which viral DNA is actively being replicated or has just completed replication. If the replication of HBV DNA involves a nuclear phase (959), then possibly the presence of large amounts of empty core particles in hepatocyte nuclei suggests that they could be synthesized and assembled but not transported into the cytoplasm unless they contain a replication complex. Unfortunately, the lack of tissue culture system for growing HBV and like viruses limits the extent to which replication and maturation could be studied.

CONCLUSIONS

Characterization of the molecular components in HBV have provided sensitive techniques for detection of the various viral markers associated with infection and establishment of vaccines which will decrease infection, disease incidence and sequaelae, including possibly primary hepatocellular carcinoma. Such characterization has also had a major impact upon our understanding of the virus life cycle, virus-host interactions and treatment of disease. The role of reverse transcriptase in the replication cycle of HBV and like viruses, for example,

suggests that the life cycle of this group of viruses may be a temporally permuted version of the well known retrovirus life cycle (765). In retroviruses, genomic RNA is reverse transcribed into DNA early after infection, which is used to synthesize new genomic RNA later in infection. With HBV, pregenomic RNA is synthesized first, followed by reverse transcription of the minus DNA strand, and finished by partial plus strand synthesis (964). Further, several studies have shown that shortly after infection of susceptible fibroblasts with avian sarcoma virus (ASV), S1 sensitive linear viral DNA species were found in the cytoplasm (686, 760, 767). The finding of full-length minus strand DNA and the presence of heterogeneously sized plus strand DNA suggest the existence of replication intermediates similar to those found in the Dane particle (637, 670, 682). Similarly, using BND cellulose chromatography, it was recently shown that more than 99.5% of the unintegrated viral DNA of RSV contained at least some single-stranded regions (230). Relaxed circles of retroviral DNA are synthesized in the cytoplasm (686, 760), transported to the nucleus and converted to supercoiled forms, suggesting, as in HBV, that the linear and nicked circular DNA species could be precursors to the supercoiled forms (148). Although these similarities exist, there are also many differences in comparing the replication cycle of these two virus groups. For example, retroviruses use host tRNA to prime minus strand DNA synthesis, while HBV probably uses its attached 5' terminal protein in priming. Although AMV reverse transcriptase is active, in reconstruction experiments using duck cores it is not known how closely related the endogenous DNA polymerase enzyme is to reverse transcriptases, since it has not been isolated in soluble form from Dane particles. Other differences have been noted as well (964).

Combined with the data above, the presence of supercoiled DNA in hepatocytes replicating HBV or related viruses strongly suggests that it plays a major role in the life cycle of these viruses. DHBV supercoiled DNA has been observed in duck embryo livers within hours after experimental infection preceding the reverse transcription phase of replication (965). This suggests that the supercoiled DNA may serve as a template for synthesis of pregenomic RNA (958) and for one or more viral mRNA species, since the pregenomic RNA of DHBV is found encapsidated

within immature core particles by the time minus strand DNA synthesis is detected (965). In light of other experiments, the appearance of supercoiled DNA early after infection and during periods of viral replication may signify that such a DNA conformation is necessary for the expression of one or more genes in the initial step(s) of infection. In the absence of a replicating system in tissue culture, these types of experiments in experimentally infected animals will continue to elucidate the life cycle of these viruses.

A variety of independent observations suggest that the extent of virus replication slows and finally stops during the chronic course of type B hepatitis progressing to PHC (812, 822, 842, 843, 963). While the underlying reasons for this are likely to be varying and complex, their understanding will undoubtly elucidate virus-host factors modulating replication as well as the mechanism by which viral persistence is involved in progression toward chronic disease and malignancy. Certainly, the immune response against the virus and/or infected cells will alter viral spread and the number of cells actively replicating virus. For example, an immune response directed against the receptor for polymerized albumin, if it is the viral receptor, will eliminate 22 nm spherical HBsAg and Dane particles bearing such a receptor. Since cells actively replicating virus often express membranous HBsAg, presumably also containing the receptor, they too may be eliminated. This would leave only the cells which are not actively replicating virus, and over time, probably containing only integrated sequences. Other viral gene products may serve as immunological targets, either for elimination or for antigenic modulation. In either case, the host immune system would play an important role in determining the outcome of virus-host interaction. Viral spread might also slow over time as a consequence of increasing numbers of DI particles, many of which are described above. Their generation may be the consequence of an inadequate immune response (793, 966). The relative difficulty in achieving HBcAg expression in vitro using conditions in which surface antigen is easily expressed implies that the state of cellular differentiation may be important for core expression. Since the presence of HBcAg often correlates with active virus replication, it is possible that only a subset of hepatocytes at the appropriate state of differentiation is

susceptible to infection and supports replication. If an immune response eliminates most of these cells during the course of infection, then the fraction of cells capable of supporting viral replication will decrease over time. With the development of a successful hepatitis B vaccine, one of the major unresolved areas is to understand the nature of the chronic carrier state so that effective intervention could be devised to ameliorate or eliminate it.

Recent research has shown that one or more markers of HBV have been found in association with cells and tissues other than hepatocytes. For example, one of the characteristics of hepatitis is proliferation and enlargement of Kupffer cells, the differentiated monocytes making up the reticuloendothelial system within the liver (967). Antigen-antibody complexes containing HBsAg have been found in such cells (968). Combined with the observation that mouse macrophages passively uptake HBsAg reactive material (969) and that Kupffer cells line liver sinusoids, thereby forming a barrier between the blood and hepatocytes, it is possible that a macrophage-like cell may play an important role in the immunopathogenesis of HBV by passive transfer of the virus to hepatocytes or by providing a site of replication (966). Other work demonstrates both HBsAg and HBcAg localized within the cytoplasm of pancreatic acinar cells in autopsy material from HBV infected individuals (970), and hybridization shows the presence of integrated HBV DNA in cellular DNA derived from pancreas (971). The finding that radiolabeled HBsAg binds specifically to human liver but not to pancreas, lung or kidney suggests that the HBV receptor on liver cells is not present on the cells of these other tissue types (972), even though infection of some of these tissues apparently occurs. Congenitally infected ducks demonstrated DHBV antigens in the cytoplasm of pancreatic acinar cells as well as replication specific forms of viral nucleic acid from pancreatic tissue extracts, strongly suggesting that the pancreas supports DHBV replication (811). Hybridization analysis has also shown integrated HBV sequences in human kidney (971) and replicative forms of DHBV DNA in experimentally infected duck kidney (811), similarly suggesting that one or more cell types in the kidney may support viral replication. Recent results have also documented HBV infection in bone marrow cells from HBV infected patients (947, 962),

in peripheral blood lymphocytes of patients with chronic hepatitis or hepatocellular carcinoma (835, 946), and in peripheral blood leukocytes (829). If further studies reveal that HBV is likely to replicate in one or more of these cells types, then the chances of finally culturing HBV or another related virus becomes a real possibility. And if this goal is achieved, our knowledge of the replication cycle, the mechanism(s) of persistence, and the relationship of HBV to hepatocellular carcinoma can be addressed much more completely on the molecular level.

REFERENCES

1. Blumberg, B. S. Bull. N. Y. Acad. Med. 40:377-386, 1964.
2. Blumberg, B. S., H. J. Alter and S. Visnich. JAMA 191:541-546, 1965.
3. Blumberg, B. S. Science 197:17-25, 1977.
4. Blumberg, B. S., B. J. S. Gerstley, D. A. Hungerford, W. T. London and A. I. Sutnick. Ann. Intern. Med. 66:924-931, 1967.
5. Okochi, K. and S. Murakami. Vox. Sang. 15:374-385, 1968.
6. London, W. T., A. I. Sutnick and B. S. Blumberg. Ann. Intern. Med. 70:55-59, 1969.
7. Prince, A. M. Proc. Natl. Acad. Sci. (USA) 60:814-821, 1968.
8. Sutnick, A. I., W. T. London, B. J. S. Gerstley, M. M. Cronlund and B. S. Blumberg. JAMA 205:670-674, 1968.
9. Sutnick, A. I., J. J. Cerda, P. P. Toskes, W. T. London and B. S. Blumberg. Arch. Intern. Med. 127:939-941, 1971.
10. Cherubin, C. E., R. L. Hargrove and A. M. Prince. Am. J. Epidemiol. 91:510-517, 1970.
11. Szmuness, W., A. M. Prince, G. F. Grady, M. K. Mann, R. W. Levine, E. A. Friedman, M. J. Jacobs, A. Josephson, S. Ribot, F. L. Shapiro, K. H. Stenzel, W. N. Suki and G. N. Vyas. JAMA 227:901-906, 1974.
12. Garibaldi, R. A., J. A. Bryan, J. N. Forest, B. F. Hanson and W. E. Dismukes. JAMA 225:384-389, 1973.
13. Gocke, D. J., J. G. Greenberg and N. B. Kavey. Lancet ii:248-249, 1969.
14. Gocke, D. J. and N. B. Kavey. Lancet i:1055-1059, 1969.
15. Gocke, D. J., J. G. Greenberg and N. B. Kavey. JAMA 212:877-879, 1970.
16. Knodell, R. G., M. E. Conrad, J. E. Dienstag and C. J. Bell. Gastroenterology 69:1278-1285, 1975.
17. Senior, J. R., A. I. Sutnick, E. Goeser, W. T. London, M. D. Dahlke and B. S. Blumberg. Am. J. Med. Sci. 267:171-177, 1974.
18. Bayer, M. E., B. S. Blumberg and B. Werner. Nature 218:1057-1059, 1968.
19. Dane, D. S., C. H. Cameron and M. Biggs. Lancet i:695-698, 1970.
20. Almeida, J. D., D. Rubenstein and J. E. Stott. Lancet ii: 1225-1227, 1971.
21. Levene, C. and B. S. Blumberg. Nature 221:195-196, 1969.
22. LeBouvier, G. L. J. Infect. Dis. 123:671-675, 1971.
23. Kim, C. Y. and J. G. Tilles. J. Infect. Dis. 123:618-628, 1971.
24. van Kooten Kok-Doorschodt, H. J., R. van de Akker and R. Gispen. J. Infect. Dis. 126:117-122, 1972.
25. Soulier, J. P. and A. M. Courouce-Pauty. Vox Sang. 25:212-234, 1973.
26. Holland, P. V. Am. J. Med. Sci. 270:161-164, 1975.
27. Courouce-Pauty, A. M. and J. P. Soulier. Vox Sang. 27:533-549, 1974.
28. Bancroft, W. H., F. K. Mundo and P. K. Russel. J. Immunol. 109: 842-848, 1972.
29. Howard, C. R. and C. J. Burrell. Prog. Med. Virol. 22:36-103, 1976.
30. Kim, C. Y. and D. M. Bissel. J. Infect. Dis. 123:470-476, 1971.

31. Steiner, S., T. Huebner and G. R. Dreesman. J. Virol. 14:572-577, 1974.
32. Burrell, C. J. J. Gen. Virol. 27:117-126, 1975.
33. Vnek, J., A. M. Prince, N. Hashimoto and H. Ikram. J. Med. Virol. 2:319-333, 1978.
34. Neurath, A. R., A. M. Prince and A. Lippin. Proc. Natl. Acad. Sci. (USA) 71:2663-2667, 1974.
35. O'Neill, S. P. J. Med. Virol. 4:177-185, 1979.
36. Shih, J. W-K., P. L. Tan and J. L. Gerin. Infect. Immun. 28: 459-463, 1980.
37. Vierucci, A., W. T London and B. S. Blumberg. Lancet ii:344, 1974.
38. Neurath, A. R., A. M. Prince, A. Lippin and M. Chen. Lancet ii: 1394-1395, 1973.
39. Blumberg, B. S., A. Vierucci, W. T. London and A. I. Sutnick. Nature 236:28-30, 1972.
40. Ziegenfuss, J. F., Jr., J. Miller and D. Rossman. N. Engl. J. Med. 284:1104, 1971.
41. Hruska, J. F. and W. S. Robinson. J. Med. Virol. 1:119-131, 1977.
42. Robinson, W. S. Ann. Rev. Microbiol. 31:357-377, 1977.
43. Neurath, A. R., S-N. Huang and N. Strick. J. Gen. Virol. 39: 91-101, 1978.
44. Budkowska, A., J. W.-K. Shih and J. L. Gerin. J. Immunol. 118: 1300-1305, 1977.
45. Fields, H. A., F. B. Hollinger, J. Desmyter, J. L. Melnick and G. R. Dreesman. Intervirology 8:336-350, 1977.
46. Robinson, W. S., D. A. Clayton and R. L. Greenman. J. Virol. 14: 384-391, 1974.
47. Summers, J. A., A. O'Connell and I. Millman. Proc. Natl. Acad. Sci. (USA) 72:4597-4601, 1975.
48. Kaplan, P. M., R. L. Greenman, J. L. Gerin, R. H. Purcell and W. S. Robinson. J. Virol. 12:995-1005, 1973.
49. Robinson, W. S. and R. L. Greenman. J. Virol. 13:1231-1236, 1974.
50. Hirschman, S. Z., S. Vernace and F. Schaffner. Lancet i:1099-1103, 1971.
51. Magnius, L. O. and J. A. Espmark. J. Immunol. 109:1017-1021, 1972.
52. Magnius, L. O. Clin. Exp. Immunol. 20:209-216, 1975.
53. Magnius, L. O. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, PA, p. 177, 1978.
54. Albin, C. and W. S. Robinson. J. Virol. 34:297-302, 1980.
55. Feitelson, M. A., P. L. Marion and W. S. Robinson. J. Virol. 43: 741-748, 1982.
57. Huang, S.-N. Am. J. Med. Sci. 270:131-139, 1975.
58. Gudat, F. and L. Bianchi. Gastroenterology 73:1194-1197, 1977.
59. Murphy, B. L., J. M. Peterson, J. W. Ebert, K. R. Berquist, J. E. Maynard and R. H. Purcell. Intervirology 6:207-211, 1976.
60. Nowoslawski, A., W. J. Brzosko, K. Madalinski and K. Krawczynski. Lancet i:494-498, 1970.
61. Cappel, R., D. V. Beers, F. Maes, M. Toppet and S. Cadranel. J. Med. Virol. 8:201-205, 1981.
62. Lemon, S. M., N. L. Gates, T. E. Simms and W. H. Bancroft. J. Infect. Dis. 143:803-809, 1981.
63. Kryger, P., L. R. Mathiesen, J. Aldershvile, J. O. Nielsen, and the Copenhagen Hepatitis Acuta Programme. Hepatology 1:233-237, 1981.

64. Krugman, S., J. H. Hoofnagle, R. J. Gerety, P. M. Kaplan and J. L. Gerin. N. Engl. J. Med. 290:1331-1335, 1974.
65. Kaplan, P. M., J. L. Gerin and H. J. Alter. Nature 249:762-764, 1974.
66. Rizzetto, M., M. G. Canese, S. Arico, O. Crivelli, C. Trepo, F. Bonino and G. Verme. Gut 18:997-1003, 1977.
67. Rizzetto, M., M. G. Canese, R. H. Purcell, W. T. London, L. D. Sly and J. L. Gerin. Hepatology 1:567-574, 1981.
68. Rizzetto, M., C. Morello, P. M. Mannucci, D. J. Gocke, J. A. Spero, J. H. Lewis, D. H. van Thiel, C. Scaroni and F. Peyretti. J. Infect. Dis. 145:18-22, 1982.
69. Bonino, F., B. Hoyer, E. Ford, J. W.-K. Shih, R. H. Purcell and J. L. Gerin. Hepatology 1:127-131, 1981.
70. Schaffner, F. Am. J. Med. 49:658-668, 1970.
71. Popper, H. Am. J. Pathol. 81:609-628, 1975.
72. Warnatz, H. Acta Hepatogastroenterology 21:237-244, 1974.
73. Gocke, D. Am. J. Med. Sci. 270:49-52, 1975.
74. London, W. T. N. Engl. J. Med. 296:1528-1529, 1977.
75. Redeker, A. G. Am. J. Med. Sci. 270:9-16, 1975.
76. Fye, K. H., M. J. Becker, A. N. Theofilopoulos, H. Moutsopoulos, J.-L. Feldman and N. Talal. Am. J. Med. 62:783-791, 1977.
77. Gocke, D. J., K. Hsu, C. Morgan, S. Bombardieri, M. Lockshin and C. L. Christian. J. Exp. Med. 134 Suppl:330-336, 1971.
78. Combes, B., P. Stastny, J. Shorey, E. H. Eigenbrodt, A. Barrera, A. R. Hull and N. W. Carter. Lancet ii:234-237, 1971.
79. Kohler, P. F., R. E. Cronin and W. S. Hammond. Ann. Intern. Med. 81:488, 1974.
80. Knieser, M. R., E. H. Jenis, D. T. Lowenthal, W. H. Bancroft, W. Burns and R. Shalhoub. Arch. Pathol. 97:193-200, 1974.
81. Gianotti, F. Arch. Dis. Child. 48:794-799, 1973.
82. Levo, Y., P. D. Gorevic, H. J. Kassab, D. Zucker-Franklin and E. C. Franklin. N. Engl. J. Med. 296:1501-1504, 1977.
83. Tiku, M. L., K. R. Beutner and P. L. Ogra. Clin. Exp. Immunol. 36:54-62, 1979.
84. Popp, J. W., Jr., J. L. Dienstag, J. R. Wands and K. J. Bloch. Ann. Intern. Med. 92:379-383, 1980.
85. Meyer zum Buschenfelde, K-H., T. H. Hutteroth, W. Arnold and U. Hopf. In Progress in Liver Disease, Vol. 4, edited by Popper, H. and F. Schaffner, Grune and Stratton, San Francisco, CA, pp. 407-424, 1979.
86. Zinkernagel, R. M. Ann. Rev. Microbiol. 33:201-213, 1979.
87. Edgington, T. S. and F. V. Chisari. Am. J. Med. Sci. 270:213-227, 1975.
88. Krugman, S. and D. J. Gocke. In Major Problems in Internal Medicine, edited by Smith, L. H., Jr., W. B. Saunders Company, Philadelphia, PA, Chapter 2, p. 17, 1978.
89. Szmuness, W. Prog. Med. Virol. 24:40-69, 1978.
90. Beasley, R. P., C.-C. Lin, L.-Y. Hwang and C.-S. Chien. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, PA, Chapter 23, pp. 261-270, 1982.
91. Delong, S. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, PA, Chapter 22, pp. 253-259, 1982.

92. Obata, H., N Hayashi, Y. Motoike, T. Hisamitsu, H. Okuda, S. Kobayashi and K. Nishioka. Int. J. Cancer 25:741-747, 1980.
93. Williams, A. O. Am. J. Med. Sci. 270:53-56, 1975.
94. Marion, P. L. and W. S. Robinson. In Viruses in Naturally Occurring Cancers, Cold Spring Harbor Conferences on Cell Proliferation, Vol. VII, edited by Essex, M., G. Todaro and H. zur Hausen, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 423-434, 1980.
95. Okuda, K. and T. Nakashima. In Progress in Liver Disease, Vol. 4, edited by Popper, H. and F. Schaffner, Grune and Stratton, San Francisco, CA, Chapter 35, pp. 639-650, 1979.
96. Larouze, B., B. S. Blumberg, W. T. London, E. D. Lustbader, M. Sankale and M. Payet. J. Natl. Cancer Inst. 58:1557-1561, 1977.
97. Blumberg, B. S., B. Larouze, W. T. London, B. Werner, J. E. Hesser, I. Millman, G. Saimot and M. Payet. Am. J. Pathol. 81: 669-682, 1975.
98. Steiner, P. E. and J. N. P. Davies. Br. J. Cancer 11:523-534, 1957.
99. Sherlock, S., R. A. Fox, S. P. Niazi and P. J. Scheuer. Lancet i: 1243-1247, 1970.
100. Vogel, C. L., P. P. Anthony, N. Mody and L. F. Barker. Lancet ii: 621-624, 1970.
101. Sumithran, E. and K. Prathap. Cancer 40:1618-1620, 1977.
102. Tabor, E., R. J. Gerety, C. L. Vogel, A. C. Bayley, P. P. Anthony, C. H. Chan and L. F. Barker. J. Natl. Cancer Inst. 58:1197-1200, 1977.
103. Maupas, Ph., P. Coursaget, A. Goudeau, J. Drucker, M. Sankale, J. Linhard and G. Diebolt. Ann. Microbiol. (Inst. Pasteur) 128A: 245-253, 1977.
104. Maupas, P., B. Werner, B. Larouze, I. Millman, W. T. London, A. O'Connell and B. S. Blumberg. Lancet ii:9-11, 1975.
105. Brechot, C., M. Hadchouel, J. Scotto, F. Degos, P. Charnay, C. Trepo and P. Tiollais. Lancet ii:765-768, 1981.
106. Brechot, C., C. Pourcel, A. Louise, B. Rain and P. Tiollais. Nature 286:533-535, 1980.
107. Shafritz, D. A., D. Shouval, H. I. Sherman, S. J. Hadziyannis and M. C. Kew. N. Engl. J. Med. 305:1067-1073, 1981.
108. Koshy, R., P. Maupas, R. Muller and P. H. Hofschneider. J. Gen. Virol. 57:95-102, 1981.
109. Shafritz, D. A. and M. C. Kew. Hepatology 1:1-8, 1981.
110. Twist, E. M., H. F. Clark, D. P. Aden, B. B. Knowles and S. A. Plotkin. J. Virol. 37:239-243, 1981.
111. Brechot, C., C. Pourcel, A. Louise, B. Rain and P. Tiollais. Prog. Med. Virol. 27:99-102, 1981.
112. Brechot, C., M. Hadchouel, J. Scotto, M. Fonck, F. Potet, G. N. Vyas and P. Tiollais. Proc. Natl. Acad. Sci. (USA) 78:3906-3910, 1981.
113. Macnab, G. M., J. J. Alexander, G. Lecatsas, E. M. Bey and J. M. Urbanowicz. Br. J. Cancer 34:509-515, 1976.
114. Marion, P. L., F. H. Salazar, J. J. Alexander and W. S. Robinson. J. Virol. 33:795-806, 1980.
115. Edman, J. C., P. Gray, P. Valenzuela, L. B. Rall and W. J. Rutter. Nature 286:535-538, 1980.
116. Chakraborty, P. R., N. Ruiz-Opazo, D. Shouval and D. A. Shafritz. Nature 286:531-533, 1980.

117. Shouval, D., L. M. Reid, P. R. Chakraborty, N. Ruiz-Opazo, R. Morecki, M. A. Gerber, S. N. Thung and D. A. Shafritz. Cancer Research 41:1342-1350, 1981.
118. Desmyter, J., G. D. Groote, M. B. Ray, A. F. Bradburne, V. Desmet, P. D. Somer and J. Alexander. Prog. Med. Virol. 27: 103-108, 1981.
119. Bassendine, M. F., B. A. M. Arborgh, N. Shipton, J. Monjardino, J. Arangelbel, H. C. Thomas and S. Sherlock. Gastroenterology 79: 528-532, 1980.
120. Summers, J., J. M. Smolec and R. Snyder. Proc. Natl. Acad. Sci. (USA) 75:4533-4537, 1978.
121. Popper, H., J. W.-K., Shih, J. L. Gerin, D. C. Wong, B. H. Hoyer, W. T. London, D. L. Sly and R. H. Purcell. Hepatology 1:91-98, 1981.
122. Snyder, R. L. and J. Summers. Cold Spring Harbor Symposia on Quantitative Biology, Vol. VII, Cold Spring Harbor Labs, Cold Spring Harbor, NY, pp. 447-457, 1980.
123. Summers, J., C. W. Ogston, G. J. Jonak, S. M. Astrin, G. V. Tyler and R. L. Snyder. Submitted for publication, 1982.
124. Noyes, W. F. Proc. Soc. Exp. Biol. Med. 144:845-851, 1973.
125. Jenson, A. B., R. M. McCombs and N. Sakurad. Exp. Molec. Pathol. 13:217-230, 1970.
126. Zuckerman, A. J., P. M. Baines and J. D. Almeida. Nature 236: 78-81, 1972.
127. Coyne, V. E., B. S. Blumberg and I. Millman. Proc. Soc. Exp. Biol. Med. 138:1051-1057, 1971.
128. Zuckerman, A. J. Am. J. Med. Sci. 270:197-204, 1975.
129. Brighton, W. D., P. E. Taylor and A. J. Zuckerman. Nature New Biology 232:57-58, 1971.
130. Watanabe, M., T. Umenai, H. Ohori and N. Ishida. Br. J. Exp. Pathol. 57:211-216, 1976.
131. Ananiev, A. V., V. M. Stakhanova and N. N. Zhulina. Vopr. Virusol. 19:301-304, 1974.
132. Blumberg, B. S., A. I. Sutnick and W. T. London. Bull. N. Y. Acad. Med. 44:1566-1586, 1968.
133. Maynard, J. E., W. V. Hartwell and K. R. Berquist. J. Infect. Dis. 126:660-664, 1971.
134. Lichter, E. A. Nature 224:810, 1969.
135. Barker, L. F., F. Chisari, P. P. McGrath, D. W. Dalgard, R. L. Kirschstein, J. D. Almedia, T. S. Edgington, D. G. Sharp and M. R. Peterson. J. Infect. Dis. 127:648-662, 1973.
136. Prince, A. M. In Hepatitis and Blood Transfusions, edited by Vyas, G. N., H. A. Perkins and R. Schmid, Grune and Stratton, New York, NY, p. 403, 1972.
137. Bancroft, W. H., R. Snitbhan, R. M. Scott, M. Tingpalapong, W. T. Watson, P. Tauticharoenyos, J. J. Karwacki and S. Srimarut. J. Infect. Dis. 135:79-85, 1977.
138. London, W. T., I. Millman and A. I. Sutnick. Clin. Res. 18:536, 1970.
139. Barker, L. F., J. E. Maynard, R. H. Purcell, J. H. Hoofnagle, K. R. Berquist and W. T. London. Am. J. Med. Sci. 270:189, 1975.
140. Viral hepatitis: Report of a scientific group. WHO technical report series 512, 1973.
141. Knowles, B. B., C. C. Howe and D. P. Aden. Science 209:497-499, 1980.

142. Hofschneider, P. H., V. Zaslavsky, F. Deinhardt, G. G. Frosner and J. J. Alexander. J. Med. Virol. 4:171-175, 1979.
143. Monjardino, J. and E. Crawford. Virology 96:652-655, 1979.
144. Skelly, J., J. A. Copeland, C. R. Howard and A. J. Zuckerman. Nature 282:617-618, 1979.
145. Marion, P. L., F. H. Salazar, J. J. Alexander and W. S. Robinson. J. Virol. 32:796-802, 1979.
146. Aden, D. P., A. Fogel, S. Plotkin, I. Damjanov and B. B. Knowles. Nature 282:615-616, 1979.
147. Hofschneider, P. H., O. Marquardt, W.-K. Wong and V. Zaslavsky. Cold Spring Harbor Symposia on Quantitative Biology, Vol. VII, Cold Spring Harbor Labs, Cold Spring Harbor, NY, pp. 435-446, 1980.
148. Baltimore, D., E. Gilboa, E. Rothenberg and F. Yoshimura. Cold Spring Harbor Symposium on Quantitative Biology 43:869-874, 1978.
149. Johnson, P. J., M. H. Wansbrough-Jones, B. Portmann, A. L. Eddleston, R. Williams, W. D. Maycook and R. Y. Calne. Br. Med. J. 1:216, 1978.
150. Hoefs, J. C., I. G. Renner, M. Ashcanai and A. G. Redeker. Gastroenterology 79:191-194, 1980.
151. Mason, W. S., G. Seal and J. Summers. J. Virol. 36:829-836, 1980.
152. Marion, P. L., L. S. Oshiro, D. C. Regnery, G. H. Scullard and W. S. Robinson. Proc. Natl. Acad. Sci. (USA) 77:2941-2945, 1980.
153. Summers, J. Hepatology 1:179-183, 1981.
154. Summers, J., J. M. Smolec, B. G. Werner, T. J. Kelly, Jr., G. V. Tyler and R. L. Snyder. Cold Spring Harbor Symposium on Quantitative Biology, Vol. VII, Cold Spring Harbor Labs, Cold Spring Harbor, New York, NY, pp. 459-470, 1980.
155. Valenzuela, P., P. Gray, M. Quiroga, J. Zaldivar, H. M. Goodman and W. J. Rutter. Nature 280:815-819, 1979.
156. Galibert, F., E. Mandart, F. Fitoussi, P. Tiollais and P. Charnay. Nature 281:646-650, 1979.
157. Charnay, P., E. Mandart, A. Hampe, F. Fitoussi, P. Tiollais and F. Galibert. Nucleic Acids Research 7:335-346, 1979.
158. Galibert, F. In Hepatitis B Vaccine, INSERM Symposium No. 18, edited by Maupas, P. and P. Guesry, Elsevier/North Holland Biomedical Press, pp. 267-273, 1981.
159. Galibert, F., T. N. Chen and E. Mandart. J. Virol. 41:51-65, 1982.
160. Galibert, F., T. N. Chen and E. Mandart. Proc. Natl. Acad. Sci. (USA) 78:5315-5319, 1981.
161. Robinson, W. S. Ann. N. Y. Acad. Sci. 354:371-378, 1980.
162. Price, P., S. Z. Hirschman and E. Garfinkel. J. Med. Virol. 6: 139-145, 1980.
163. Siddiqui, A., P. L. Marion and W. S. Robinson. J. Virol. 38: 393-397, 1981.
164. Siddiqui, A., F. Sattler and W. S. Robinson. Proc. Natl. Acad. Sci. (USA) 76:4664-4668, 1979.
165. Sninsky, J. J., A. Siddiqui, W. S. Robinson and S. N. Cohen. Nature 279:346-348, 1979.
166. Cummings, I. W., J. K. Browne, W. A. Salser, G. V. Tyler, R. L. Snyder, J. M. Smolec and J. Summers. Proc. Natl. Acad. Sci. (USA) 77:1842-1846, 1980.

167. Murray, K., P. Mackay, C. J. Burrell, H. Schaller, S. Stahl and M. Pasek. In Hepatitis B Vaccine, INSERM Symposium No. 18, edited by Maupas, P. and P. Guesry, Elsevier/North Holland Biomedical Press, pp. 289-304, 1981.
168. Pourcel, C., A. Louise, M. Gervais, N. Chenciner, M. F. Dubois and P. Tiollais. J. Virol. 42:100-105, 1982.
169. Mackay, P., M. Pasek, M. Magazin, R. T. Kovacic, B. Allet, S. Stahl, W. Gilbert, H. Schaller, S. A. Bruce and K. Murray. Proc. Natl. Acad. Sci. (USA) 78:4510-4514, 1981.
170. Pourcel, C., M.-F. Dubois, S. Rousset, C. Chany and P. Tiollais. Prog. Med. Virol. 27:93-98, 1981.
171. Moriarty, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin and D. H. Hamer. Proc. Natl. Acad. Sci. (USA) 78:2606-2610, 1981.
172. Mackay, P., J. Lees and K. Murray. J. Med. Virol. 8:237-243, 1981.
173. Dubois, M.-F., C. Pourcel, S. Rousset, C. Chany and P. Tiollais. Proc. Natl. Acad. Sci. (USA) 77:4549-4553, 1980.
174. Charnay, P., M. Gervais, A. Louise, F. Galibert and P. Tiollais. Nature 286:893-895, 1980.
175. Hirschman, S. Z., P. Price, E. Garfinkel, J. Christman and G. Acs. Proc. Natl. Acad. Sci. (USA) 77:5507-5511, 1980.
176. Hardy, K., S. Stahl and H. Kupper. Nature 293:481-483, 1981.
177. Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. Mackay, G. Leadbetter and K. Murray. Nature 282:575-579, 1979.
178. Burrell, C. J., P. Mackay, P. J. Greenaway, P. H. Hofschneider and K. Murray. Nature 279:43-47, 1979.
179. Edman, J. C., R. A. Hallewell, P. Valenzuela, H. M. Goodman and W. J. Rutter. Nature 291:503-506, 1981.
180. Gerlich, W. H., M. A. Feitelson, P. L. Marion and W. S. Robinson. J. Virol. 36:787-795, 1980.
181. Feitelson, M. A., P. L. Marion and W. S. Robinson. J. Virol. 39: 447-454, 1981.
182. Lerner, R. A., N. Green, A. Olson, T. Shinnick and J. G. Sutcliffe. Hospital Practice 16:55-62, 1981.
183. Lerner, R. A., N. Green, H. Alexander, F.-T. Liu, J. G. Sutcliffe and T. M. Shinnick. Proc. Natl. Acad. Sci. (USA) 78:3403-3407, 1981.
184. Hopp, T. P. Molec. Immunol. 18:869-872, 1981.
185. Hopp, T. P. and K. R. Woods. Proc. Natl. Acad. Sci. (USA) 78: 3824-3828, 1981.
186. Dreesman, G. R., Y. Sanchez, I. Ionescu-Matiu, J. T. Sparrow, H. R. Six, D. L. Peterson, F. B. Hollinger and J. L. Melnick. Nature 295:158-160, 1982.
187. Prince, A. M., H. Ikram and T. P. Hopp. Proc. Natl. Acad. Sci. (USA) 79:579-582, 1982.
188. Mason, W. S., C. Aldrich, J. Summers and J. M. Taylor. Proc. Natl. Acad. Sci. (USA) 79:3997-4001, 1982.
189. Summers, J. and W. S. Mason. Cell 29:403-415, 1982.
190. Gerin, J. L., P. V. Holland and R. H. Purcell. J. Virol. 7: 569-576, 1971.
191. Gerlich, W. and G. May. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A. 224:49-60, 1973.
192. Vyas, G. N., E. W. Williams, G. G. B. Klaus and H. E. Bond. J. Immunol. 108:1114-1118, 1972.

193. Gerin, J. L. In Hepatitis and Blood Transfusion, edited by Vyas, G. N., H. A. Perkins and R. Schmid, Grune and Stratton, New York, NY, pp. 205-219, 1972.
194. Gerin, J. L. In Mechanisms of Virus Disease, edited by Robinson, W. S. and C. F. Fox, W. A. Benjamin, Inc., Menlo Park, CA, pp. 215-223, 1974.
195. Chairez, R., S. Steiner, J. L. Melnick and G. R. Dreesman. Intervirology 1:224-228, 1973.
196. Chairez, R., F. B. Hollinger, J. P. Brunschwig and G. R. Dreesman. J. Virol. 15:182-190, 1975.
197. Howard, C. R. and A. J. Zuckerman. Intervirology 4:31-44, 1974.
198. Chairez, R., F. B. Hollinger, J. L. Melnick and G. R. Dreesman. Intervirology 3:129-140, 1974.
199. Dreesman, G. R., F. B. Hollinger, J. R. Suriano, R. S. Fujioka, J. P. Brunschwig and J. L. Melnick. J. Virol. 10:469-476, 1972.
200. Rao, K. R. and G. N. Vyas. Nature (London) New Biology 241: 240-241, 1973.
201. Sanchez, Y., I. Ionescu-Matiu, F. B. Hollinger, J. L. Melnick and G. R. Dreesman. J. Gen. Virol. 48:273-283, 1980.
202. Gold, J. W. M., J. W.-K. Shih, R. H. Purcell and J. L. Gerin. J. Immunol. 117:1404-1406, 1976.
203. Dreesman, G. R., F. B. Hollinger and J. L. Melnick. Am. J. Med. Sci. 270:123-129, 1975.
204. Neurath, A. R., N. Strick and C. Y. Huang. Intervirology 10: 265-275, 1978.
205. Hubert, M., F. Barin and A. Goudeau. J. Virol. Methods 3: 349-354, 1982.
206. Koistinen, V. U. J. Virol. 35:20-23, 1980.
207. Dreesman, G. R., R. Chairez, M. Suarez, F. B. Hollinger, R. J. Courtney and J. L. Melnick. J. Virol. 16:508-515, 1975.
208. Sanchez, Y., I. Ionescu-Matiu, G. R. Dreesman, F. B. Hollinger and J. L. Melnick. Virology 114:71-80, 1981.
209. Shih, J. W.-K. and J. L. Gerin. J. Immunol. 115:634-639, 1975.
210. Skelly, J., C. R. Howard and A. J. Zuckerman. J. Gen. Virol. 44: 679-689, 1979.
211. Shih, J. W.-K. and J. L. Gerin. J. Virol. 21:347-357, 1977.
212. Almeida, J. D., A. J. Zuckerman, P. E. Taylor and A. P. Waterson. Microbios. 2:117-123, 1969.
213. Hirschman, S. Z., J. Schwartz, S. Vernace, F. Schaffner and C. Ganz. J. Infect. Dis. 128:605-617, 1973.
214. Barker, L. F., K. O. Smith, W. D. Gehle and N. P. Shulman. J. Immunol. 102:1529-1532, 1969.
215. Drovet, J., A. M. Courouce, A. M. Therenoux, J. P. Soulier, J. Chanard, G. Vallee and J. L. Funck. Biomedicine 22:158-166, 1975.
216. Gerin, J. L., R. H. Purcell, M. D. Hoggan, P. V. Holland and R. M. Chanock. J. Virol. 4:763-768, 1969.
217. Howard, C. R. In Human Viral Hepatitis: Hepatitis-associated Antigen and Viruses, edited by Zuckerman, A. J., North-Holland, Amsterdam, pp. 108-121, 1975.
218. Schober, A., U. Kaboth and R. Thomssen. Arch. Ges. Virusforsch. 33:364-374, 1971.
219. Bohme, U., P. G. Hoher and E. Kowert. Arch. Ges. Virusforsch. 39:393-395, 1972.
220. Kim, C. Y. and J. G. Tilles. J. Clin. Invest. 52:1176-1186, 1973.

221. Takahashi, T. J. Japan. Med. Assoc. 73:225-234, 1975.
222. Bourbonnais, R., R. M. Guevin and E. E. Delvin. Vox Sang 29:269-279, 1975.
223. Ling, C. M. and L. R. Overby. J. Immunol. 109:834-841, 1972.
224. Skinhøj, P. and J. F. Hansen. Scand. J. Lab. Invest. 32:199-203, 1973.
225. Barinsky, I. F. and A. F. Bocharov. Acta Virol., Prague 18:363, 1974.
226. Barker, L. F., J. D. Almeida, J. H. Hoofnagle, R. J. Gerety, D. R. Jackson and P. P. McGrath. J. Virol. 14:1552-1558, 1974.
227. London, W. T., J. S. Drew, B. S. Blumberg, R. A. Grossman and P. J. Lyons. N. Engl. J. Med. 296:241-244, 1977.
228. Lipman, M. B., W. J. Hierholzer and A. Schluederberg. J. Infect. Dis. 128:664-667, 1973.
229. Moritsugu, Y., J. W. M. Gold, J. Wagner, R. Y. Dodd and R. H. Purcell. J. Immunol. 114:1792-1798, 1975.
230. Hsu, T. W. and J. M. Taylor. J. Virol. 44:47-53, 1982.
231. Portocala, R., L. M. Popa and R. Repanovici. Acta Virol., Prague 16:191-200, 1972.
232. Alter, H. J. and B. S. Blumberg. Blood 27:297-309, 1966.
233. Sukeno, N., R. Shirachi, J. Yamaguchi and N. Ishida. J. Virol. 9:182-183, 1972.
234. Neurath, A. R., L. Cosio, A. M. Prince, A. Lippin and H. Ikram. Proc. Soc. Exp. Biol. Med. 143:440-445, 1973.
235. Howard, C. P. and A. J. Zuckerman. J. Gen. Virol. 20:253-256, 1973.
236. Tripatzis, I. and H. G. Horst. Nature 231:266-267, 1971.
237. Neurath, A. R., A. M. Prince and A. Lippin. J. Gen. Virol. 19: 391-395, 1973.
238. Grabow, W. O. K. and W. O. Prozesky. J. Infect. Dis. 127: 183-186, 1973.
239. Vyas, G. N., K. R. Rao and A. B. Ibrahim. Science 178:1300-1301, 1972.
240. Dreesman, G. R., F. B. Hollinger, R. M. McCombs and J. L. Melnick. J. Gen. Virol. 19:129-134, 1973.
241. Imai, M., A. Gotoh, K. Nishioka, S. Kurashina, Y. Miyakawa and M. Mayumi. J. Immunol. 112:416-419, 1974.
242. Sukeno, N., R. Shirachi, H. Shiraishi and N. Ishida. J. Virol. 10: 157-158, 1972.
243. Neurath, A. R. and N. Strick. J. Med. Virol. 6:309-322, 1980.
244. Mishiro, S., M. Imai, T. Gotanda, K. Takahashi, T. Sano, H. Yoshizawa, Y. Miyakawa and M. Mayumi. J. Med. Virol. 6:269-278, 1980.
245. Hirschman, S. Z., S. Kochwa, R. Rosenfield and J. Schwartz. Vox Sang. 26:222-226, 1974.
246. Millman, I., L. A. Loeb, M. E. Bayer and B. S. Blumberg. J. Exp. Med. 131:1190-1199, 1970.
247. Sugimoto, Y. and S. Toyoshima. Antimicrobial Agents and Chemotherapy 16:329-332, 1979.
248. Horst, H., I. Tripatzis and I. Konstantinidis. Zbl. Bakt. Hyg., 1 Abt. Orig. A 219:1-6, 1972.
249. Rao, K. R. and G. N. Vyas. J. Gen. Virol. 54:571-573, 1974.
250. Gerin, J. L. and G. N. Vyas. In Progress in Transfusion and Transplantation, edited by Schmidt, P. J., American Association of Blood Banks, Washington, D.C., pp. 167-187, 1972.

250a. Shechter, Y., A Patchornik and Y. Burstein. Biochemistry 15:5071-5075, 1976.
251. Shiraishi, H., R. Shirachi, N. Ishida and T. Sekine. J. Gen. Virol. 38:363-367, 1978.
252. Burrell, C. J., E. Proudfoot, G. A. Keen and B. P. Marmion. Nature New Biology 243:260-262, 1973.
253. Nicolet, B. H., and L. A. Shinn. J. Am. Chem. Soc. 61:1615, 1939.
254. Atassi, M. Z. Biochem. J. 102:478-487, 1967.
255. Dolby, L. J. and D. L. Booth. J. Am. Chem. Soc. 88:1049-1051, 1966.
256. Atassi, M. Z. Arch. Biochem. Biophys. 120:56-59, 1967.
257. Horton, H. R. and D. E. Koshland, Jr. J. Am. Chem. Soc. 87: 1126-1132, 1965.
257a. Rao, K. R. and G. N. Vyas. Microbios. 9:239-245, 1974.
258. Koshland, D. E., Jr., Y. D. Karkhanis and H. G. Latham. J. Am. Chem. Soc. 86:1448-1450, 1964.
259. Oza, N. B. and C. J. Martin. Biochem. Biophys. Res. Comm. 26: 7-13, 1967.
260. Neurath, A. R., N. Strick and W. R. Oleszko. J. Virol. Methods 3:115-125, 1981.
261. Mackay, P. and C. J. Burrell. J. Gen. Virol. 33:181-191, 1976.
262. Means, G. E. and R. E. Feeney. Biochemistry 7:2192-2201, 1968.
263. Reichlin, M. Adv. Immunol. 20:71-123, 1975.
264. Peterson, D. L., I. M. Roberts and G. N. Vyas. Proc. Natl. Acad. Sci. (USA) 74:1530-1534, 1977.
265. Jeansson, S., O. Vesterberg and T. Wadstrom. Life Sciences 11: 929-937, 1972.
266. Millman, I., H. Hutanen, F. Merino, M. E. Bayer and B. S. Blumberg. Res. Commun. Chem. Path. Pharm. 2:667-685, 1971.
267. Bolton, A. E. and W. M. Hunter. Biochem. J. 133:529-539, 1973.
268. Courouce-Pauty, A. M., J. M. Lemaire and J. F. Roux. Vox Sang. 35:304-308, 1978.
269. Courouce, A. M., P. V. Holland, J. Y. Muller and J. P. Soulier, eds. Bibliotheca Haematologica 42:1-158, 1976.
270. LeBouvier, G. and A. Williams. Am. J. Med. Sci. 270:165-171, 1975.
271. Hess, G., J. W.-K. Shih, W. Arnold, J. L. Gerin and K.-H. Meyer zum Buschenfelde. J. Immunol. 123:1189-1194, 1979.
272. Nordenfelt, E. and G. LeBouvier. Intervirology 2:65-75, 1974.
273. Mazzur, S., S. Burgert and G. LeBouvier. J. Immunol. 114: 1510-1512, 1975.
274. Maupas, P., A. Goudeau, P. Coursaget, J. Drucker, F. Barin and M. Andre. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 539-556, 1978.
275. Krugman, S., J. P. Giles and J. Hammond. JAMA 217:41-45, 1971.
276. Hilleman, M. R., A. U. Bertland and E. B. Buynak. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 525-538, 1978.
277. Szmuness, W., C. E. Stevens, E. A. Zang, E. J. Harley and A. Kellner. Hepatology 1:377-385, 1981.
278. Szmuness, W., C. E. Stevens, E. J. Harley, E. A. Zang, W. R. Oleszko, D. C. Williams, R. Sadovsky, J. M. Morrison and A. Kellner. N. Engl. J. Med. 303:833-841, 1980.

279. Purcell, R. H. and J. L. Gerin. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 491-505, 1978.
280. Seeff, L. B., H. J. Zimmerman, E. C. Wright, B. F. Felsher, J. D. Finkelstein, P. Garci-Pont, H. B. Greenlee, A. A. Dietz, J. Hamilton, R. S. Koff, C. M. Leevy, T. Kiernan, C. H. Tamburro, E. R. Schiff, Z. Vlahcevic, R. Zemel, D. S. Zimmon and N. Nath. Lancet ii:939-941, 1975.
281. Redeker, A. G., J. W. Mosley, D. J. Gocke, A. P. McKee and W. Pollack. N. Engl. J. Med. 293:1055, 1975.
282. Rao, K. R. and G. N. Vyas. Microbios. 10:233-238, 1974.
283. Burrell, C. J., G. Leadbetter, P. Mackay and B. P. Marmion. J. Gen. Virol. 33:41-50, 1976.
284. Morein, B., A. Helenius, K. Simons, R. Pettersson, L. Kaariainen and V. Schirrmacher. Nature 276:715-718, 1978.
285. Cappel, R. and F. DeCyper. Arch. Virol. 50:207-213, 1976.
286. Hillemann, M. R. Cancer Res. 36:857-858, Part 2, 1976.
287. Hunsmann, G., V. Moening and W. Schafer. Virology 66:327-329, 1976.
288. Helenius, A. and K. Simons. Biochim. Biophys. Acta 415:29-79, 1975.
289. Tanford, C. and J. Reynolds. Biochim. Biophys. Acta 457:133-170, 1976.
290. Mishiro, S., M. Imai, K. Takahashi, A. Machida, T. Gotanda, Y. Miyakawa and M. Mayumi. J. Immunol. 124:1589-1593, 1980.
291. Shih, J. W.-K. and J. L. Gerin. J. Virol. 21:1219-1222, 1977.
292. Peterson, D. L. J. Biol. Chem. 256:6975-6983, 1981.
293. Chou, P. Y. and G. D. Fasman. Biochemistry 13:222-245, 1974.
294. Sternberg, M. J. E. and J. M. Thornton. Nature 271:15-20, 1978.
295. Skelly, J., C. R. Howard and A. J. Zuckerman. J. Gen. Virol. 41: 447-457, 1978.
296. Cabral, G. A., F. Marciano-Cabral, G. A. Funk, Y. Sachez, F. B. Hollinger, J. L. Melnick and G. R. Dreesman. J. Gen. Virol. 38: 339-350, 1978.
297. Shih, J.-W., P. L. Tan and J. L. Gerin. J. Immunol. 120:520-525, 1978.
298. Shiraishi, H., R. Shirachi, K. Akiyama and N. Ishida. J. Gen. Virol. 48:31-38, 1980.
299. Werner, B. G., J. M. Smolec, R. Snyder and J. Summers. J. Virol. 32:314-322, 1979.
300. Millman, I., T. Halbherr and H. Simmons. Infect. Immun. 35: 752-757, 1982.
301. Cote, P. J., Jr., G. M. Dapolito, J. W.-K. Shih and J. L. Gerin. J. Virol. 42:135-142, 1982.
302. Wands, J. R. and V. R. Zurawski, Jr. Gastroenterology 80: 225-232, 1981.
303. Shih, J. W.-K., P. J. Cote, Jr., G. M. Dapolito and J. L. Gerin. J. Virol. Methods 1:257-273, 1980.
304. Gerin, J. L., J. W.-K. Shih and B. H. Hoyer. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 5, pp. 49-55, 1982.
305. Wands, J. R., R. I. Carlson, H. Schoemaker, K. J. Isselbacher and V. R. Zurawski, Jr. Proc. Natl. Acad. Sci. (USA) 78:1412-1218, 1981.

306. Zurawski, V. R., Jr., B. C. DelVillano and J. R. Wands. In Monoclonal Antibodies and T Cell Hybridomas, edited by Hammerling, H., G. J. Hammerling and J. J. Kearney, Elsevier-North Holland, New York, pp. 273-282, 1981.
307. Fairbanks, G., T. L. Steck and D. F. H. Wallach. Biochemistry 10: 2606-2617, 1971.
308. Parish, C. R. J. Exp. Med. 134:21-47, 1971.
309. Thompson, K., M. Harris, E. Benjamini, G. Mitchell and M. Noble. Nature New Biology 238:20-21, 1972.
310. Kremastinou, T., C. R. Howard, J. Skelly and A. J. Zuckerman. Intervirology 16:176-183, 1981.
311. Ionescu-Matiu, I., Y. Sanchez, F. B. Hollinger, J. L. Melnick and G. R. Dreesman. J. Med. Virol. 6:175-178, 1980.
312. Milich, D. R., P. K. Bhatnagar, E. D. Papas and G. N. Vyas. J. Med. Virol. 7:193-204, 1981.
313. Milich, D. R., E. D. Papas, P. K. Bhatnagar and G. N. Vyas. J. Med. Virol. 7:181-192, 1981.
314. Thung, S. N. and M. A. Gerber. Hepatology 1:132-136, 1981.
315. Hollinger, F. B. and G. R. Dreesman. Gastroenterology 76: 641-643, 1979.
316. Hansson, B. G. and R. H. Purcell. Infect. Immun. 26:125-130, 1979.
317. Imai, M., Y. Yanase, T. Nojiri, Y. Miyakawa and M. Mayumi. Gastroenterology 76:242-247, 1979.
318. Neurath, R. A. and N. Strick. Intervirology 11:128-132, 1979.
319. Krugman, S., H. P. Holley, Jr., M. Davidson, M. S. Simberkoff and N. Matsaniotis. J. Med. Virol. 8:119-121, 1981.
320. Szmuness, W., C. E. Stevens, E. J. Harley, E. A. Zang, P. E. Taylor, H. J. Alter and the Dialysis Vaccine Trial Group. J. Med. Virol. 8:123-129, 1981.
321. Funakoshi, S., T. Ohmura, T. Fujiwara, T. Suyama and R. Naito. Prog. Med. Virol. 27:163-167, 1981.
322. Rizzetto, M., B. Hoyer, M. G. Canese, J. W.-K. Shih, R. Purcell and G. Gerin. Proc. Natl. Acad. Sci. (USA) 77:6124-6128, 1980.
323. Rizzetto, M., M. G. Canese, J. L. Gerin, W. T. London, D. L. Sly and R. H. Purcell. J. Infect. Dis. 141:590-602, 1980.
324. Jozwiak, W., J. Koscielak, K. Madalinski, W. J. Brzosko, A. Nowoslawski and M. Kloczewiak. Nature New Biology 229:92-94, 1971.
324a. Schuurs, A. H. W. M. and G. Wolters. Am. J. Med. Sci. 270: 173-177, 1975.
324b. Werner, B. and W. T. London. Ann. Intern. Med. 83:113-114, 1975.
324c. Neurath, A. R., N. Strick, C. Y. Huang and A. M. Prince. J. Med. Virol. 2:231-245, 1978.
325. Melnick, J. L., G. R. Dreesman and F. B. Hollinger. J. Infect. Dis. 133:210-229, 1976.
326. London, W. T., A. I. Sutnick, I. Millman, V. Coyne, B. S. Blumberg and A. Vierucci. Can. Med. Assoc. J. 106:480-484, 1971.
327. Hesser, J. E., J. Economidou and B. S. Blumberg. Hum. Biol. 47: 415-425, 1975.
328. Drew, J. S., W. T. London, E. D. Lustbader, J. E. Hesser and B. S. Blumberg. Science 201:687-692, 1978.
329. London, W. T., J. S. Drew, E. D. Lustbader, B. G. Werner and B. S. Blumberg. Kidney Internat. 12:51-58, 1977.
330. Wallace, J. J. Clin. Pathol. 25:553, 1972.

331. Szmuness, W., R. L. Hirsch, A. M. Prince, R. W. Levine, E. J. Harley and H. Ikram. J. Infect. Dis. 131:111-118, 1975.
332. Goodman, M., R. L. Wainwright, H. F. Weir and J. C. Gall. Pediatrics 48:907-913, 1971.
333. Terrier, E., M. Simonneau and B. Jaulmes. Vox Sang. 19:352-356, 1970.
334. Tevaluoto-Aarnio, M., B. Werner and W. T. London. Med. Biol. 53:51-59, 1975.
335. Soulier, J. P., A. M. Courouce-Pauty and D. Benamon-Djiane. Vox Sang. 19:345-351, 1970.
336. Lous, P., P. Skinhøj and H. Olesen. Vox Sang. 19:379-383, 1970.
337. Mazzur, S. and N. Jones. J. Infect. Dis. 133:331-333, 1976.
338. Blumberg, B. S., A. I. Sutnick, W. T. London and L. Melartin. Arch. Intern. Med. 130:227-231, 1972.
339. Cherubin, C. E., J. J. Lander, R. H. Purcell, T. G. McGinn and L. A. Cone. Lancet ii:149-151, 1972.
340. Okochi, K., M. Mayumi, Y. Haguino and N. Saito. Vox Sang. 19: 332-337, 1970.
341. Farrow, L. J., S. G. Lamb, N. F. Coghill, R. L. Lindon, J. Preece, A. J. Zuckerman and J. S. Stewart. Br. Med. J. 3:83-86, 1974.
342. London, W. T. and J. S. Drew. Proc. Natl. Acad. Sci. (USA) 74: 2561-2563, 1977.
343. Werner, B. G., W. T. London and S. S. Wachtel. Immunogenetics 8: 561-565, 1979.
344. London, W. T., H. Jarvinen, L. Jasko, R. Grossman, P. Lyons and N. Lasker. In Virus and the Liver, edited by Bianchi, L., W. Gerok, K. Sickinger and G A. Stalder, MTP Press, Ltd., Lancaster, pp. 267-276, 1980.
345. Craxi, A., L. Montano, A. Goodall and H. C. Thomas. J. Med. Virol. 9:117-123, 1982.
346. Almeida, J. D. and A. P. Waterson. Lancet ii:983-986, 1969.
347. Good, R. A. and A. R. Page. Am. J. Med. 29:804-810, 1960.
348. Bianchi, L. and F. Gudat. In Progress in Liver Disease, Vol. 4, edited by Popper, H. and F. Schaffner, Grune and Stratton, San Francisco, CA, Chapter 20, pp. 371-392, 1979.
349. Millman, I. and J. C. McMichael. Infect. Immun. 21:879-885, 1978.
350. Skelly, J., C. R. Howard and A. J. Zuckerman. Nature 290:51-54, 1981.
351. Dreesman, G. R., F. B. Hollinger, Y. Sanchez, P. Oefinger and J. L. Melnick. Infect. Immun. 32:62-67, 1981.
352. Sanchez, Y., I. Ionescu-Matiu, G. R. Dreesman, W. Kramp, H. R. Six, F. B. Hollinger and J. L. Melnick. Infect. Immun. 30:728-733, 1980.
353. Purcell, R. H. and J. L. Gerin. Am. J. Med. Sci. 270:395-399, 1975.
354. Hollinger, F. B., J. Werch and J. L. Melnick. N. Engl. J. Med. 290:1104-1109, 1974.
355. Hollinger, F. B., G. R. Dreesman, Y. Sanchez, G. A. Cabral and J. L. Melnick. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 557-567, 1978.
356. Gregoriadis, G. N. Engl. J. Med. 295:704-710, 765-770, 1976.
357. Weissman, G. Infect. Dis. Rev. 10:4-22, 1980.
358. Tiollais, P., P. Charnay and G. N. Vyas. Science 213:406-411, 1981.

359. Rose, G. D. and S. Roy. Proc. Natl. Acad. Sci. (USA) 77: 4643-4647, 1980.
360. Erickson, B. W. and R. B. Merrifield. In The Proteins, Vol. 2, edited by Neurath, H. and R. L. Hill, Academic Press, New York, p. 257, 1976.
361. Berzofsky, J. A. and A. N. Schechter. Molec. Immunol. 18: 751-763, 1981.
362. Hopp, T. P. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 761, 1982. Abstract.
363. Prince, A. M., H. Ikram and T. P. Hopp. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 761-762, 1982. Abstract.
364. Vyas, G. N. In Hepatitis B Vaccine, INSERM Symposium No. 18, edited by Maupas, P. and P. Guesry, Elsevier/North-Holland Biomedical Press, pp. 227-237, 1981.
365. Neurath, A. R. and N. Strick. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 760-761, 1982. Abstract.
366. Dreesman, G. R., I. Ionescu-Matiu, Y. Sanchez, F. B. Hollinger and J. L. Melnick. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 760, 1982. Abstract.
367. Howard, C. R., J. Skelly, K. N. Tsiquaye, A. J. Zuckerman, E. Tabor, R. J. Gerety and T. Kremastinou. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 36, pp. 411-424, 1982.
368. Shih, J. W.-K., G. M. Dapolito and J. L. Gerin. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 712-713, 1982. Abstract.
369. Lutwick, L. I., M. B. Hebert and J. M. Sywassink. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 711-712, 1982. Abstract.
370. Robinson, W. S., P. L. Marion, M. A. Feitelson and A. A. Siddiqui. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 6, pp. 57-68, 1982.
371. Glossman, H. and D. M. Neville, Jr. J. Biol. Chem. 246: 6339-6346, 1971.
372. Cowley, L. P. Am. J. Clin. Pathol. 57:253, 1972.
373. Bond, H. E. In Hepatitis and Blood Transfusion, edited by Vyas, G. N., H. A. Perkins and R. S. Schmid, Grune and Stratton, New York and London, pp. 221-222, 1972.
374. Goldstein, J. J. and L. S. Lucy. Arch. Biochem. Biophys. 111: 407-414, 1969.
375. Shiraishi, H., T. Kohama, R. Shirachi and N. Ishida. J. Gen. Virol. 36:207-210, 1977.
376. Neurath, A. R., N. Hashimoto and A. M. Prince. J. Gen. Virol. 27:81-91, 1975.

377. Morell, A. G., R. A. Irvine, I. Sternlieb, I. H. Scheinberg and G. Ashwell. J. Biol. Chem. 243:155-159, 1968.
378. Morell, A. G., G. Gregoriadis, I. H. Scheinberg, J. Hickman and G. Ashwell. J. Biol. Chem. 246:1461-1467, 1971.
379. Massey, R. J. and G. Schochetman. Science 213:447-449, 1981.
380. Lenkei, R., V. T. Babes, A. Mustea and I. Dobre. J. Med. Virol. 4:137-145, 1979.
381. Lenkei, R., V. T. Babes, M. E. Dan, A. Mustea and I. Dobre. J. Med. Virol. 1:29-34, 1977.
382. Dudley, F. J., R. A. Fox and S. Sherlock. Lancet i:723-726, 1972.
382a. Segrest, J. P., R. J. Jackson, E. P. Andrews and V. T. Marchesi. Biochem. Biophys. Res. Comm. 44:390-395, 1971.
383. Russ, G. and K. Polakova. Biochem. Biophys. Res. Comm. 55: 666-672, 1973.
384. Peterson, D. L. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 707-708, 1982. Abstract.
385. Reading, C. L., E. E. Penhoet and C. E. Ballou. J. Biol. Chem. 253:5600-5612, 1978.
386. Struck, D. K. and W. J. Lennarz. J. Biol. Chem. 252:1007-1013, 1977.
387. Johnson, I. and J. R. Clamp. Biochem. J. 123:739-745, 1971.
388. Benson, J. R., G. A. Funk, Y. Sanchez and G. R. Dreesman. Fed. Proc. 39:1611, 1980. Abstract.
389. Alberti, A., S. Diana, G. H. Scullard, A. L. Eddleston and R. Williams. Br. Med. J. 2:1056-1059, 1978.
390. Alberti, A. In Virus and the Liver, edited by Bianchi, L., W. Gerok, K. Sickinger and G. A. Stalder, MTP Press, Ltd., Lancaster, pp. 59-66, 1980.
391. Moodie, J., L. M. Stannard and A. Kipps. J. Gen. Virol. 24: 375-379, 1974.
392. Zalan, E., J. J. Hamvas, B. A. Tobe, O. Kuderewko and N. A. Labzoffsky. Can. Med. Assoc. J. 104:145-147, 1971.
393. Stannard, L. M., J. Moodie, G. A. Keen and A. Kipps. J. Clin. Pathol. 26:209-216, 1973.
394. Field, A. M. and Y. E. Cossart. Lancet ii:91, 1971.
395. Trepo, C., O. Hantz, L. Vitvitski, B. Blanchy and C. Pichoud. In Hepatitis B Vaccine, INSERM Symposium No. 18, edited by Maupas, P. and P. Guesry, Elsevier/North-Holland Biomedical Press, pp. 67-77, 1981.
396. Wands, J. R., R. R. Bruns, R. I. Carlson, A. Ware, J. E. Menitove and K. J. Isselbacher. Proc. Natl. Acad. Sci. (USA) 79:1277-1281, 1982.
397. Wands, J. R., R. A. Marciniak, K. J. Isselbacher, M. Varghese, G. Don, J. W. Halliday and L. W. Powell. Lancet i:977-980, 1982.
398. Hirschman, S. Z., M. Gerber and E. Garfinkel. J. Med. Virol. 2: 61-76, 1978.
399. Brzosko, W. J., K. Madalinski, K. Krawczynski and A. Nowoslawski. J. Infect. Dis. 127:424-428, 1973.
400. Budkowska, A. J. Infect. Dis. 135:463-467, 1977.
401. Takahashi, K., M. Imai, F. Tsuda, T. Takahashi, Y. Miyakawa and M. Mayumi. J. Immunol. 117:102-105, 1976.
402. Werner, B. G., A. P. O'Connell and J. Summers. Proc. Natl. Acad. Sci. (USA) 74:2149-2151, 1977.
403. Neurath, A. R. and N. Strick. J. Gen. Virol. 42:645-649, 1979.

404. Yoshizawa, H., Y. Itoh, J. P. Simonetti, T. Takahashi, A. Machida, Y. Miyakawa and M. Mayumi. J. Gen. Virol. 42:513-519, 1979.
405. Takahashi, K., Y. Akahane, T. Gotanda, T. Mishiro, M. Imai, Y. Miyakawa and M. Mayumi. J. Immunol. 122:275-279, 1979.
406. Ohori, H., S. Onodera and N. Ishida. J. Gen. Virol. 43:423-427, 1979.
407. Takahashi, K., M. Imai, T. Gotanda, T. Sano, A. Oinuma, S. Mishiro, Y. Mikayawa and M. Mayumi. J. Gen. Virol. 50:49-57, 1980.
408. Lam, K. C., M. J. Tong and J. Rakela. Infect. Immun. 16:403-404, 1977.
409. Gerlich, W. H., U. Goldmann, R. Muller, W. Stibbe and W. Wolff. J. Virol. 42:761-766, 1982.
410. Kaplan, P. M., E. C. Ford, R. H. Purcell and J. L. Gerin. J. Virol. 17:885-893, 1976.
411. Hess, G., J. W.-K. Shih, P. M. Kaplan and J. L. Gerin. J. Immunol. 119:1542-1544, 1977.
412. Shih, J. W.-K., G. Hess, P. M. Kaplan and J. L. Gerin. J. Virol. Methods 1:47-59, 1980.
413. Hess, G., W. Arnold and K.-H. Meyer zum Buschenfelde. J. Med. Virol. 7:241-250, 1981.
414. Hoofnagle, J. H., R. J. Gerety and L. F. Barker. Lancet ii:869-873, 1973.
414a. Ohori, H., M. Yamaki, S. Onodera, E. Yamada and N. Ishida. Intervirology 13:74-82, 1980.
415. Hansson, B. J. Clin. Microbiol. 6:209-211, 1977.
415a. Desmyter, J., W. Liu and J. Creemers. Am. J. Dis. Child. 123: 315-318, 1972.
415b. Almeida, J. D. Am. J. Dis. Child. 123:303-308, 1972.
415c. Stannard, L. M. and M. Hodgkiss. J. Gen. Virol. 45:509-514, 1979.
416. Schalm, S. W., R. A. Heijtink, C. H. Gips and J. R. Huizenga. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 611, 1982. Abstract.
417. Lemon, S. M. and J. H. Hoofnagle. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 723-724, 1982. Abstract.
418. Takahashi, T., S. Nakagawa, T. Hashimoto, K. Takahashi, M. Imai, Y. Miyakawa and M. Mayumi. J. Immunol. 117:1392-1397, 1976.
419. Howard, C. R. and A. J. Zuckerman. J. Immunol. Methods 14: 291-301, 1977.
420. Gerin, J. L. and J. W.-K. Shih. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, Chapter 13, pp. 147-153, 1978.
421. Gerin, J. L., E. C. Ford and R. H. Purcell. Am. J. Pathol. 81: 651-668, 1975.
422. Takahashi, K., M. Imai, Y. Miyakawa, S. Iwakiri and M. Mayumi. Proc. Natl. Acad. Sci. (USA) 75:1952-1956, 1978.
423. Howard, C. R. and A. J. Zuckerman. J. Med. Virol. 4:303-314, 1979.
424. Yamada, E., H. Ohori and N. Ishida. J. Med. Virol. 4:33-42, 1979.
425. Neurath, A. R., N. Strick, W. Szmuness, C. E. Stevens and E. J. Harley. J. Gen. Virol. 42:493-504, 1979.
426. McAuliffe, V. J. and R. H. Purcell. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 161-171, 1978.

427. Tedder, R. S. and F. G. Bull. Clin. Exp. Immunol. 35:380-389, 1979.
428. Howard, C. R., A. R. Zanetti and A. J. Zuckerman. J. Med. Virol. 3:59-66, 1978.
429. Fields, H. A., D. W. Bradley, C. L. Davis and J. E. Maynard. Infect. Immun. 20:792-803, 1978.
430. Yamada, E., H. Ohori and N. Ishida. J. Gen. Virol. 55:75-86, 1981.
431. Katz, D., J. L. Melnick and F. B. Hollinger. J. Med. Virol. 5: 87-100, 1980.
432. Takahashi, K., M. Imai, M. Nomura, A. Oinuma, A. Machida, G. Funatsu, Y. Miyakawa and M. Mayumi. J. Gen. Virol. 57:325-330, 1981.
433. Blanchy, B., O. Hantz, L. Vitvitski and C. Trepo. J. Med. Virol. 5:39-46, 1980.
434. Nakano, M., T. Tohita and T. Ando. Int. J. Peptide & Protein Res. 8:565-578, 1976.
435. Laub, O., J. C. Edman, P. Valenzuela, L. Rall, M. Ziemer and W. J. Rutter. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 812, 1982. Abstract.
436. Tiollais, P., C. Pourcel, C. Brechot, P. Charnay, M.-F. Dubois, A. Fritsch, M. Gervais, A. Louise and S. Wain-Hobson. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 8, pp. 81-96, 1982.
437. Miyakawa, Y. and M. Mayumi. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 16, pp. 183-194, 1982.
438. Hoofnagle, J. H., L. B. Seeff, Z. B. Bales, H. J. Zimmerman and the V.A. Hepatitis Cooperative Study Group. N. Engl. J. Med. 298: 1379-1383, 1978.
439. Katchaki, J. N., R. Brouwer and T. H. Siem. N. Engl. J. Med. 298: 1421-1422, 1978.
440. Dienstag, J. L. Gastroenterology 75:1172-1174, 1978.
441. Hoofnagle, J. H., R. J. Gerety, L. Y. Ni and L. F. Barker. N. Engl. J. Med. 290:1336-1340, 1974.
442. Gerlich, W. H., W. Luer and R. Thomssen. J. Infect. Dis. 142: 95-101, 1980.
443. Niermeijer, P., C. H. Gips, J. R. Huizenga, H. J. Houthoff and J. A. Snijder. Hepato-Gastroenterology 27:271-276, 1980.
444. Omata, M., A. Afroudakis, C.-T. Liew, M. Ashcavai and R. L. Peters. Gastroenterology 75:1003-1009, 1978.
445. Kojima, M., K. Udo, Y. Takahashi, H. Yoshizawa, F. Tsuda, Y. Itoh, Y. Miyakawa and M. Mayumi. Gastroenterology 73:664-667, 1977.
446. Bianchi, L., F. Gudat and M. Schmid. In The Liver: Quantitative Aspects of Structure and Function, edited by Preisig, R., J. Bircher and G. Paumgartner, Aulendorf, Editio Cantor, pp. 99-105, 1976.
447. Endo, Y., F. Gudat, L. Bianchi, M. Mihatsch, M. Gasser, G. A. Stalder and M. Schmid. Schweiz und Wochenschr 108:363-373, 1978.
448. Niermeijer, P., C. Gips, J. Huizenga, J. Ringers, S. Verkerk, H. Houthoff, B. Houwen, J. Snijder and J. Nielsen. Acta Hepato-Gastroenterologica 25:360-364, 1978.

449. Trevisan, A., G. Realdi, A. ALberti, M. Baraldini, F. Miglio and G. Ongaro. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 787-788, 1982. Abstract.
450. Trevisan, A., G. Realdi, A. Alberti, G. Ongaro, E. Pornaro and R. Meliconi. Gastroenterology 82:218-222, 1982.
451. Trevisan, A., G. Realdi, A. Alberti and F. Noventa. Gastroenterology 77:209-214, 1979.
452. Ray, M. B., V. J. Desmet, A. F. Bradburne, J. Desmyter, J. Fevery and J. DeGroote. Gastroenterology 71:462-469, 1976.
453. Alberti, A., G. Realdi, F. Tremolada and G. P. Spina. Clin. Exp. Immunol. 25:396-402, 1976.
454. Trevisan, A., G. Realdi and A. Alberti. In Virus and the Liver, edited by Bianchi, L., W. Gerok, K. Sickinger and G. A. Stalder, MTP Press, Ltd., Lancaster, pp. 97-103, 1980.
455. Costa, J., A. S. Rabson, C. Yee and T. S. Tralka. Nature 269: 251-252, 1977.
456. Oldstone, M. B. A. and A. Tishon. Clin. Immunol. Immunopathol. 9:55-62, 1978.
457. Burns, H. W. and A. C. Allison. In Virus Infection and the Cell Surface, edited by Poste, G. and G. L. Nicolson, Amsterdam, North Holland, pp. 213-247, 1977.
458. Hoofnagle, J. H., R. J. Gerety and L. F. Barker. Am. J. Med. Sci. 270:179-187, 1975.
459. Magnius, L. O., A. Lindholm, P. Lundin and S. Iwarson. JAMA 231: 356-359, 1975.
460. Nielsen, J. O., O. Dietrichson and E. Juhl. Lancet ii:913-915, 1974.
461. Feinman, S. V., B. Berris, J. C. Sinclair, D. M. Wrobel, B. L. Murphy and J. E. Maynard. Lancet ii:1173-1174, 1975.
462. Alter, H. J., L. B. Seeff, P. M. Kaplan, V. J. McAuliffe, E. C. Wright, J. L. Gerin, R. H. Purcell, P. V. Holland and H. J. Zimmerman. N. Engl. J. Med. 295:909-913, 1976.
463. Grady, G. F. and members of the U.S. National Heart and Lung Institute Collaborative Study Group. Lancet ii:492-494, 1976.
464. Berquist, K. R., J. E. Maynard and B. L. Murphy. Lancet i: 1026-1027, 1976.
465. Ling, C.-M., I. K. Mushahwar, L. R. Overby, K. R. Berquist and J. E. Maynard. Infect. Immun. 24:352-356, 1979.
466. Ohbayashi, A., Y. Nakamura, Y. Matsuo, Y. Miyakawa, K. Baba and M. Mayumi. Lancet i:433, 1977.
467. Tong, M. J., M. W. Thursby, J.-H. Lin, J. Y. Weissman and C. M. McPeak. Prog. Med. Virol. 27:137-147, 1981.
468. Beasley, R. P., C. Trepo, C. E. Stevens and W. Szmuness. Am. J. Epidemiol. 105:94-97, 1977.
469. Okada, K., I. Kamiyama, M. Inomata, M. Imai, Y. Miyakawa and M. Mayumi. N. Engl. J. Med. 294:746-749, 1976.
470. Murata, S., K. Nomoto, T. Hattori, M. Imai, Y. Miyakawa and M. Mayumi. N. Engl. J. Med. 296:692-693, 1977.
471. Nordenfelt, E. and L. Kjellen. Intervirology 5:225-232, 1975.
472. Nordenfelt, E. and M. Andren-Sandberg. J. Infect. Dis. 134: 85-89,, 1976.
473. Tong, M. J., D. Stevenson and I. Gordon. J. Infect. Dis. 135: 980-984, 1977.

474. Hindman, S. H., C. R. Gravelle, B. L. Murphy, D. W. Bradley, W. R. Budge and J. E. Maynard. Ann. Intern. Med. 85:458-460, 1976.
475. Schweitzer, I. L., V. M. Edwards and M. Brezina. N. Engl. J. Med. 293:940, 1975.
476. E. Sheikh, N., I. L. Woolf, R. M. Galbraith, A. L. W. F. Eddleston, I. W. Dymock and R. Williams. Br. Med. J. 4:252-253, 1975.
477. Eleftheriou, N., J. Heathcote, H. C. Thomas and S. Sherlock. Lancet ii:1171-1173, 1975.
478. Alberti, A., S. Diana, G. H. Scullard, A. L. W. F. Eddleston and R. Williams. Gastroenterology 75:869-874, 1978.
479. Cappel, R., F. D. Cuyper and D. V. Beers. J. Infect. Dis. 136: 617-622, 1977.
480. Arnold, W., J. O. Nielsen, F. Hardt and K. H. Meyer zum Buschenfelde. Gut 19:994-996, 1977.
481. Huang, A. S. and D. Baltimore. Nature 226:325-327, 1970.
482. Takahashi, K., M. Fukuda, K. Baba, M. Imai, Y. Miyakawa and M. Mayumi. J. Immunol. 119:1556-1559, 1977.
483. Fields, H. A., D. W. Bradley, C. L. Davis, B. L. Murphy, C. A. Schable and J. E. Maynard. J. Immunol. 121:930-935, 1978.
484. Mushahwar, I. K., L. R. Overby, G. Frosner, F. Deinhardt and C.-M. Ling. J. Med. Virol. 2:77-87, 1978.
485. Frosner, G. G., M. Brodersen, G. Papaevangelou, U. Sugg, H. Haas, I. K. Mushahwar, C.-M. Ling, L. R. Overby and F. Deinhardt. J. Med. Virol. 3:67-76, 1978.
486. Trepo, C. G., L. O. Magnius, R. A. Schaefer and A. M. Prince. Gastroenterology 71:804-808, 1976.
487. Williams, A. and G. LeBouvier. Bibliotheca Haematologica 42: 71-75, 1976.
488. Courouce-Pauty, A. M. and A. Plancon. Vox Sang. 34:231-238, 1978.
489. Trepo, C., O. Hantz, L. Vitvitski, P. Chevallier, A. Williams, J. M. Lemaire and M. Sepetjian. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, Chapter 20, p. 203-209, 1978.
490. Miller, D. J., A. E. Williams, G. L. LeBouvier, J. M. Dwyer, J. Grant and G. Klatskin. Gastroenterology 74:1208-1213, 1978.
491. Anderson-Visona, K. and V. M. Villarejos. J. Infect. Dis. 141: 603-608, 1980.
492. Risenberg, A., A. Gomez and U. Rife. Ann. Rheum. Dis. 28:428-430, 1969.
493. Kacari, J. N., T. H. Siem and R. Brouwer. Lancet i:1008-1009, 1977.
494. Ohori, H., E. Yamada, A. Tateda and N. Ishida. J. Med. Virol. 6: 61-74, 1980.
495. Takekoshi, Y., M. Tanaka, N. Shida, Y. Satake, Y. Saheki and S. Matsumoto. Lancet ii:1065-1068, 1978.
496. Takekoshi, Y., M. Tanaka, Y. Miyakawa, H. Yoshizawa, K. Takahashi and M. Mayumi. N. Engl. J. Med. 300:814-819, 1979.
497. Neurath, A. R. and N. Strick. Proc. Natl. Acad. Sci. (USA) 74: 1702-1706, 1977.
498. Takahashi, K., Y. Miyakawa, T. Gotanda, S. Mishiro, M. Imai and M. Mayumi. Gastroenterology 77:1193-1199, 1979.
499. Miyakawa, Y. and M. Mayumi. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, Chapter 19, pp. 193-201, 1978.

500. Vyas, G. N., D. L. Peterson, R. M. Townsend, S. R. Damle and L. O. Magnius. Science 198:1068-1070, 1977.
501. Neurath, A. R., C. Trepo, M. Chen and A. M. Prince. J. Gen. Virol. 30:277-285, 1976.
502. Vnek, J., A. M. Prince, C. Trepo, A. E. Williams, I. K. Mushahwar, C.-M. Ling and L. R. Overby. J. Med. Virol. 4:187-199, 1979.
503. Takahashi, K., S. Yamashita, M. Imai, Y. Miyakawa and M. Mayumi. J. Gen. Virol. 38:431-436, 1978.
504. Gerin, J. L., J. W.-K. Shih, V. J. McAuliffe and R. H. Purcell. J. Gen. Virol. 38:561-566, 1978.
505. Neurath, A. R., N. Strick and C.-Y. Huang. J. Gen. Virol. 48: 53-61, 1980.
506. Chien, D. and G. Vyas. N. Engl. J. Med. 299:1253-1254, 1978.
507. Lenkei, R., D. Onica and V. Ghetie. Experimentia 33:1046-1047, 1977.
508. Wager, O. and A. M. Teppo. Scand. J. Immunol. 7:503-509, 1978.
509. Wadsworth, C., L. A. Hanson and J. Holmgren. In Protides of the Biological Fluids, Proceedings of the 20th Colloquium, Brugge, 1972, edited by Peeters, H., Pergamon Press, Oxford, pp. 595-601, 1973.
510. Matuhashi, T. and Z. Hosokawa. Jap. J. Exp. Med. 42:183-184, 1972.
511. Budkowska, A., B. Kalinowska and A. Nowoslawski. J. Immunol. 123: 1415-1416, 1979.
512. Huang, S. N. and A. R. Neurath. Lab. Invest. 40:1-17, 1978.
513. Rubin, C. S. and O. M. Rosen. Ann. Rev. Biochem. 44:831-887, 1975.
514. Greengard, P. Science 199:146-152, 1978.
515. Tan, K. B. Virology 64:566-570, 1975.
516. Siddell, S. G., A. Barthel and V. T. Meulen. J. Gen. Virol. 52: 235-243, 1981.
517. Downer, D. N., H. W. Rogers and C. C. Randall. Virology 52:13-21, 1973.
518. Paoletti, E. and B. Moss. J. Virol. 10:417-424, 1972.
519. Silberstein, H. and J. T. August. J. Biol. Chem. 251:3176-3184, 1976.
520. Sokol, F. and H. F. Clark. Virology 52:246-263, 1973.
521. Moyer, S. A. and D. F. Summers. J. Virol. 13:455-465, 1974.
522. Imblum, R. L. and R. R. Wagner. J. Virol. 13:113-124, 1974.
523. Gravell, M. and T. L. Cromeans. Virology 48:847-851, 1972.
524. Hatanaka, M., E. Twiddy and R. V. Gilden. Virology 47:536-538, 1972.
525. Strand, M. and J. T. August. Nature New Biology 233:137-140, 1971.
526. Randall, C. C., H. W. Rogers, D. N. Downer and G. A. Gentry. J. Virol. 9:216-222, 1972.
527. Rubenstein, A. S., M. Gravell and R. Darlington. Virology 50: 287-290, 1972.
528. Tan, K. B. and F. Sokol. J. Virol. 13:1245-1253, 1974.
529. Kit, S. Pharmac. Ther. 4:501-585, 1979.
530. Smith, A. E., R. Smith, B. Griffin and M. Fried. Cell 18:915-924, 1979.
531. Eckhart, W., M. A. Hutchinson and T. Hunter. Cell 18:925-933, 1979.
532. Sefton, B. M., T. Hunter, K. Beemon and W. Eckhart. Cell 20: 807-816, 1980.
533. Hunter, T. and B. M. Sefton. Proc. Natl. Acad. Sci. (USA) 77: 1311-1315, 1980.

534. Griffin, J. D., G. Sprangler and D. M. Livingston. Proc. Natl. Acad. Sci. (USA) 76:2610-2614, 1979.
535. Witte, O. N., A. Dasgupta and D. Baltimore. Nature 283:826-831, 1980.
536. Blumberg, B. S. and W. T. London. In Viruses in Naturally Occurring Cancers, Cold Spring Harbor Conferences on Cell Proliferation, Vol. VII, edited by Essex, M., G. Todaro and H. zur Hausen, Cold Spring Harbor Laboratory, New York, pp. 401-421, 1980.
536a. Valenzuela, P., M. Quironga, J. Zaldivar, P. Gray and W. J. Rutter. In Animal Virus Genetics, edited by Fields, V., R. Jaenisch and C. F. Fox, Academic Press, Inc., New York, pp. 57-70, 1980.
537. Maupas, P. and J. L. Melnick. Prog. Med. Virol. 27:1-5, 1981.
538. Prince, A. M. Hepatology 1:73-75, 1981.
539. Bradley, D. W., B. L. Murphy, J. L. Smith, J. E. Maynard and G. L. Gitnick. Nature 259:594-596, 1976.
540. Hirschman, S. Z. and E. Garfinkel. J. Infect. Dis. 135:897-910, 1977.
541. Hirschman, S. Z. Trans. N. Y. Acad. Sci. 33:595-606, 1971.
542. Hirschman, S. Z., M. Gerber and E. Garfinkel. Am. J. Med. Sci. 270:141-149, 1975.
543. Hirschman, S. Z. Nature (New Biology) 238:277-279, 1972.
544. Hirschman, S. Z. and E. Garfinkel. Nature 271:681-683, 1978.
545. Nordenfelt, E., E. Helgstrand and B. Oberg. Acta Pathol. Microbiol. Scand., Sect. B. 87:75-76, 1979.
546. Hess, G., W. Arnold and K. H. Meyer zum Buschenfelde. J. Med. Virol. 5:309-316, 1980.
547. Hess, G., W. Arnold, B. Moller, G. M. Gahl and K. H. Meyer zum Buschenfelde. Med. Microbiol. Immunol. 168:25-34, 1980.
548. Nath, N., C. T. Fang and R. Y. Dodd. J. Med. Virol. 10:131-140, 1982.
549. Hess, G., W. Arnold and K. H. Meyer zum Buschenfelde. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 708-709, 1982. Abstract.
550. Kornberg, A. In DNA Replication, W. H. Freeman and Company, San Francisco.
551. Weissbach, A. Ann. Rev. Biochem. 46:25-47, 1977.
552. Dube, D. K., E. C. Travaglini and L. A. Loeb. In Methods in Cell Biology, Vol. 19, edited by Stein, G., J. Stein and L. J. Kleinsmith, Academic Press, San Francisco, Chapter 2, pp. 27-42, 1978.
553. Silberstein, H. and J. T. August. J. Biol. Chem. 251:3185-3190, 1976.
554. Kleiman, J. H. and B. Moss. J. Biol. Chem. 250:2420-2429, 1975.
554a. Kingsford, L. and S. U. Emerson. J. Virol. 33:1097-1105, 1980.
555. Kleiman, J. H. and B. Moss. J. Biol. Chem. 250:2430-2437, 1975.
556. Lee, S. G., M. V. Miceli, R. A. Jungmann and P. P. Hung. Proc. Natl. Acad. Sci. (USA) 72:2945-2949, 1975.
557. Hirschman, S. Z. Molec. Cell. Biochem. 26:47-67, 1979.
558. Kingsbury, D. W., C.-H. Hsu and E. M. Morgan. In The Replication of Negative Strand Virus, Vol. 7, edited by Bishop, D. H. L. and R. W. Compans, Elsevier/North Holland Publishing Co., New York, pp. 821-827, 1981.
559. Naito, S. and A. Ishihama. J. Biol. Chem. 251:4307-4314, 1976.
560. Emerson, S. U. and H.-Y. Yu. J. Virol. 15:1348-1356, 1975.

561. Clinton, G. M., B. W. Burge and A. S. Huang. J. Virol. 27: 340-346, 1978.
562. Tsiapalis, C. M. Nature 266:27-31, 1977.
563. Sen, A., C. J. Sherr and G. J. Todaro. Cell 7:21-32, 1976.
564. Sen, A., C. J. Sherr and G. J. Todaro. Cell 10:489-496, 1977.
565. Bolen, J. B., D. G. Anders, J. Trempy and R. A. Consigli. J. Virol. 37:80-91, 1981.
566. Brady, J. N., C. A. Lavialle, M. F. Radonovich and N. P. Salzman. J. Virol. 39:432-437, 1981.
567. Shaw, S. B. and P. Tegtmeyer. Virology 115:88-96, 1981.
568. Prives, C., B. Barnet, A. Scheller, G. Khoury and G. Jay. J. Virol. 43:73-82, 1982.
569. Khandjian, E. W., M. Loche, J.-L. Darlix, R. Cramer, H. Turler and R. Weil. Proc. Natl. Acad. Sci. (USA) 79:1139-1143, 1982.
570. Tjian, R. Cell 26:1-2, 1981.
571. Oren, M., E. Winocour, and C. Prives. Proc. Natl. Acad. Sci. (USA) 77:220-224, 1980.
572. Bradley, M. K., J. D. Griffin and D. M. Livingston. Cell 28: 125-134, 1982.
573. Greenspan, D. S. and R. B. Carroll. Virology 99:413-416, 1979.
574. Tegtmeyer, P., B. Andersen, S. B. Shaw and V. G. Wilson. Virology 115:75-87, 1981.
575. Fanning, E., C. Burger and E. G. Gurney. J. Gen. Virol. 55: 367-378, 1981.
576. Jay, G., G. Khoury, A. B. DeLeo, W. G. Dippold and L. J. Old. Proc. Natl. Acad. Sci. (USA) 78:2932-2936, 1981.
577. Sarnow, P., Y. S. Ho, J. Williams and A. J. Levine. Cell 28: 387-394, 1982.
578. Prives, C., Y. Beck and H. Shore. J. Virol. 33:689-696, 1980.
579. Carter, T. H., J.-C. Nicolas, C. S. H. Young and P. B. Fisher. Virology 117:519-521, 1982.
580. Bremerskov, V. Nature New Biology 246:170-174, 1973.
581. Klessig, D. F. and T. Grodzicker. Cell 17:957-966, 1979.
582. Jeng, Y.-H., W. S. M. Wold, K. Sugawara, Z. Gilead and M. Green. J. Virol. 22:402-411, 1977.
582a. Montenarh, M. and R. Henning. FEBS Letters 114:107-110, 1980.
583. Levinson, A. D., E. H. Postel and A. J. Levine. Virology 79: 144-159, 1977.
584. Mar, E.-C., P. C. Patel and E.-S. Huang. J. Gen. Virol. 57: 419-156, 1981.
585. Gibson, W., T. L. Murphy and C. Roby. Virology 111:251-262, 1981.
586. Gergely, L., J. Czegledy and L. Vaczi. Intervirology 13:352-356, 1980.
587. van der Vliet, P. C., J. Landberg and H. S. Jansz. Virology 80: 98-110, 1977.
588. van der Vliet, P. C., A. J. Levine, M. J. Ensinger and H. S. Ginsberg. J. Virol. 15:348-354, 1975.
589. Luka, J., W. Siegert and G. Klein. J. Virol. 22:1-8, 1977.
590. Roubal, J., B. Kallin, J. Luka and G. Klein. Virology 113: 285-292, 1981.
591. Kamata, T., K. Takaki, Y. Hinuma and Y. Watanabe. Virology 113: 512-520, 1981.
592. Spelsberg, T. C., T. B. Sculley, G. M. Pikler, J. A. Gilbert and G. R. Pearson. J. Virol. 43:555-565, 1982.

593. Kit, S., M. Kurchak, W. Wray and D. R. Dobbs. Proc. Natl. Acad. Sci. (USA) 75:3288-3291, 1978.
594. Powell, K. L., E. Littler and D. J. M. Purifoy. J. Virol. 39: 894-902, 1981.
595. Alberts, B. M. and L. Frey. Nature (London) 227:1313-1318, 1970.
595a. Herrick, G. and B. Alberts. J. Biol. Chem. 251:2133-2141, 1976.
596. Herrick, G. and B. Alberts. J. Biol. Chem. 251:2124-2132, 1976.
597. Herrick, G., H. Delius and B. Alberts. J. Biol. Chem. 251: 2142-1246, 1976.
598. Gallo, R. C. Nature 234:194-198, 1971.
599. Ting, R. C., S. S. Yang and R. C. Gallo. Nature New Biology 236: 163-166, 1972.
600. Hirschman, S. Z. In The Liver and its Diseases, edited by Schaffner, F., S. Sherlock and C. M. Leevy, Intercontinental Medical Book Corp., New York, NY, pp. 126-132, 1974.
601. Gerin, J. L. In Australia Antigen, edited by Prier, J. E. and H. Friedman, University Park Press, Baltimore, MD, pp. 31-53, 1973.
602. Villarejos, V. M., J. Serra C., K. A. Visona and C. E. Eduarte. J. Med. Virol. 4:97-101, 1979.
603. Mao, J. C. H., E. R. Otis, I. K. Mushahwar and L. R. Overby. J. Med. Virol. 6:285-299, 1980.
604. Cappel, R., J. Desmyter and A. F. Badburne. Biomedicine 25: 209-211, 1976.
605. Lutwick, L. I. and W. S. Robinson. J. Virol. 21:96-104, 1977.
606. Hruska, J. F., D. A. Clayton, J. L. Rubenstein and W. S. Robinson. J. Virol. 21:666-672, 1977.
607. Landers, T. A., H. B. Greenberg and W. S. Robinson. J. Virol. 23:368-376, 1977.
608. Hirschman, S. Z. and E. Garfinkel. Clin. Res. 25:377A, 1977.
609. Poiesz, B. J., G. Seal and L. A. Loeb. Proc. Natl. Acad. Sci. (USA) 71:4892-4896, 1974.
610. Auld, D. S., H. Kawaguchi, D. M. Livingston and B. L. Vallee. Biochem. Biophys. Res. Comm. 62:296-302, 1975.
611. Chang, L. M. S. and F. J. Bollum. Proc. Natl. Acad. Sci. (USA) 65:1041-1048, 1970.
612. Slater, J. P., A. S. Mildvan and L. A. Loeb. Biochem. Biophys. Res. Comm. 44:37-43, 1971.
613. Renis, H. E. Antimicrobial Agents and Chemotherapy 1969:167-172, 1970.
614. Hahn, F. E. and J. Ciak. Antimicrobial Agents and Chemotherapy 9:77-80, 1976.
615. Hahn, F. E. and J. Ciak. Ann. N. Y. Acad. Sci. 182:295-304, 1971.
616. Leinbach, S. S., J. M. Reno, L. F. Lee, A. F. Isbell and J. A. Boezi. Biochemistry 15:426-430, 1976.
617. Helgstrand, E., B. Eriksson, N. G. Johannssen, B. Lannero, A. Larsson, A. Misiorny, J. O. Noren, B. Sjoberg, K. Stenberg, G. Stening, S. Stridh, B. Oberg, S. Alenius and L. Philipson. Science 201:819-821, 1978.
618. Mao, J. C. H. and E. E. Robinshaw. Biochemistry 14:5475-5479, 1975.
619. Muller, W. E. G., R. K. Zahn, R. Beyer and D. Falke. Virology 76:787-796, 1977.
620. Muller, W. E. G., R. K. Zahn, K. Bittlingmaier and D. Falke. Ann. N. Y. Acad. Sci. 284:34-48, 1977.

621. Craxi, A., I. V. D. Weller, A. K. Murray, M. F. Bassendine, H. C. Thomas and S. Sherlock. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 649-650, 1982. Abstract.
622. Weller, I. V. D., M. F. Bassendine, A. K. Murray, A. Craxi, H. C. Thomas and S. Sherlock. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 649, 1982. Abstract.
623. Scullard, G. H., R. B. Pollard, J. L. Smith, S. L. Sacks, P. B. Gregory, W. S. Robinson and T. C. Merigan. J. Infect. Dis. 143: 772-783, 1981.
624. Scullard, G. H., H. B. Greenberg, J. L. Smith, P. B. Gregory, T. C. Merigan and W. S. Robinson. Hepatology 2:39-49, 1982.
625. Merigan, T. C. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 45, pp. 537-541, 1982.
626. Soloway, R. D., W. H. J. Summerskill, A. H. Baggenstoss, M. G. Geall, G. L. Gitnick, L. R. Elveback and L. J. Schoenfield. Gastroenterology 63:820-833, 1972.
627. Cook, C. G., R. Mulligan and S. Sherlock. Quar. J. Med. 40: 159-185, 1971.
628. Weller, I. V. D., M. F. Bassendine, A. K. Murray, H. C. Thomas and S. Sherlock. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 647-648, 1982. Abstract.
629. Hess, G., W. Arnold, T. H. Hutteroth, B. Palmowski and K. H. Meyer zum Buschenfelde. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 614, 1982. Abstract.
630. Stevens, J. G. and M. L. Cook. J. Immunol. 113:1685-1693, 1974.
630a. Muller, R., I. Vido and F. W. Schmidt. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 647, 1982. Abstract.
631. Scullard, G. H., P. B. Gregory, W. S. Robinson and T. C. Merigan. Gastroenterology 77:A40, 1979.
631a. Hirschman, S. Z., M. Gerber and E. Garfinkel. Nature 251: 540-542, 1974.
632. Mondelli, M., G. M. Vergani, A. Alberti, D. Vergani, B. Portmann, A. L. W. F. Eddleston and R. Williams. J. Immunol. 129:2773-2778, 1982.
632a. Overby, L. R., P. P. Hung, J. C.-H. Mao, C. M. Ling and T. Kakefuda. Nature 255:84-85, 1975.
633. Gilbert, W. and D. Dressler. Cold Spring Harbor Symposium on Quantitative Biology 33:473-484, 1968.
634. Sattler, F. and W. S. Robinson. J. Virol. 32:226-233, 1979.
635. Varmus, H. E. Science 216:812-820, 1982.
636. Hirschman, S. Z., M. Gerber and E. Garfinkel. Proc. Natl. Acad. Sci. (USA) 71:3345-3349, 1974.
637. Varmus, H. E., S. Heasley, H.-J. Kung, H. Opperman, V. C. Smith, J. M. Bishop and P. R. Shank. J. Molec. Biol. 120:55-82, 1978.
638. Robinson, W. S. and L. I. Lutwick. In Animal Virology, edited by Baltimore, D. and C. F. Huang, Academic Press, New York, NY, pp. 787-811, 1976.

639. Hirschman, S. Z. Lancet ii:436-438, 1975.
640. Bonino, F., B. Hoyer, J. Nelson, R. Engle, G. Verme and J. Gerin. Hepatology 1:386-391, 1981.
641. Hung, P. P., J. C.-H. Mao, C. M. Ling and L. R. Overby. Nature 253:571-572, 1975.
642. Charnay, P., C. Pourcel, A. Louise, A. Fritsch and P. Tiollais. Proc. Natl. Acad. Sci. (USA) 76:2222-2226, 1979.
643. Ruiz-Opazo, N., P. R. Chakraborty and D. A. Shafritz. J. Cell. Biochem. 19:281-292, 1982.
644. Ruiz-Opazo, N., P. R. Chakraborty and D. A. Shafritz. Cell 29: 129-138, 1982.
645. Shouval, D., P. R. Chakraborty, N. Ruiz-Opazo, S. Baum, I. Spigland, E. Muchmore, M. A. Gerber, S. N. Thung, H. Popper and D. A. Shafritz. Proc. Natl. Acad. Sci. (USA) 77:6147-6151, 1980.
646. Monjardino, J., M. J. F. Fowler, L. Montano, I. Weller, K. N. Tsiquaye, A. J. Zuckerman, D. M. Jones and H. C. Thomas. J. Med. Virol. 9:189-199, 1982.
647. Law, M.-F., D. R. Lowry, I. Dvoretzky and P. M. Howley. Proc. Natl. Acad. Sci. (USA) 78:2727-2731, 1981.
648. Moar, M. H., M. S. Campo, H. M. Laird and W. F. H. Jarrett. J. Virol. 39:945-949, 1981.
649. Moar, M. H., M. S. Campo, H. Laird and W. F. H. Jarrett. Nature 293:729-751, 1981.
650. Lancaster, W. D. Virology 108:251-255, 1981.
651. Wettstein, F. O. and J. G. Stevens. In Viruses in Naturally Occurring Cancers, Vol. VII, Cold Spring Harbor Laboratory Conferences on Cell Proliferation, edited by Essex, M., G. Todaro and H. zur Hausen, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 301-307, 1980.
652. Wettstein, F. O. and J. G. Stevens. Proc. Natl. Acad. Sci. (USA) 79:790-794, 1982.
653. Kam, W., L. B. Rall, E. A. Smuckler, R. Schmid and W. J. Rutter. Proc. Natl. Acad. Sci. (USA) 79:7522-7526, 1982.
654. Kam, W., L. Rall, R. Schmid and W. J. Rutter. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 809, 1982. Abstract.
655. Gowans, E. J., C. J. Burrell, A. R. Jilbert and B. P. Marmion. J. Med. Virol. 8:67-78, 1981.
656. Gowans, E., C. J. Burrell, A. R. Jilbert and B. P. Marmion. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 807-808, 1982. Abstract.
657. Risser, R. and C. Mulder. Virology 58:424-438, 1974.
658. Fried, M. J. Virol. 13:939-946, 1974.
659. Blackstein, M. E., C. P. Stanners and A. J. Farmilio. J. Molec. Biol. 42:301-313, 1969.
660. Yoshiike, K. Virology 34:391-401, 1968.
661. Ben-Porat, T., J. M. Demarchi and A. S. Kaplan. Virology 61: 29-37, 1974.
662. Bronson, D. L., G. R. Dreesman, N. Biswal and M. Benyesh-Melnick. Intervirology 1:141, 1973.
663. Wagner, M., J. Skare and W. C. Summers. Cold Spring Harbor Symposium on Quantitative Biology 39:683-686, 1975.

664. Huang, A. S. and D. Baltimore. In Comprehensive Virology, edited by Fraenkel-Conrat, H. and R. R. Wagner, Plenum Press, New York, pp. 73-116, 1977.
665. Uchida, S., S. Watanabe and M. Kato. Virology 28:135-141, 1966.
666. Nayak, D. P., K. Tobita, J. M. Janda, A. R. Davis and B. K. De. J. Virol. 28:375-386, 1978.
667. Weinberg, R. A. Ann. Rev. Biochem. 49:197-226, 1980.
668. Trichopoulos, D., J. Kremastinou and A. Tzonou. In Host Factors in Human Carcinogenesis, edited by Bartsch, H. and B. Armstrong, International Agency for Research on Cancer (W.H.O.) Scientific Publication No. 39, Lyon, France, pp. 317-332, 1982.
669. Summers, J., A. O'Connell, P. Maupas, A. Goudeau, P. Coursaget and J. Drucker. J. Med. Virol. 2:207-214, 1978.
670. Kung, H. J., Y. K. Fung, J. E. Majors, J. M. Bishop and H. E. Varmus. J. Virol. 37:127-138, 1981.
671. Hadziyannis, S., A. Guistozi, A. Moussouros and G. Merikas. In Diseases of the Liver and Biliary Tract, edited by Leevy, C. M., S. Karger (publisher), Basel, pp. 174-178, 1976.
672. Hadziyannis, S. J. Clin. Gastroenterol. 9:117-134, 1980.
673. Nayak, N. C., A. Dhar, R. Sachdeva, A. Mittal, N. H. Seth, D. Sudarsoram, B. Reddy, U. L. Wahgolikar and C. R. R. Reddy. Int. J. Cancer 20:643-654, 1977.
674. Nazarewicz, T., K. Krawczynski, J. Slusarcauk and A. Nowaslawski. J. Infect. Dis. 135:298-302, 1977.
675. Thung, S. N., M. A. Gerber, E. Sarno and H. Popper. Lab. Invest. 41:101-105, 1979.
676. Koshy, R., R. Muller, Ph. Maupas and P. H. Hofschneider. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 694, 1982. Abstract.
677. Shafritz, D., D. Shouval, H. Sherman, S. J. Hadziyannis and M. Kew. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 690-691, 1982. Abstract.
678. Brechot, C., M. Fonck, M. Hadchouel, J. Scotto, B. Rain and P. Tiollais. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 691-692, 1982. Abstract.
679. Aden, D. P., J. Abramczuk, D. Searls, G. C. Overton, E. M. Twist and B. B. Knowles. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 784, 1982. Abstract.
680. Pourcel, C., A. Louise, M. Gervais, M.-F. Dubois, N. Chenciner and P. Tiollais. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 810, 1982. Abstract.
681. Snyder, R. L. and J. Summers. In Viruses in Naturally Occurring Cancers, Vol. VII, Cold Spring Harbor Conferences on Cell Proliferation, edited by Essex, M., G. Todaro and H. zur Hausen, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, pp. 447-457, 1980.
682. Baltimore, D. Bacteriol. Review 35:235-241, 1971.
683. Dejean, A., L. Vitvitski, C. Brechot, C. Trepo, P. Tiollais and P. Charnay. Virology 121:195-199, 1982.
684. Shafritz, D. A. and I. M. Arias. Hepatology 2:106-107, 1982.

685. Snyder, R. L. In 1977-78 Regional Workshop Proceedings of American Association of Zoological Parks and Aquariums, Wheeling, WV, 1978.
686. Shank, P. R. and H. E. Varmus. J. Virol. 25:104-114, 1978.
687. Rogler, C. E. and J. Summers. J. Virol. 44:852-863, 1982.
688. Ogston, C. W., G. J. Jonak, C. E. Roger, S. M. Astrin and J. Summers. Cell 29:385-394, 1982.
689. Stringer, J. R. J. Virol. 38:671-679, 1981.
690. Botchan, M., J. Stringer, T. Mitchison and J. Sanbrook. Cell 20: 143-152, 1980.
691. Clayton, C. E. and P. W. J. Rigby. Cell 25:547-559, 1981.
692. Sambrook, J., R. Green, J. Stringer, T. Mitchison, S.-L. Hu and M. Botchan. Cold Spring Harbor Symposium on Quantitative Biology 44:569-584, 1980.
693. Rovigatti, U. G., C. E. Rogler, B. G. Neel, W. S. Hayward and S. M. Astrin. In Tumor Cell Heterogeneity: Origins and Implications, Fourth Annual Bristol-Myers Symposium on Cancer Research, New York, Academic Press, 1982.
694. Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang and S. M. Astrin. Cell 23:323-334, 1981.
695. Weiss, R. A. In Host Factors in Human Carcinogenesis, edited by Bartsch, H. and B. Armstrong, International Agency for Research on Cancer (W.H.O.), IARC Scientific Publication No. 39, Lyon, France, pp. 307-316, 1982.
696. Hayward, W. S., B. G. Neel and S. M. Astrin. Nature 290:475-480, 1981.
697. Cooper, G. M. and P. E. Neiman. Nature 287:656-658, 1980.
698. Lane, M.-A., A Sainten and G. M. Cooper. Proc. Natl. Acad. Sci. (USA) 78:5185-5189, 1981.
699. Simon, D., D. P. Aden and B. B. Knowles. Int. J. Cancer 30: 27-33, 1982.
700. Atkin, N. B. and V. Brito-Babapulle. Cancer Genet. Cytogenet. 3: 261-272, 1981.
701. Wang, N., B. Trend, D. L. Bronson and E. E. Fraley. Cancer Res. 40:796-802, 1980.
702. Trent, J. M. and S. E. Salmon. Cancer Genet. Cytogenet. 3: 279-291, 1981.
703. Atkin, N. B. and V. J. Pickthall. Hum. Genet. 38:25-33, 1977.
704. Atkin, N. B. and M. C. Baker. Cancer 44:604-613, 1979.
705. Whang-Peng, J., P. A. Bunn, Jr., C. S. Kao-Shan, E. C. Lee, D. N. Carney, A. Gazdar and J. D. Minna. Cancer Genet. and Cytogenet. 6:119-134, 1982.
706. Jones Cruciger, Q. V., S. Pathak and R. Cailleau. Cytogenet. Cell Genet. 17:231-235, 1976.
707. Atkin, N. B. and M. C. Baker. Cytobios. 18:101-109, 1977.
708. Kovacs, G. Int. J. Cancer 21:688-694, 1978.
709. Rechavi, G., D. Givol and E. Canaani. Nature 300:607-611, 1982.
710. Klein. G. Nature 294:313-318, 1981.
711. Cooper, G. M. Science 218:801-806, 1982.
712. Maxam, A. M. and W. Gilbert. Proc. Natl. Acad. Sci. (USA) 74: 560-564, 1977.
713. Maat, J. and A. J. H. Smith. Nucleic Acids Research 5:4537-4545, 1978.
714. Sanger, F., S. Nicklen and A. R. Coulson. Proc. Natl. Acad. Sci. (USA) 74:5463-5467, 1977.

715. Onodera, S., H. Ohori, M. Yamaki and N. Ishida. J. Med. Virol. 10:147-155, 1982.
716. Yamaki, M., H. Ohori, S. Onodera, N. Ishida and H. Maeda. Biochim. et Biophys. Acta 706:165-173, 1982.
717. Axelrod, N. Virology 87:366-383, 1978.
718. Reddy, V. B., B. Thimmappaya, R. Dhar, K. N. Subramanian, B. S. Zain, J. Pan, P. K. Ghosh, M. L. Celma and S. M. Weissman. Science 200:494-502, 1978.
719. Fiers, W., R. Contreras, G. Haegemann, R. Rogiers, A. van de Voorde, H. van Heuverswyn, J. Herreweghe, G. Volckaert and M. Ysebaert. Nature 273:113-120, 1978.
720. Alexander, J. J., E. M. Bey, E. Geddes and G. Lecatsas. S. Afr. Med. J. 50:2124-2128, 1976.
721. Moriarty, A., B. Hoyer, J. W.-K. Shih, J. Gerin and D. Hamer. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 811, 1982. Abstract.
722. Gough, N. M. and K. Murray. J. Molec. Biol. 162:43-67, 1982.
723. Copeland, J. A., J. Skelly, G. F. Mann, C. R. Howard and A. J. Zuckerman. J. Med. Virol. 5:257-264, 1980.
724. Valenzuela, P., A. Medina, W. J. Rutter, G. Ammerer and B. D. Hall. Nature 298:347-350, 1982.
725. Ray, M. B. Hepatitis B Virus Antigens in Tissues, University Park Press, Baltimore, MD, 1979.
726. Stratowa, C., J. Doehmer, Y. Wang and P. H. Hofschneider. EMBO J. 1:1573-1578, 1982.
727. Christman, J. K., M. Gerber, P. M. Price, C. Flordellis, J. Edelman and G. Acs. Proc. Natl. Acad. Sci. (USA) 79:1815-1819, 1982.
728. Wang, Y., M. Schafer-Ridder, C. Stratowa, T.-K. Wong and P. H. Hofschneider. EMBO J. 1:1213-1216, 1982.
729. Pribnow, D. Proc. Natl. Acad. Sci. (USA) 72:784-788, 1975.
730. Gannon, F., K. O'Hare, F. Perrin, J. P. LePennec, C. Benoist, M. Cochet, R. Breathnach, A. Royal, A. Garapin, B. Cami and P. Chambon. Nature 278:428-434, 1979.
731. Ziff, E. B. and R. M. Evans. Cell 15:1463-1475, 1978.
732. Pellicer, A., D. Robins, B. Wold, R. Sweet, J. Jackson, I. Lowy, J. M. Roberts, K. G. Sim, S. Silverstein and R. Axel. Science 209:1414-1422, 1980.
733. Anderson, R. A., T. Krakauer and R. D. Camerini-Otero. Proc. Natl. Acad. Sci. (USA) 79:2748-2752, 1982.
734. Acheson, N. H. Proc. Natl. Acad. Sci. (USA) 75:4754-4758, 1978.
735. Treisman, R. Nucleic Acids Res. 8:4867-4888, 1980.
736. Chakraborty, P. R., N. Ruiz-Opazo and D. A. Shafritz. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 811-812, 1982. Abstract.
737. Stahl, S., P. Mackay, M. Magazin, S. A. Bruce and K. Murray. Proc. Natl. Acad. Sci. (USA) 79:1606-1610, 1982.
738. Cohen, B. J. and J. E. Richmond. Nature 296:677-678, 1982.
739. Ryoji, M., K. Hsia and A. Kaji. Trends Biochem. Sci. 8:88-90, 1983.
740. Salas, M. and E. Vinuela. Trends Biomed. Sci. 5:191-193, 1980.
741. Wimmer, E. Cell 28:199-201, 1982.
742. Gerlich, W. H. and W. S. Robinson. Cell 21:801-809, 1980.

743. Coombs, D. H. and G. D. Pearson. Proc. Natl. Acad. Sci. (USA) 75:5291-5295, 1978.
744. Molnar-Kimber, K. L., J. Summers, J. M. Taylor and W. S. Mason. J. Virol. 45:165-172, 1983.
745. Stillman, B. W. and J. O. Bellet. Virology 93:69-79, 1979.
746. Rekosh, D. J. Virol. 40:329-333, 1981.
747. Ganem, D., L. Greenbaum and H. E. Varmus. J. Virol. 44:374-383, 1982.
748. Rothberg, P. G., T. J. R. Harris, A. Nomoto and E. Wimmer. Proc. Natl. Acad. Sci. (USA) 75:4868-4872, 1978.
749. Gumport, R. I. and I. R. Lehman. Proc. Natl. Acad. Sci. (USA) 68: 2559-2563, 1971.
750. Waldeck, W., B. Fohring, K. Chowdhury, P. Gruss and G. Sauer. Proc. Natl. Acad. Sci. (USA) 75:5964-5968, 1978.
751. Kasamatsu, H. and M. Wu. Proc. Natl. Acad. Sci. (USA) 73:1945-1949, 1976.
752. Rekosh, D. M. K., W. C. Russell, A. J. D. Bellet and A. J. Robinson. Cell 11:283-295, 1977.
753. Challberg, M. D., J. M. Ostrove and T. J. Kelly, Jr. J. Virol. 41:265-270, 1982.
754. Challberg, M. D., S. V. Desiderio and T. J. Kelley, Jr. Proc. Natl. Acad. Sci. (USA) 77:5105-5109, 1980.
755. Horwitz, M. S. and H. Ariga. Proc. Natl. Acad. Sci. (USA) 78: 1476-1480, 1981.
756. Will, H., R. Cattaneo, H.-G. Koch, G. Darai, H. Schaller, H. Schellekens, P. M. C. A. van Eerd and F. Deinhardt. Nature 299: 740-742, 1982.
757. Sharp, P. A., C. Moore and J. L. Haverty. Virology 75:442-456, 1976.
758. Montano, L., G. Miescher, A. Goodall, I. V. D. Weller, A. Craxi, H. C. Thomas and S. Sherlock. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 608-609, 1982. Abstract.
759. Kinoshita, R., K. Miura, H. Uchino, A. Konishi, T. Nakagawa and Y. Imai. J. Infect. Dis. 144:303-306, 1981.
760. Guntaka, R. V., O. C. Richards, P. R. Shank, H.-J. Kung, N. Davidson, E. Fritsch, J. M. Bishop and H. E. Varmus. J. Molec. Biol. 106:337-357, 1976.
761. Stillman, B. W., F. Tamanoi and M. B. Mathews. Cell 31:613-623, 1982.
762. Marquardt, O., V. Zaslavsky and P. H. Hofschneider. J. Gen. Virol. 61:105-109, 1982.
763. Zaslavsky, V., O. Marquardt, T.-K. Wong and P. H. Hofschneider. J. Gen. Virol. 51:341-349, 1980.
764. Yamada, G., Y. Sakamoto, M. Mizuno, T. Nishihara, T. Kobayashi, T. Takahashi and H. Nagashima. Gastroenterology 83:348-356, 1982.
765. Varmus, H. E. Nature 299:204-205, 1982.
766. Mason, W. S., J. M. Taylor, G. Seal and J. Summers. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 10, pp. 107-116, 1982.
767. Gianni, A. M. and R. A. Weinberg. Nature (London) 255:646-648, 1975.

768. Feitelson, M. A., P. L. Marion and W. S. Robinson. Virology 130: 76-90, 1983.
769. Gavilanes, F., J. M. Gonzalez-Ros and D. L. Peterson. J. Biol. Chem. 257:7770-7777, 1982.
770. Goudeau, A., F. Barin, P. Coursaget, P. Y. Sizaret, M. Andre and P. Maupas. In Viral Hepatitis, edited by Vyas, G. N., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 707-708, 1978. Abstract.
771. Peterson, D. L., N. Nath, and F. Gavilanes. J. Biol. Chem. 257: 10414-10420, 1982.
772. Aoki, N., S. N. Thung and M. A. Gerber. Laboratory Investigation 47:465-470, 1982.
773. Yamada, G. and P. K. Nakane. Laboratory Investigation 36:649-659, 1977.
774. Shikata, T. Jap. J. Exp. Med. 43:231-245, 1973.
775. Gerber, M. A., S. Hadziyannis, C. Vissoulis, F. Schaffner, F. Paronetto and H. Popper. Proc. Soc. Exp. Biol. Med. 145:863-867, 1974.
776. Gerber, M. A., S. Hadziyannis, C. Vissoulis, F. Schaffner, F. Paronetto and H. Popper. Am. J. Pathol. 75:489-502, 1974.
777. Neurath, A. R., S. B. H. Kent and N. Strick. Proc. Natl. Acad. Sci. USA 79:7871-7875, 1982.
778. Bhatnagar, P. K., E. Papas, H. E. Blum, D. R. Milich, D. Nitecki, M. J. Karels and G. N. Vyas. Proc. Natl. Acad. Sci. (USA) 79: 4400-4404, 1982.
779. Ionescu-Matiu, I., R. C. Kennedy, J. T. Sparrow, A. R. Culwell, Y. Sanchez, J. L. Melnick and G. R. Dreesman. J. Immunol. 130: 1947-1952, 1983.
780. Kennedy, R. C. and G. R. Dreesman. J. Immunol. 130:385-389, 1983.
781. Kennedy, R. C., Y. Sanchez, I. Ionescu-Matiu, J. L. Melnick and G. R. Dreesman. Virology 122:219-221, 1982.
782. Kennedy, R. C., I. Ionescu-Matiu, Y. Sanchez and G. R. Dreesman. Eur. J. Immunol. 13:232-235, 1983.
783. Kennedy, R. C., K. Adler-Storthz, R. D. Henkel, Y. Sanchez, J. L. Melnick and G. R. Dreesman. Science 221:853-855, 1983.
784. Gerin, J. L., H. Alexander, J. W.-K. Shih, R. H. Purcell, G. Dapolito, R. Engle, N. Green, J. G. Sutcliffe, T. M. Shinnick and R. A. Lerner. Proc. Natl. Acad. Sci. (USA) 80:2365-2369, 1983.
785. Williams, N. Nature 306:427, 1983.
786. Machida, A., S. Kishimoto, H. Ohnuma, H. Miyamoto, Y. Baba, K. Oda, T. Nakamura, G. Funatsu, Y. Miyakawa and M. Mayumi. Molec. Immunol. 19:1087-1093, 1982.
787. Shouval, D., J. R. Wands, V. R. Zurawski, Jr., K. J. Isselbacher and D. A. Shafritz. Proc. Natl. Acad. Sci. (USA) 79:650-654, 1982.
788. Gerber, M. A., E. Garfinkel, S. Z. Hirschman, S. N. Thung and T. Panagiotatos. J. Immunol. 126:1085-1089, 1981.
789. Chisari, F. V., M. S. Bieber, C. A. Josepho, C. Xavier and D. S. Anderson. J. Immunol. 126:45-49, 1981.
790. Shouval, D., D. A. Shafritz, V. R. Zurawski, Jr., K. J. Isselbacher and J. R. Wands. Nature 298:567-569, 1982.
791. Dusheiko, G. M., J. H. Hoofnagle, W. G. Cooksley, S. P. James and E. A. Jones. J. Clin. Invest. 71:1104-1113, 1983.
792. Feitelson, M. A. Unpublished observations.
793. Eddleston, A. L. W. F. and R. Williams. Lancet ii:1543-1545, 1974.

794. Heijtink, R. A., J. van Hattum, S. W. Schalm and N. Masurel. J. Med. Virol. 10:83-90, 1982.
795. Schlicht, I., J. Gadow, H. Ortmans, L. Jorno, H.-D. Gentz, G. Wolter and I. Hoppe. Acta Hepato-Gastroenterology 26:450-456, 1979.
796. Trepo, C. G., D. Robert, J. Motin, D. Trepo, M. Sepetjian and A. M. Prince. Gut 17:10-13, 1976.
797. Chakraborty, P. R., N. Ruiz-Opazo and D. A. Shafritz. Virology 111:647-652, 1981.
798. Yoakum, G. H., B. E. Korba, J. F. Lechner, T. Tokiwa, A. F. Gazdar, T. Seeley, M. Siegel, L. Leeman, H. Autrup and C. C. Harris. Science 222:385-389, 1983.
799. Roggendorf, M., Deinhardt, R. Zachoval and K. von der Helm. J. Virol. Methods 6:61-70, 1983.
800. Koziol, D. E., H. J. Alter, J. P. Kirchner and P. V. Holland. J. Immunol. 117:2260-2262, 1976.
801. Linnemann, C. C. and P. A. Askey. Lancet i:346-347, 1984.
802. Feinmann, S. V., L. R. Overby, B. Berris, K. Chau, C. A. Schable and J. E. Maynard. Hepatology 2:795-799, 1982.
803. Banninger, P., J. Altorfer, G. G. Frosner, M. Pirovino, F. Gudat, L. Bianchi, P. J. Grob and M. Schmid. Hepatology 3:337-342, 1983.
804. Lavarini, C., P. Farci, E. Chiaberge, V. Veglio, D. Giacobbi, G. Bedarida, G. Susani, M. Toti, P. Almi, N. Caporaso, C. del Vecchio Blanco and M. Rizzetto. Brit. Med. J. 287:1254-1256, 1983.
805. Perrillo, R. P., K. H. Chau, L. R. Overby and R. H. Decker. Gastroenterology 85:163-167, 1983.
806. Goudeau, A., G. Lesage, F. Denis, J. P. Chiron, B. Yvonnet, F. Barin, P. Coursaget and I. Diop Mar. Lancet ii:1103-1104, 1983.
807. Cohen, B. J. J. Med. Virol. 3:141-149, 1978.
808. Tedder, R. S. and R. Wilson-Croome. J. Med. Virol. 6:235-247, 1980.
809. Roggendorf, M., F. Dienhardt, G. G. Frosner, R. Scheid, B. Bayeri and R. Zachoval. J. Clin. Microbiol. 13:618-626, 1981.
810. Hawkes, R. A., C. R. Boughton, V. Ferguson and N. I. Lehmann. J. Clin. Microbiol. 11:581-583, 1980.
811. Halpern, M. S., J. M. England, D. T. Deery, D. J. Petcu, W. S. Mason and K. L. Molnar-Kimber. Proc. Natl. Acad. Sci. (USA) 80: 4865-4869, 1983.
812. Omata, M., J. Mori, O. Yokosuka, S. Iwama, Y. Ito and K. Okuda. Liver 2:125-132, 1982.
813. Tedder, R. S. and R. Wilson-Croome. J. Hygiene (London) 86: 163-172, 1981.
814. Shimizu, M., M. Ohyama, Y. Takahashi, K. Udo, M. Kojima, M. Kametani, F. Tsuda, E. Takai, Y. Miyakawa and M. Mayumi. Gastroenterology 84:604-610, 1983.
815. Shiraki, K., N. Yoshihara, T. Kawana, H. Yasui and M. Sakurai. Am. J. Dis. Children 131:644-647, 1977.
816. Arakawa, K., F. Tsuda, K. Takahashi, I. Ise, S. Naito, E. Kosugie, Y. Miyakawa and M. Mayumi. Pediatric Res. 16:247-250, 1982.
817. Derso, A., E. H. Boxall, M. J. Tarlow and T. H. Flewett. Brit. Med. J. 1:949-952, 1978.
818. Dupuy, J. M., E. Kostewicz and D. Alagille. J. Pediatrics 92: 17-20, 1978.

819. Seeff, L. B., E. C. Wright, H. J. Zimmerman, H. J. Alter, A. A. Dietz, B. F. Felsher, J. D. Finkelstein, P. Garcia-Pont, J. L. Gerin, H. B. Greenlee, J. Hamilton, P. V. Holland, P. M. Kaplan, T. Kiernan, R. S. Koff, C. M. Leevy, V. J. McAuliffe, N. Nath, R. H. Purcell, E. R. Schiff, C. C. Schwartz, C. H. Tamburro, Z. Vlahcevic, R. Zemel and D. S. Zimmon. Ann. Intern. Med. 88: 285-293, 1978.
820. Stevens, C. E., R. A. Neurath, R. P. Beasley and W. Szmuness. J. Med. Virol. 3:237-241, 1979.
821. Perrillo, R. P., L. Gelb, C. Campbell, W. Wellinghoff, F. R. Ellis, L. Overby and R. D. Aach. Gastroenterology 76:1319-1325, 1979.
822. Omata, M., K. Uchiumi, Y. Ito, O. Yokosuka, J. Mori, K. Terao, Y. Wei-Fa, A. P. O'Connell, W. T. London and K. Okuda. Gastroenterology 85:260-267, 1983.
823. Hoofnagle, J. H., G. M. Dusheiko, L. B. Seeff, E. A. Jones, J. G. Waggoner and Z. B. Bales. Ann. Intern. Med. 94:744-748, 1981.
824. Stevens, C. E. and W. Szmuness. In Virus and the Liver, edited by Bianchi, L., W. Gerok, K. Sickinger and G. A. Stalder, MTP Press, Lancaster, pp. 285-291, 1980.
825. Barin, F., J. Perrin, J. Chotard, F. Denis, R. N'Doye, I. Diop Mar, J. P. Chiron, P. Coursaget, A. Goudeau and P. Maupas. Prog. Med. Virol. 27:148-162, 1981.
826. Hoofnagle, J. H. Gastroenterology 84:422-423, 1983.
827. Hadziyannis, S. J., H. M. Lieberman, G. G. Karvountzis and D. A. Shafritz. Hepatology 3:656-662, 1983.
828. Hadziyannis, S. J., I. Weller, G. Karvountzis and H. Thomas. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 710-711, 1982. Abstract.
829. Lie-Injo, L. E., M. Balasegaram, C. G. Lopez and A. R. Herrera. DNA 2:301-308, 1983.
830. Liaw, Y.-F., C.-M. Chu, I.-J. Su, M.-J. Huang, D.-Y. Lin and C.-S. Chang-Chien. Gastroenterology 84:216-219, 1983.
831. Lofgren, B. and E. Nordenfelt. J. Med. Virol. 5:323-330, 1980.
832. Realdi, G., A. Alberti, M. Rugge, F. Bortolotti, A. M. Rigoli, F. Tremolada and A. Ruol. Gastroenterology 79:195-199, 1980.
833. Kumada, H., A. Yoshiba and E. Oyake. Acta Hepatol. Japan 22: 820-826, 1981.
834. Suzuki, H., T. Matsushima, T. Miyazaki, J. Toyota, Y. Okuuchi and H. Itoh. Acta Hepatol. Japan 22:1518-1524, 1981.
835. Ding, J. L. and C. J. Oon. Cytobios 39:29-33, 1984.
836. Davis, G. L., J. H. Hoofnagle and J. G. Waggoner. Gastroenterology 86:230-235, 1984.
837. Hoofnagle, J. H., G. M. Dusheiko and D. F. Schafer. Ann. Intern. Med. 96:447-449, 1982.
838. Lightdale, C. J., H. Ikram and C. Pinsky. Cancer 46:1117-1122, 1980.
839. Wands, J. R., C. M. Chura, F. J. Roll and W. C. Maddrey. Gastroenterology 68:105-112, 1975.
840. Dusheiko, G., E. Song, S. Bowyer, M. Witcutt, G. Maier, A. Meyers and M. C. Kew. Hepatology 3:330-336, 1983.
841. Nagington, J., Y. E. Cossart and B. J. Cohen. Lancet i:558-560, 1977.

842. Andres, L. L., V. K. Sawhney, G. H. Scullard, J. L. Smith, T. C. Merigan, W. S. Robinson and P. B. Gregory. Hepatology 1:583-585, 1981.
843. Musca, A., C. Cordova, V. Barnaba, M. S. Bonavita, M. Levrero, C. Zaccari and F. Balsano. Hepatogastroenterology 30:3-5, 1983.
844. Bull, F. G., R. G. Chadwick, R. M. Cockerell, I. W. Davidson, M. Glyn and R. Wright. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 790, 1982. Abstract.
845. Imai, M., M. Nomura, T. Gotanda, T. Sano, K. Tachibana, H. Miyamoto, K. Takahashi, S. Toyama, Y. Miyakawa and M. Mayumi. J. Immunol. 128:69-72, 1982.
846. Ohori, H., N. Shimizu, E. Yamada, S. Onodera and N. Ishida. J. Gen. Virol. 65:405-414, 1984.
847. Takahashi, K., A. Machida, G. Funatsu, M. Nomura, S. Usuda, S. Aoyagi, K. Tachibana, H. Miyamoto, M. Imai, T. Nakamura, Y. Miyakawa and M. Mayumi. J. Immunol. 130:2903-2907, 1983.
848. Raimondo, G., S. Recchia, C. Lavarini, O. Crivelli and M. Rizzetto. Hepatology 2:449-454, 1982.
849. Yamada, E., N. Ishida and H. Ohori. J. Gen. Virol. 64:895-903, 1983.
850. Lenkei, R., G. Mota, M. E. Dan and M. Laky. Rev. Roum. Biochim. 11:271-276, 1974.
851. Schultze, H. E. and J. F. Heremans. In Physiology and Pathology of Plasma Proteins, Vol. 2, edited by Schultze, H. E. and J. F. Heremans, Elsevier Publishing Company, Amsterdam, pp. 648-649, 1970.
852. Peters, T. Adv. Clin. Chem. 13:37-111, 1970.
853. Rothschild, M. A. and S. S. Schreiber. Am. J. Dig. Dis. 14: 711-744, 1969.
854. Lenkei, R. and V. Ghetie. J. Immunol. Methods 16:23-30, 1977.
855. Onica, D., A. M. Dobre and R. Lenkei. Immunochemistry 15: 941-944, 1978.
856. Onica, D., R. Lenkei and V. Ghetie. Immunochemistry 15:687-693, 1978.
857. Lenkei, R. Revue Roum. Med. Interne 18:129-147, 1980.
858. Lenkei, R., L. Buligescu, I. Belascu, D. Pospai and I. Dobre. Clin. Exp. Immunol. 43:381-389, 1981.
859. Onica, D., A. Calugaru, I. Margineanu, G. Zamfir and I. Belascu. Clin. Immunol. Immunopathol. 26:223-231, 1983.
860. Ghetie, V., D. Onica, R. Lenkei and I. Margineanu. Mech. Aging Develop. 17:27-30, 1981.
861. Alberti, A., P. Pontisso and G. Realdi. J. Med. Virol. 10: 141-146, 1982.
862. Pontisso, P., A. Alberti, E. Schiavon, F. Tremolada, F. Bortolotti and G. Realdi. J. Virol. Methods 6:151-159, 1983.
863. Hadziyannis, S., T. Feizi, and P. J. Scheuer. Clin. Exp. Immunol. 5:499-514, 1969.
864. Ballieux, R. E. and J. W. Imhof. IX Colloquium on Protides of the Biological Fluids, Elsevier Press, Inc., Amsterdam, p. 226, 1961.
865. Hauptman, S. and T. B. Tomasi, Jr. J. Clin. Invest. 54:122-127, 1974.
866. Mannik, M. J. Immunol. 99:899-906, 1967.
867. Thung, S. N. and M. A. Gerber. Infect. Immun. 32:1292-1294, 1981.

868. Thung, S. N. and M. A. Gerber. Gastroenterology 80:260-264, 1981.
869. Pontisso, P., A. Alberti, F. Bortolotti and R. Guiseppe. Gastroenterology 84:220-226, 1983.
870. Thung, S. N. and M. A. Gerber. Gastroenterology 85:466-468, 1983.
871. Trevisan, A., F. Gudat and R. Guggenheim. Hepatology 2:832-835, 1982.
872. Stevens, C., R. Neurath, W. Szmuness, R. P. Beasley and H. Ikram. In Viral Hepatitis, edited by Vyas, G., S. N. Cohen and R. Schmid, Franklin Institute Press, Philadelphia, pp. 211-215, 1978.
873. Mayumi, M., M. Imai and Y. Miyakawa. Gastroenterology 77:203, 1979.
874. Machida, A., S. Kishimoto, H. Ohnuma, H. Miyamoto, K. Baba, K. Oda, T. Nakamura, Y. Miyakawa and M. Mayumi. Gastroenterology 85:268-274, 1983.
875. Stibbe, W. and W. H. Gerlich. Virology 123:436-442, 1982.
876. Stibbe, W. and W. H. Gerlich. J. Virol. 46:626-628, 1983.
877. Hoofnagle, J. H., D. F. Schafer, P. Ferenci, J. G. Waggoner, J. Vergalla, M. April and L. Phillips. Gastroenterology 84:1478-1482, 1983.
878. Franklin, S. G., I. Millman and B. S. Blumberg. Proc. Natl. Acad. Sci. (USA) 81:564-567, 1984.
879. Toti, M., R. Rizzi, P. Almi, M. Palla and F. Bonino. J. Med. Virol. 11:139-145, 1983.
880. Palla, M., R. Rizzi, M. Toti, P. Almi, M. Rizzetto, F. Bonino and R. H. Purcell. Infect. Immun. 41:950-958, 1983.
881. Mills, P. R., E. A. C. Follet, G. E. D. Urquhart, G. Clements, G. Watkinson and R. N. M. MacSween. Brit. Med. J. 282:437-438, 1981.
882. Dienstag, J. L. In Frontiers in Liver Disease, edited by Berk, P. D. and T. C. Chalmers, Thieme-Stratton, Inc., New York, pp. 176-195, 1981.
883. Nordenfelt, E., A. Widell, B. G. Hansson, B. Lofgren, C. Moller-Nielsen and B. Oberg. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 90:449-451, 1982.
884. Lin, H. J., J. P.-W. Kwan, P.-C. Wu and W. Chak. J. Med. Virol. 12:61-70, 1983.
885. Nordenfelt, E., B. Oberg, E. Helstrand and E. Miller. Acta Pathol. Microbiol. Scand. Sect. B 88:169-175, 1980.
886. Stenberg, K. and A. Larsson. Antimicrobial Agents and Chemotherapy 14:727-730, 1978.
887. Reno, J. M., L. F. Lee and J. A. Boezi. Antimicrobial Agents and Chemotherapy 13:188-192, 1978.
888. Pumpen, P. P., T. M. Kozlovskaya, A. V. Dishler, V. V. Eychko, Y. V. Kalis, N. V. Pudova, E. Y. Gren, M. B. Rivkina and R. A. Kukain. Molec. Biol. 16:1059-1065, 1983.
889. Delius, H., N. M. Gough, C. H. Cameron and K. Murray. J. Virol. 47:337-343, 1983.
890. Burrell, C. J., E. J. Gowans, A. R. Jilbert, J. R. Lake and B. P. Marmion. Hepatology 2:85S-91S, 1982.
891. Monjardino, J. P., M. J. F. Fowler and H. C. Thomas. J. Gen. Virol. 64:2299-2303, 1983.
892. Koike, K., M. Kobayashi, H. Mizusawa, E. Yoshida, K. Yaginuma and M. Taira. Nucleic Acids Res. 11:5391-5402, 1983.
893. Dejean, A., C. Brechot, P. Tiollais and S. Wain-Hobson. Proc. Natl. Acad. Sci. (USA) 80:2505-2509, 1983.

894. Koshy, R., S. Koch, A. Freytag von Loringhoven, R. Kahmann, K. Murray and P. H. Hofschneider. Cell 34:215-223, 1983.
895. Kam, W., L. Rall, R. Schmid and W. J. Rutter. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, p. 809, 1982. Abstract.
896. Shafritz, D. A. Hepatology 2:35S-41S, 1982.
897. Shafritz, D. A. J. Cell. Biochem. 20:303-316, 1982.
898. Chen, D.-S., B. H. Hoyer, J. Nelson, R. H. Purcell and J. L. Gerin. Hepatology 2:42S, 1982.
899. Miller, R. H. and W. S. Robinson. Proc. Natl. Acad. Sci. (USA) 80:2534-2538, 1983.
900. Ono, Y., H. Onda, R. Sasada, K. Igarashi, Y. Sugino and K. Nishioka. Nucleic Acids Res. 11:1747-1757, 1983.
901. Kobayashi, K., K. Fukuoka, F. Matsushita, H. Morimoto, Y. Hinoue, H. Honjo, N. Tamaka, T. Sugimoto, Y. Kato, N. Hattori, S. Ueda and S. Kato. Hepatology 3:663-666, 1983.
902. Mitamora, K., B. H. Hoyer, A. Ponzetto, J. Nelson, R. H. Purcell and J. L. Gerin. Hepatology 2:47S-50S, 1982.
903. Johnson, R. A., K. Mitamura, M. C. Kew, D.-S. Chen and R. H. Purcell. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, pp. 713-714, 1982. Abstract.
904. Wen, Y.-M., K. Mitamura, B. Merchant, Z. Y. Tang and R. H. Purcell. Infect. Immun. 39:1361-1367, 1983.
905. Rall, L. B., D. N. Standring, O. Laub and W. J. Rutter. Molec. Cell. Biol. 3:1766-1773, 1983.
906. Nishioka, Y., A. Leder and P. Leder. Proc. Natl. Acad. Sci. (USA) 77:2806-2809, 1980.
907. Wilde, C. D., C. E. Crowther, N. J. Cowan, C. E. Rogler, S. M. Astrin and J. Summers. Cell 29:385-394, 1982.
908. Vanin, E. F., G. I. Goldberg, P. W. Tucker and O. Smithies. Nature 286:222-226, 1980.
909. Malpiece, Y., M. L. Michel, G. Carloni, M. Revel, P. Tiollais and J. Weissenbach. Nucleic Acids Res. 11:4645-4654, 1983.
910. Lange, M., E. May and P. May. J. Virol. 38:940-951, 1981.
911. Fausto, N. and P. R. Shank. Hepatology 3:1016-1023, 1983.
912. Mandart, E., A. Kay and F. Galibert. J. Virol. 49:782-792, 1984.
913. Seeger, C., D. Ganem and H. E. Varmus. J. Virol., in press, 1984.
914. Sninsky, J. J. and S. N. Cohen. Hepatology 2:72S-78S, 1982.
915. Fujisawa, Y., Y. Ito, R. Sasada, Y. Ono, K. Igarashi, R. Marumoto, M. Kikuchi and Y. Sugino. Nucleic Acids Res. 11:3581-3591, 1983.
916. Miyanohara, A., A. Toh-e, C. Nozaki, F. Hamada, N. Ohtomo and K. Matsubara. Proc. Natl. Acad. Sci. (USA) 80:1-5, 1983.
917. Liu, C. C., D. Yansura and A. D. Levinson. DNA 1:213-221, 1982.
918. Liu, C. C. and A. D. Levinson. In Eukaryotic Viral Vectors, edited by Gluzman, Y., Cold Spring Harbor Labs, Cold Spring Harbor, New York, pp. 55-60, 1982.
919. Siddiqui, A. Molec. Cell. Biol. 3:143-146, 1983.
920. Crowley, C. W., C. C. Liu and A. D. Levinson. Molec. Cell. Biol. 3:44-55, 1983.
921. Laub, O., L. B. Rall, M. Truett, Y. Shaul, D. N. Standring, P. Valenzuela and W. J. Rutter. J. Virol. 48:271-280, 1983.
922. Simonsen, C. C. and A. D. Levinson. Molec. Cell. Biol. 3:2250-2258, 1983.

923. Myers, R. M. and R. Tjian. Proc. Natl. Acad. Sci. (USA) 77: 6491-6495, 1980.
924. Mellon, P., V. Parker, Y. Gluzman and T. Maniatis. Cell 27: 279-288, 1981.
925. Lusky, M. and M. Botchan. Nature (London) 293:79-81, 1981.
926. Gruss, P., R. Dhar and G. Khoury. Proc. Natl. Acad. Sci. (USA) 78:943-947, 1981.
927. Colbere-Garapin, F., F. Horodniceanu, P. Kourilsky and A. C. Garapin. EMBO Journal 2:21-25, 1983.
928. Hitzeman, R. A., C. Y. Chen, F. E. Hagie, E. J. Patzer, C. C. Liu, D. A. Estell, J. V. Miller, A. Yaffe, D. G. Kleid, A. D. Levinson and H. Oppermann. Nucleic Acids Res. 11:2745-2763, 1983.
929. McAleer, W. J., E. B. Buynak, R. Z. Maigetter, D. E. Wampler, W. J. Miller and M. R. Hilleman. Nature 307:178-180, 1984.
930. Stenlund, A., D. Lamy, J. Moreno-Lopez, H. Ahola, U. Pettersson and P. Tiollais. EMBO Journal 2:669-673, 1983.
931. Wang, Y., C. Stratowa, M. Schaefer-Ridder, J. Doehmer and P. H. Hofschneider. Molec. Cell. Biol. 3:1032-1039, 1983.
932. Sarver, N., P. Gruss, M. F. Law, G. Khoury and P. M. Howley. Molec. Cell. Biol. 1:486-496, 1981.
933. Hirschman, S. Z. and E. Garfinkel. Hepatology 2:79S-84S, 1982.
934. Smith, G. L., M. Mackett and B. Moss. Nature 302:490-495, 1983.
935. Paoletti, E., B. R. Lipinskas, C. Samsonoff, S. Mercer and D. Panicali. Proc. Natl. Acad. Sci. (USA) 81:193-197, 1984.
936. Beale, A. J. Nature 302:476-477, 1983.
937. Cattaneo, R., H. Will, N. Hernandez and H. Schaller. Nature 305: 336-338, 1983.
938. Brady, J. Cell 31:625-633, 1982.
939. Contreras, R., D. Gheysen, J. Knowland, A. van de Voorde and W. Fiers. Nature 300:500-505, 1982.
940. Cattaneo, R., H. Will, G. Darai, E. Pfaff and H. Schaller. EMBO Journal 2:511-514, 1983.
941. Standring, D. N., L. B. Rall, O. Laub and W. J. Rutter. Molec. Cell. Biol. 3:1774-1782, 1983.
942. Gough, N. M. J. Molec. Biol. 165:683-699, 1983.
943. Early, P., J. Rogers, M. Davis, K. Calame, M. Bond, R. Wall and L. Hood. Cell 20:313-320, 1980.
944. Alt, F. W., A. L. M. Bothwell, M. Knapp, E. Siden, E. Mather, M. Koshland and D. Baltimore. Cell 20:293-301, 1980.
945. Shaw, A. R. and E. B. Ziff. Cell 22:905-916, 1980.
946. Chong-Jin, O. and D. Jeak-Ling. Lancet i:395-396, 1984.
947. Romet-Lemonne, J. L., M. F. McLane, E. Elfassi, W. A. Haseltine, J. Azocar and M. Essex. Science 221:667-669, 1983.
948. London, W. T. and B. S. Blumberg. Hepatology 2:10S-14S, 1982.
949. Price, P., S. Ostrove, C. Flordellis, M. A. Sells, S. Thung, M. Gerber, J. Christman and G. Acs. Bioscience Reports 3:1017-1026, 1983.
950. Korba, B. E., G. H. Yoakum, V. L. Wilson and C. C. Harris. Federation Proceedings 43:1048, 1984. Abstract.
951. Alberti, A., F. Tremolada, G. Fattovich, F. Bortolotti and G. Realdi. Dig. Dis. Sci. 28:962-966, 1983.
952. Toh, H., H. Hayashida and T. Miyata. Nature 305:827-829, 1983.
953. Baker, C. C. and E. B. Ziff. J. Molec. Biol. 149:189-221, 1981.

954. Varmus, H. and R. Swanstrom. In RNA Tumor Viruses, edited by Weiss, R., N. Teich, H. Varmus and J. Coffin, Cold Spring Harbor Labs, Cold Spring Harbor, New York, pp. 369-512, 1982.
955. Golomb, M., D. P. Grandgenett and W. Mason. J. Virol. 38: 548-555, 1981.
956. Grandgenett, D. P., M. Golomb and A. C. Vola. J. Virol. 33: 264-271, 1980.
957. Thimmappaya, B., C. Weinberger, R. J. Schneider and T. Shenk. Cell 31:543-551, 1982.
958. Weiser, B., D. Ganem, C. Seeger and H. E. Varmus. J. Virol. 48: 1-9, 1983.
959. Fowler, M. J. F., J. Monjardino, K. N. Tsiquaye, A. J. Zuckerman and H. C. Thomas. J. Med. Virol. 13:83-91, 1984.
960. Gowans, E. J., C. J. Burrell, A. R. Jilbert and B. P. Marmion. J. Gen. Virol. 64:1229-1239, 1983.
961. Weller, I. V. D., M. J. F. Fowler, J. Monjardino and H. C. Thomas. J. Med. Virol. 9:273-280, 1982.
962. Romet-Lemonne, J. L., E. Elfassi, W. Haseltine and M. Essex. Lancet i:732, 1983.
963. Lieberman, H. M., D. R. Labrecque, M. C. Kew, S. J. Hadziyannis and D. A. Shafritz. Hepatology 3:285-291, 1983.
964. Summers, J. and W. S. Mason. Hepatology 2:61S-66S, 1982.
965. Mason, W. S., M. S. Halpern, J. M. England, G. Seal, J. Egan, L. Coates, C. Aldrich and J. Summers. Virology 131:375-384, 1983.
966. Howard, C. R. Spring Seminars in Immunopathology 3:397-419, 1981.
967. Ishak, K. G. In The Liver, edited by Gall, E. A. and F. K. Mostofi, Williams and Wilkins, Baltimore, Md., p. 218, 1973.
968. Nowoslawski, A., K. Krawczynski, W. J. Brzosko and K. Madalinski. Am. J. Pathol. 68:31-56, 1972.
969. Pepe, G., A. Cifarelli, F. Paradisi and F. de Ritis. Experimentia 35:382-384, 1979.
970. Shimoda, T., T. Shikata, T. Tsutomu, S. Tsukagoshi, M. Yoshimura and I. Sakurai. Gastroenterology 81:998-1005, 1981.
971. Dejean, A., C. Lugassy, S. Zafrani, P. Tiollais and C. Brechot. J. Gen. Virol. 65:651-655, 1984.
972. Lutwick, L. I., M. B. Hebert and T. Sklamberg. J. Med. Virol. 9: 101-109, 1982.
973. Alberti, A., P. Pontisso, L. Chemello, E. Schiavon and G. Realdi. In Viral Hepatitis and Delta Infection, edited by Verme, G., F. Bonino and M. Rizzetto, Alan R. Liss, Inc., New York, pp. 327-336, 1983.
974. Robinson, W. S. In Comprehensive Virology, Vol. 14, edited by Fraenkel-Conrat, H. and R. R. Wagner, Plenum Publishing Corp., New York, Chapter 8, pp. 471-526, 1979.
975. Overby, L. R., C.-M. Ling, R. H. Decker, I. K. Mushahwar and K. Chau. In Viral Hepatitis, 1981 International Symposium, edited by Szmuness, W., H. J. Alter and J. E. Maynard, Franklin Institute Press, Philadelphia, Chapter 15, pp. 169-182, 1982.
976. Tabor, E., L. F. Barker and R. J. Gerety. J. Med. Virol. 6: 279-284, 1980.