JOURNAL OF VIROLOGY, June 1990, p. 2802–2809
0022-538X/90/062802-08$02.00/0

Vol. 64, No. 6

Mapping of B-Cell Epitopes of the Human Hepatitis B Virus X Protein

MARIETTA STEMLER,[1] THOMAS WEIMER,[1] ZHEN-XING TU,[1]† DA-FANG WAN,[1]‡ MASSIMO LEVRERO,[2] CHRISTINA JUNG,[3] GERD R. PAPE,[3] AND HANS WILL[1]*

Max-Planck-Institut für Biochemie, Am Klopferspitz 18a, 8033 Martinsried, Munich, Federal Republic of Germany[1]; Clinica Medica, Universita di Roma "La Sapienza," 00161 Rome, Italy[2]; and Institut für Immunologie, 8000 Munich, Federal Republic of Germany[3]

Received 2 January 1990/Accepted 26 February 1990

The immune response to the X protein of human hepatitis B virus (HBV) was studied by epitope mapping by using a set of MS2-HBx fusion proteins and synthetic peptides. Antibodies in sera of patients with acute and chronic HBV infection showed a multispecific immune response. Each serum contained antibodies to a different set of epitopes, which taken together cover most of the HBx sequence. Some of the epitopes were detectable only by immunoblotting with fusion proteins; others were detectable only by an enzyme-linked immunosorbent assay (ELISA) with synthetic peptides. The carboxy-terminal half of the HBx protein was preferentially recognized by antibodies from patients with chronic hepatitis and contained a short immunodominant antigenic region with at least two major nonoverlapping epitopes. Anti-HBx antibody titers as revealed by peptide ELISAs were highest and most frequent in patients with chronic hepatitis and usually low in acutely infected patients and asymptomatic carriers. The data demonstrate a remarkable qualitative and quantitative heterogeneity of the humoral HBx immune response which can be monitored by HBx-specific peptide ELISAs. Such tests may become useful diagnostic tools.

Hepatitis B viruses (HBVs) have been found in humans and several animal species such as woodchucks, ground squirrels, Pekin ducks, and grey herons (14, 41, 45). Infection with these viruses often leads to acute or chronic infection and various forms of liver diseases, but it may also lead to an asymptomatic chronic carrier state (for a review, see reference 7). Moreover, chronic infection with mammalian, but not avian, HBVs is associated with a high risk for development of hepatocellular carcinoma (HCC) (1, 41).

HBVs are not cytopathogenic, and therefore it is generally assumed that viral hepatitis is mediated primarily by immune defense mechanisms. The immune response has been studied in the most detail for virus components expressed in large amounts. B- and T-cell epitopes have been identified for two antigens encoded by the viral C gene, the major nucleocapsid protein (HBcAg), and the e antigen (HBeAg) (27, 28, 39), a protein which is secreted into the serum and which is not associated with viral particles (17). Circumstantial evidence suggests an important role of the immune response to these antigens in terms of virus elimination and pathogenicity, and antibodies to both antigens are early diagnostic markers of HBV infection (27, 28).

Similar studies have been performed on the immune response to the three envelope proteins, pre-S1, pre-S2, and HBsAg. B- and T-cell epitopes have been mapped, and the participation of antibodies and T cells to envelope epitopes in immune-mediated defense mechanisms and the use of envelope proteins as vaccines have been extensively investigated (27, 28).

The role of the immune response to minor viral proteins, such as the viral polymerase and genome-linked protein (collectively designated herein as P proteins), and a viral protein with a transcriptional transactivator function, designated HBx, have been less well studied. In several reports, antibodies to P gene-encoded proteins were identified in human patient sera (3, 10, 46, 50a). So far, a T-cell immune response to P proteins has not been described.

The HBx protein is currently one of the most intensively studied HBV proteins. Its function in vitro as a transcriptional transactivator of HBV or heterologous promoters invited speculations on its possible role in vivo in hepadnavirus biology and pathogenicity (5, 42, 44, 47, 48, 51, 52). It is, however, not yet clear whether HBx is essential for the virus life cycle. The X gene is present only in mammalian hepadnaviruses and is moderately conserved in sequence (23, 41). The HBx protein has been expressed successfully in several in vitro expression systems using cultured cells (2, 20, 21a, 31, 36, 37, 43).

The first indirect evidence for expression of the HBx protein in vivo was the demonstration of anti-HBx antibodies in some sera of infected patients (18). Some reports suggested preferential induction of anti-HBx antibodies in patients with hepatocellular carcinoma (31, 36); others found a low incidence in HCC patients and more frequent, high titers of anti-HBx antibodies in patients with chronic HBV infection (9, 16, 21a, 22, 26, 35). It is an open question whether the different antigens and antisera, sequence variants, or the different techniques used are responsible for these conflicting results.

In this study, we performed a detailed analysis of the humoral immune response to HBx in human patients. By epitope mapping, we tried to answer the following questions. First, are there major antigenic regions recognized by the majority of anti-HBx-positive human sera? Second, is the immune response to individual HBx epitopes correlated with the type of liver disease of the infected patient? And, third, is it possible to design HBx epitope-specific enzyme-linked

* Corresponding author.
† Present address: Institute for Hepatobiliary Surgery, Shanghai, People's Republic of China.
‡ Present address: Shanghai Cancer Institute, Shanghai 200032, People's Republic of China.

immunosorbent assays (ELISAs) to monitor the anti-HBx antibody response in clinical routine settings?

MATERIALS AND METHODS

Sera. Sera of HBV-infected patients were obtained from the Policlinico Umberto I, Rome, Italy, and Großklinikum Großhadern, Munich, Federal Republic of Germany. Blood samples negative for HBV markers were obtained from the Blood Transfusion Service, Public Health Department, Munich, Federal Republic of Germany.

Classical serological markers of HBV infection such as HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe were determined by commercial assays (Abbott Laboratories, North Chicago, Ill.).

Synthetic HBx peptides. Fifteen chemically synthesized peptides covering the whole coding region of the HBV X gene (subtype ayw [13]) were used (see Fig. 4). They were more than 90% pure, as assessed by high-pressure liquid chromatography (data not shown).

Construction of plasmids for expression of MS2-HBx fusion proteins. All constructions were made by using conventional cloning procedures (24). DNA fragments containing either the whole X gene or parts of it were expressed in *Escherichia coli* 2136 cells. Some of the vectors used (pEx 41a, pEx 41b, and pEx 41c; see Fig. 2) are derivatives of plasmid pPLc 24 (38) modified by insertion of versatile polylinkers (T. Weimer, Ph.D. thesis, Ludwig-Maximilians-Universität, Munich, Federal Republic of Germany, 1989). For expression of the full-length HBx protein, an HBV fragment containing the X gene coding region was isolated from the cloned HBV DNA (13) by restriction enzyme digestion with *Nco*I (see Fig. 1) and *Bgl*II (cleavage site within the C gene), separation by agarose gel electrophoresis, and purification by electroelution (8). The fragment was ligated to vector pEx 41a. For expression of truncated HBx protein sequences, subfragments of the X gene obtained by restriction digestion alone (Fig. 1) or a combination of restriction enzyme and BAL 31 digestion were cloned into vectors pEx 41a, pEx 41b, or pEx 41c, or into vector pPLc 24. All fragments obtained by BAL 31 digestion contained a *Hin*dIII restriction enzyme recognition site at the 5′ end which is derived from the linker region of the pSP64 vector into which the HBV DNA was previously cloned (50). Correct insertion was assessed by dideoxy sequencing (40) of the virus-plasmid junction by using a synthetic oligonucleotide as primer.

Expression of MS2-HBx fusion proteins in *E. coli*. The expression plasmids contain a heat-inducible leftward lambda promoter (38). Bacteria were grown overnight in Standard I medium (E. Merck AG, Darmstadt, Federal Republic of Germany) containing 100 μg of ampicillin per ml, and for induction of protein expression, the overnight culture was diluted 1:4 with prewarmed medium (42°C) and incubated for another 2 to 3 h at 42°C. Expression of the cloned HBV fragments resulted in MS2-HBx fusion proteins containing at their amino termini 99 amino acids of the replicase protein of bacteriophage MS2 and, in some cases, additional amino acids derived from linker and from plasmid pBR sequences (24, 61, or 66 amino acids).

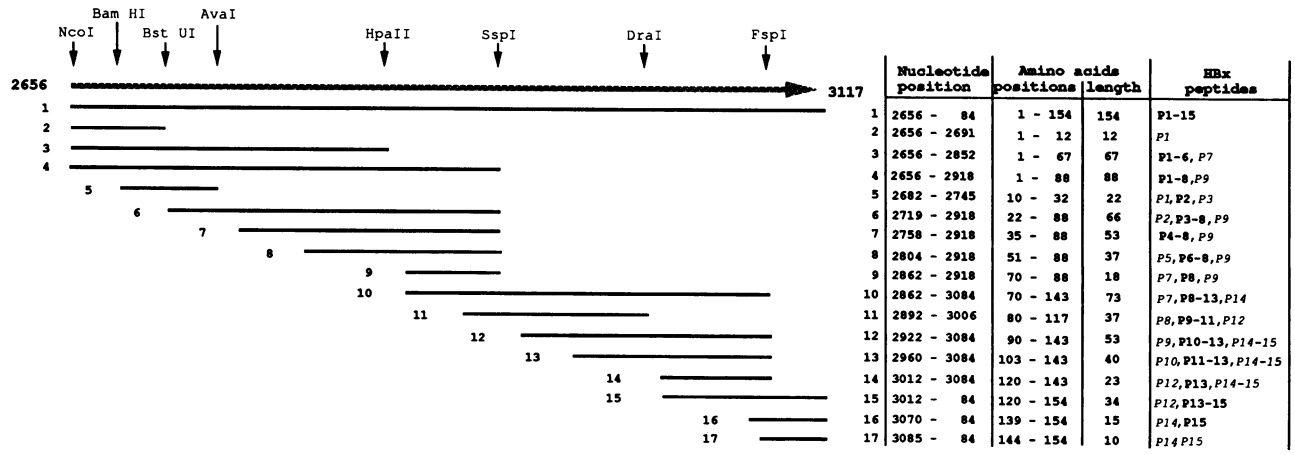
Immunoblot analysis. For immunoblotting with recombinant proteins, a protein extract was prepared by boiling the bacteria in sodium dodecyl sulfate (SDS) sample buffer (cells from a 1-ml culture in 0.3 ml of buffer) (50a). The proteins of 20 μl of these extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (21) and immunoblotted essentially as described previously (15). Proteins were transferred to nitrocellulose filters, incubated overnight in phosphate-buffered saline (PBS) (8 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 140 mM NaCl) supplemented with 5% bovine serum albumin to abolish unspecific reactivity, incubated for 2 h at room temperature with human sera at a dilution of 1:1,000 in PBS–1% bovine serum albumin, and washed three times at room temperature in PBS–0.1% Nonidet P-40. Bound human antibodies were visualized by autoradiography after incubation of the filters for 60 min with 2 μCi of iodinated protein A (30 mCi/mg; Amersham Buchler, Braunschweig, Federal Republic of Germany) in a final volume of 25 ml of PBS–1% bovine serum albumin, washing several times with PBS–0.1% Nonidet P-40, and extensive washing with water.

ELISAs with synthetic HBx peptides. Microdilution plates were coated with HBx peptides or with an unrelated control peptide (amino acid sequence TTESKLRREFEVYG PIKRIH) at a concentration of 2 μg per well in E-PBS (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 170 mM NaCl, and 3 mM KCl [pH 7.4]) for 2 h. Unbound peptide was removed by washing several times with E-PBS–5% Tween 20. Unspecific binding sites were blocked by incubation with E-PBS–5% Tween 20 at 4°C overnight. Human sera diluted 1:100 in E-PBS–5% Tween 20 were incubated for 2 h, and then the microdilution plate was washed several times with E-PBS–5% Tween 20. Bound antibodies were detected with goat anti-human antibodies coupled to horseradish peroxidase (antibody dilution, 1:3,000). After development with substrate solution (0.5 mg of *o*-phenylenediamine per ml in 0.1 M Na_2HPO_4–0.05 M citric acid, pH 6.0, and 0.5 μl of H_2O_2 per ml), the optical density was measured at 492 nm. All tests were carried out as duplicates.

Competitive inhibition assays with HBx peptides. For these assays, sera diluted 1:3,000 with PBS–1% bovine serum albumin were incubated with HBx peptides (5 μg of each peptide per μl of serum) in a final volume of 1 ml for 2 h at 28°C. The mixture was then incubated for 1 h at room temperature with the nitrocellulose filter containing the immobilized MS2-HBx fusion proteins, and unbound antibodies were removed by washing several times with PBS–0.1% Nonidet P-40. The bound antibodies were detected as described above.

RESULTS

Expression of MS2-HBx fusion proteins in *E. coli*. One type of substrate used for epitope mapping was MS2-HBx fusion proteins expressed in *E. coli*. They were obtained by cloning of the complete X gene or X gene fragments (Fig. 1) into procaryotic expression vectors (Fig. 2). The expression efficiency in *E. coli* was assessed by analyzing proteins of whole-cell extracts by SDS-PAGE and Coomassie brilliant blue staining (Fig. 3A) or immunoblotting with an anti-MS2 monoclonal antibody (Fig. 3D). With one exception (clone 5), all MS2-HBx fusion proteins were efficiently expressed (1 to 5% of the total *E. coli* proteins; Fig. 3A). The expression of the MS2-HBx fusion protein of clone 5 was low, but it could easily be visualized by immunoblotting with a monoclonal antibody to MS2 protein (Fig. 3D) or with monoclonal antibodies to HBx (data not shown). The size of the fusion proteins was in close agreement with that predicted from the constructions. Minor fusion proteins, as revealed by Coomassie brilliant blue staining and immunoblotting (Fig. 3A and D), which are smaller in size than predicted, probably represent degradation products or may derive from premature termination of translation.



	Nucleotide position	Amino acids positions	length	HBx peptides
1	2656 - 84	1 - 154	154	P1-15
2	2656 - 2691	1 - 12	12	*P1*
3	2656 - 2852	1 - 67	67	P1-6, *P7*
4	2656 - 2918	1 - 88	88	P1-8,*P9*
5	2682 - 2745	10 - 32	22	*P1,P2,P3*
6	2719 - 2918	22 - 88	66	*P2,*P3-8,*P9*
7	2758 - 2918	35 - 88	53	P4-8, *P9*
8	2804 - 2918	51 - 88	37	*P5,*P6-8,*P9*
9	2862 - 2918	70 - 88	18	*P7,*P8,*P9*
10	2862 - 3084	70 - 143	73	*P7,*P8-13,*P14*
11	2892 - 3006	80 - 117	37	*P8,*P9-11,*P12*
12	2922 - 3084	90 - 143	53	*P9,*P10-13,*P14-15*
13	2960 - 3084	103 - 143	40	*P10,*P11-13,*P14-15*
14	3012 - 3084	120 - 143	23	*P12,*P13,*P14-15*
15	3012 - 84	120 - 154	34	*P12,*P13-15
16	3070 - 84	139 - 154	15	*P14,*P15
17	3085 - 84	144 - 154	10	*P14*P15*

FIG. 1. HBx polypeptides expressed in *E. coli* and synthetic HBx peptides used for ELISAs and in competition experiments. The thick, hatched horizontal arrow indicates the HBV X gene. Some restriction sites used for molecular cloning are shown by vertical arrows. Lines below the X gene indicate X gene sequences expressed as MS2-HBx fusion proteins. Nucleotide positions of the cloned HBV DNA fragments are as described by Pasek et al. (34). The amino acid positions of the HBx sequences expressed as fusion proteins are indicated, and synthetic HBx peptides are shown as embedded totally (bold) or in part (plain) in the HBx fusion protein sequences.

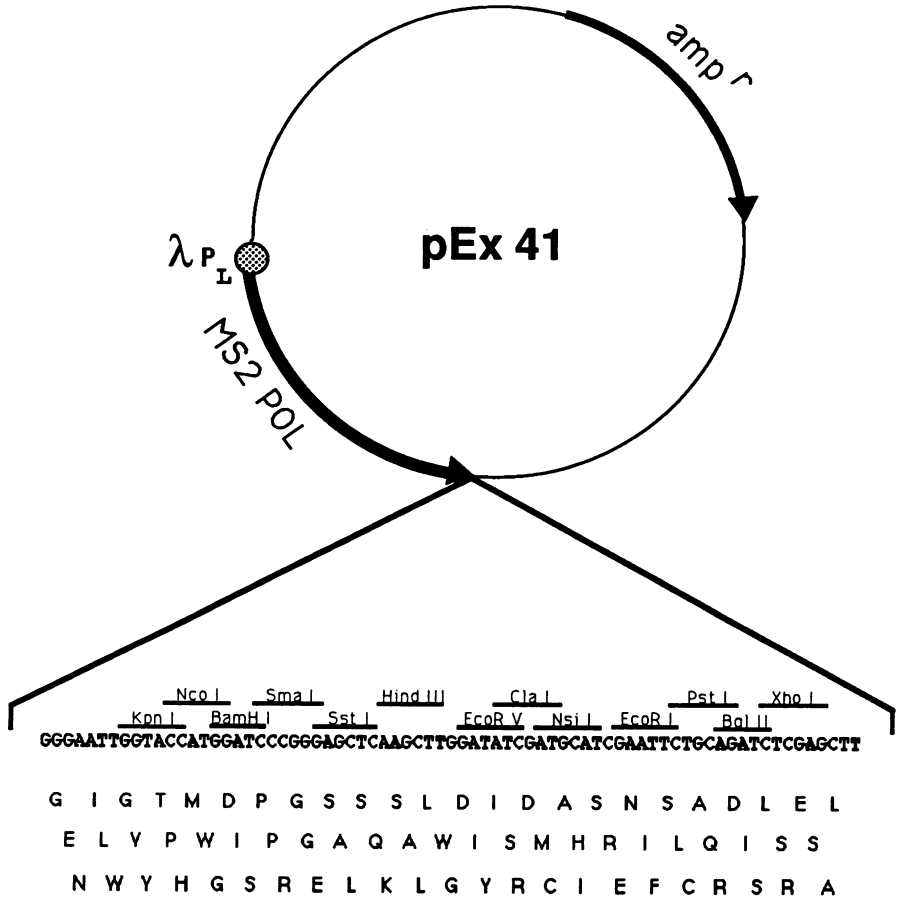


FIG. 2. Map of expression vectors pEx 41a, pEx 41b, and pEx 41c. HBV fragments were inserted in different cloning sites of the linker region downstream of the MS2 gene sequences. Amino acid sequences of the reading frames of the polylinker region of vectors pEx 41a, pEx 41b, and pEx 41c are given in the single-letter code. Abbreviations: MS2 POL, part of the replicase gene of bacteriophage MS2; lambda P_L, leftward promoter of phage lambda; amp^r, β-lactamase gene.

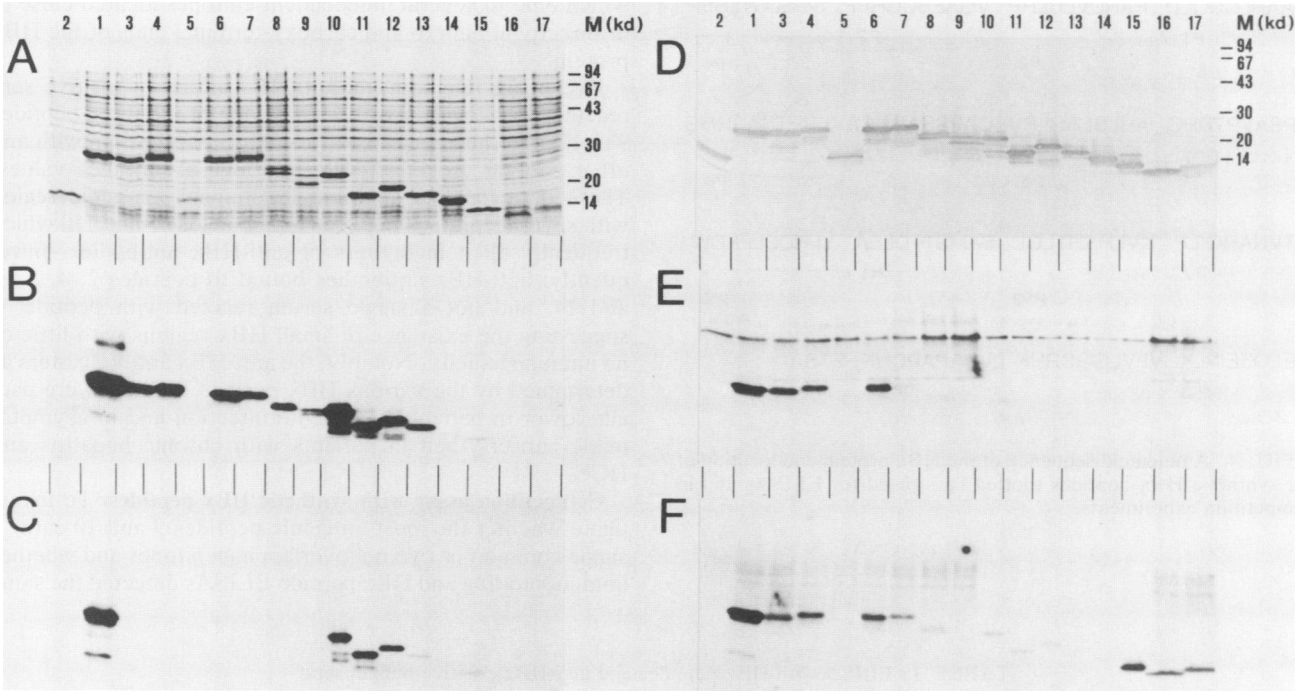


FIG. 3. Immunoblot analysis of MS2-HBx fusion proteins with an MS2-specific monoclonal mouse antibody and anti-HBx antibody-positive human sera. (A) Proteins of *E. coli* cell extracts, separated by SDS-PAGE and stained by Coomassie brillant blue. (B to F) Immunoblots with sera of a chronic HBV carrier ($HBsAg^+$ $HBeAg^+$ HBV DNA^+) (B), a patient with HCC ($HBsAg^+$ anti-HBe^+ DNA^+) (C), a mouse anti-MS2 monoclonal antibody (D), an asymptomatic HBV carrier ($HBsAg^+$) (E), and a chronic HBV carrier ($HBsAg^+$ $HBeAg^-$ DNA^-) (F). Lane numbers refer to HBx fusion proteins indicated in Fig. 1. Abbreviations: HCC, hepatocellular carcinoma; M, molecular weight marker; kd, kilodalton.

Epitope mapping by immunoblotting with MS2-HBx fusion proteins. For epitope mapping, sera from patients with acute or chronic hepatitis or HCC and from asymptomatic carriers were tested by immunoblotting with a set of 17 different MS2-HBx fusion proteins (Fig. 1). All 50 human sera used in this study had been previously screened for anti-HBx antibodies by immunoblotting or by an ELISA with full-length MS2-HBx fusion protein (data not shown). Four different reaction patterns were obtained. Most of the anti-HBx antibody-positive sera tested (43 of 50) reacted very strongly with fusion proteins 10 to 12 (for an example, see Fig. 3C). These data localize a major antigenic region in the carboxy-terminal half of the HBx protein between amino acids 89 and 102. None of 10 sera without serological HBV markers was positive (data not shown), and this demonstrates the specificity of the immunoblot assays.

Only 21 of the 50 anti-HBx antibody-positive sera reacted with amino-terminal sequences of the HBx protein (for representative examples see Fig. 3B, E, and F). Fusion protein 2 carrying the first 12 amino acids of HBx did not react with any of the sera tested. These data suggest the possible absence of antigenicity at the amino-terminal end and indicate the existence of an antigenic region with lower immunogenicity located downstream therefrom. None of the sera reacted with fusion protein 5 covering HBx amino acids 10 to 32. The lack of reactivity is probably not due to the low amount of fusion protein expressed, because a monoclonal antibody to HBx (data not shown) and to MS2 protein immunostained the fusion protein very efficiently (Fig. 3D). Thus, amino-terminal sequences 1 to 32 are apparently not antigenic in this assay.

A similar observation has been made with carboxy-terminal HBx sequences. By immunoblotting, only 2 of the 52 human sera tested reacted strongly with fusion proteins 16 and 17 covering HBx amino acids 139 to 154 of the HBx protein (one example is shown in Fig. 3F). This suggests low antigenicity for carboxy-terminal HBx sequences. Taken together, immunoblot analysis indicated one immunodominant antigenic region between amino acids 89 and 102, and less high, infrequent antigenicity of amino- and carboxy-terminal regions on the HBx protein.

Fine mapping of HBx epitopes by ELISAs with synthetic peptides. To precisely map the epitopes and to search for other epitopes which may not be detectable by immunoblotting, 15 chemically synthesized, partially overlapping peptides spanning the whole HBx protein sequence (Fig. 4) were used as substrates in ELISAs. Anti-HBx-positive sera from four different groups of patients were investigated as follows: HBV-infected patients with acute or chronic hepatitis, asymptomatic chronic HBV carriers, and chronic carriers who had developed HCC (Table 1). Sera were considered reactive with an HBx peptide when the absorbance values in an ELISA were greater than 3 standard deviations above the mean obtained with 11 serum samples of patients without any HBV markers.

Sera of patients with chronic hepatitis and HCC frequently reacted with peptides 1 and 15, representing the amino- and carboxy-terminal ends of HBx. Frequent reaction was also observed with peptide 3, whose sequence is almost completely embedded (except for three amino acids) in HBx sequences of fusion protein 5 (which was always negative by immunoblotting), and with peptides 6, 12, and 13. In contrast to the results obtained by immunoblotting, this indicates the frequent occurrence of anti-HBx antibodies in human sera

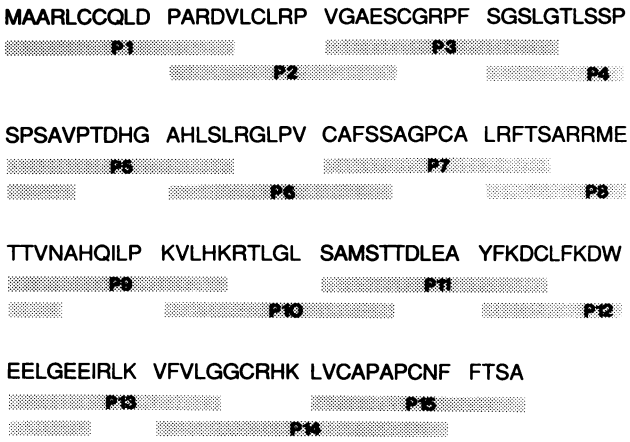


FIG. 4. Amino acid sequence of the HBx protein and location of the synthetic HBx peptides (dotted bars) used for ELISAs and in competition experiments.

which bind to several independent epitopes located close to or directly at amino- and carboxy-terminal ends of the HBx protein.

Except for five sera, all anti-HBx antibody-positive sera reacted very strongly with at least one of the three peptides 9 to 11, and this reaction was usually stronger than with any other peptide, as reflected by the high absorbance values. These data localize an immunodominant antigenic region with several epitopes between amino acids 85 and 110 which frequently elicit high titers of anti-HBx antibodies. Infrequently, anti-HBx antibodies bound to peptides 2, 4, 5, 8, and 14, and not a single serum reacted with peptide 7, suggesting the existence of small HBx regions with little or no immunogenicity. Notably, the anti-HBx antibody titers as determined by the various HBx peptide ELISAs were usually lower in patients with acute infection and in asymptomatic carriers than in patients with chronic hepatitis and HCC.

Competition assay with synthetic HBx peptides. To investigate whether the most antigenic peptides 9 and 10 carry a single common or two nonoverlapping epitopes and whether immunoblotting and HBx peptide ELISAs detected the same

TABLE 1. ELISA with HBx peptides and anti-HBx-positive human sera[a]

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15
AH											0.10	0.13	0.11		
AH											0.19				n.d
AH		1.11	0.39		1.40				1.54	0.35	1.55	0.59	0.31	1.05	0.84
AH					0.10						0.20		0.17	0.35	0.18
AH		1.48							1. 59						
AH														1.41	
AH											0.15	0.10	0.22	0.85	
ASH						0.11									
ASH					0.20				0.14						
ASH			0.15												
ASH			0.27		0.14						1.69				
ASH					0.12						0.40				
CH			0.80			0.20			0.97		0.94	0.43			0.10
CH	0.89		0.30						1.60	1.64	0.16	0.11	0.29		0.18
CH			1.25			0.38		0.27	1.40		1.19	0.82			
CH	0.75		0.20						1.43	1.67			0.11		0.16
CH			1.50			0.67		0.56	1.58		1.52	1.34			
CH	0.73		0.24						1.50	1.51		.	0.65		0.47
CH	0.88		0.24						1.53	1.59			0.17		0.20
CH						0.35			1.63						n.d.
CH	0.75		0.28						1.69	1.65			0.77		0.56
CH	0.34								1.28	0.84					
CH	0.80		0.44						1.78	1.71			0.79		0.46
CH	0.49		0.28						1.67	1.56			0.74		0.50
CH			1.44			0.37		0.30	1.59		1.29	0.99			0.50
★ CH									0.81	0.55					
CH									0.93		0.35				
CH						0.97			1.41	1.50	0.53		0.88	0.20	1.09
CH			1.24			0.20		0.16	1.44		1.21	0.78			0.11
CH	0.60		0.16						1.56	1.60			0.21		0.47
CH	0.87		0.15						1.75	1.66	0.12		0.53	0.13	0.19
CH					1.53				0.42				0.51		
CH				0.24		0.73									0.42
HCC	0.19								1.15	1.32			0.27		n.d
HCC	1.13		0.38			0.10			1.73	1.70			0.90		0.75
HCC	0.36								1.31	1.48			0.37		0.23
HCC									0.98	1.39					n.d.
HCC														0.49	1.30

[a] Sera were from patients with acute hepatitis (AH), asymptomatic chronic HBV carriers (ASH), and patients with chronic hepatitis (CH) and hepatocellular carcinoma (HCC). P, Peptide. ★, Serum which reacted with peptides 9 and 10 and which was used in the experiment described in the legend to Fig. 5.

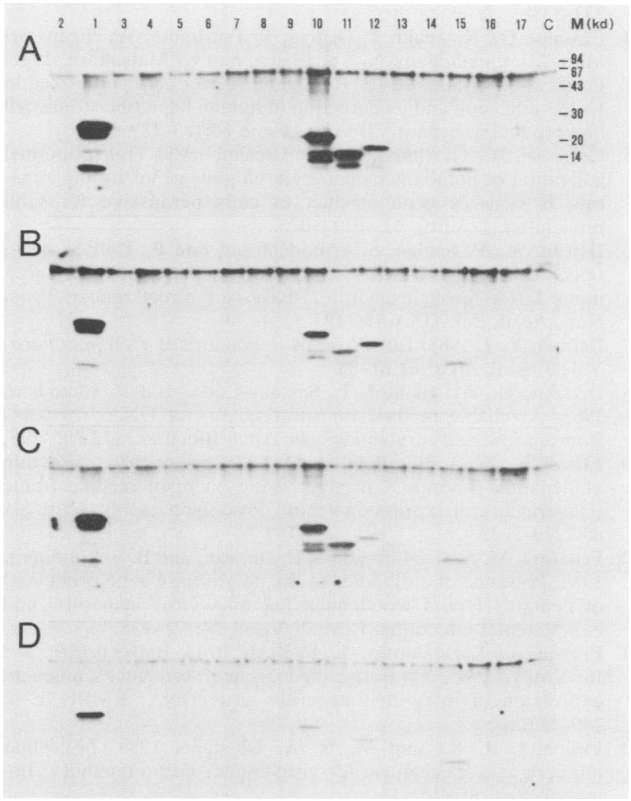


FIG. 5. Identification of HBx epitopes by immunoblot analysis by using sera preincubated with synthetic HBx peptides. Immunoblot with recombinant MS2-HBx fusion proteins and serum of a chronic HBV carrier ($HBsAg^+$ $HBeAg^+$ DNA^+) not preincubated with peptides (A); same serum preincubated with HBx peptides 9 (B) or 10 (C); same serum with a mixture of HBx peptides 9 and 10 (D). The reactivity pattern of this serum in the HBx peptide ELISA is shown in Table 1 by a star. Lanes are as described in the legend to Fig. 3.

epitopes, synthetic HBx peptides were used as competitors in immunoblots with MS2-HBx fusion proteins.

For these experiments, a serum was used which strongly reacted in immunoblotting with fusion proteins 10 to 12 and weakly reacted with fusion protein 15 (Fig. 5A) and in ELISA with the corresponding peptides 9 and 10 (Table 1, star). The reaction with fusion protein 15 but not with peptides 13 to 15 already indicates that some epitopes are detected by immunoblotting but not by our peptide ELISAs.

Preincubation of this serum with peptide 9 strongly reduced the immunoreaction with fusion proteins 10 and 11 but left virtually unchanged that with fusion protein 12 (Fig. 5B). This suggests the presence of at least two nonoverlapping epitopes in the immunodominant region. This conclusion is consistent with the reaction of the same serum after preincubation with peptide 10, which reduced strongly the signals with fusion protein 12 and also (but less strongly) fusion proteins 10 and 11 (Fig. 5C). Preincubation of the serum with both peptides completely abolished reactivity with fusion protein 11 and strongly reduced the immune reaction with fusion proteins 10 and 12 (Fig. 5D). These changes in immunoreactivity after preincubation with peptides 9 and 10 are specific, because the reaction with fusion protein 15 remained unchanged and preincubation with HBx peptides 5 and 11 did not reduce the reactivity with any of the fusion proteins (data not shown). In summary, these results demonstrate the existence of at least two major nonoverlapping epitopes in the major antigenic region which can be mimicked by peptides 9 and 10 and show that there are additional minor epitopes in this region which are not exhibited or carried by either of these peptides.

DISCUSSION

In this study, we investigated by epitope mapping the specificity of anti-HBx antibodies in sera of patients with acute and chronic HBV infection with and without HCC. Almost all sera were found to contain antibodies to more than one epitope. The carboxy-terminal half of the HBx antigen was the most frequent target for anti-HBx antibodies in sera of patients with viral hepatitis and contained an immunodominant antigenic region approximately 25 amino acids long in which two major epitopes were identified. The highest titers and more heterogeneous anti-HBx antibody specificities tended to occur in sera of patients with chronic hepatitis, whereas low titers and fewer epitopes were characteristic for sera of patients with acute hepatitis and for asymptomatic chronic carriers.

The immunodominant antigenic domain is located between amino acid positions 85 and 110. This conclusion is based on two findings. First, most of the anti-HBx-positive sera tested reacted with this domain by immunoblotting with MS2-HBx fusion protein and in ELISAs with synthetic HBx peptides. Second, the anti-HBx antibodies with the highest titers (as estimated from HBx peptide ELISA data) were directed to two epitopes of this region. The antigenicity of this region is consistent with computer algorithms which predict a relatively high antigenic index on the basis of its rather high hydrophilicity, flexibility, and probability of surface location (data not shown).

Antibodies reacting with the amino- and carboxy-terminal ends of the HBx protein were not found by immunoblotting with the corresponding MS2-HBx fusion proteins but were strongly reactive in ELISAs with peptides. The nonreactive fusion protein 2 contained only 12 X protein-specific amino-terminal amino acids, suggesting nonantigenicity of the amino-terminal HBx sequences. The 15-amino-acid amino-terminal HBx peptide, however, reacted strongly and thus contains at least one epitope. Taking into account the negative reaction with fusion protein 2, this epitope could be located at the carboxy-terminal end of peptide 1. Alternatively, it may be located at the amino-terminal end and is not recognized when fused to other sequences such as MS2. A similar reasoning can be applied to the carboxy terminus of the HBx protein. Fusion protein 16 reacted only rarely in immunoblotting (although it contains 16 carboxy-terminal HBx-specific amino acids), whereas peptide 15 containing the same HBx sequence minus two amino acids reacted frequently with anti-HBx antibody-positive sera. A simple explanation for this observation is to assume the existence of strongly conformation-dependent epitopes which cannot be built up in fusion proteins fixed to nitrocellulose, whereas these epitopes are well represented in peptide 15 in microdilution plate fixed form. The importance of conformation in epitope recognition is amply documented for other proteins and peptides (49).

The immunodominant region is highly conserved in all HBV isolate sequences described previously (23). Anti-HBx antibody-positive sera reacting with the HBx protein of a specific subtype only should therefore be rare or not exist at all.

Because of similar codon usage of the X gene and genes of eucaryotic cells, the X gene was speculated to be of cellular origin (29). The data presented show that most of the HBx sequences can be immunogenic in humans. Thus, if HBx is of cellular origin and if some sequence similarity with the cellular ancestor protein still exists, some of the HBx antibodies may, by cross-reaction, induce an autoimmune response to the cellular homolog. This would represent a special case of molecular mimicry which is known to play a role in induction of some autoimmune diseases (12). Whether the anti-HBx antibodies cross-react with a related cellular protein can be experimentally tested by using highly polyspecific anti-HBx antisera as identified in this study. Such investigations may also provide clues to our understanding of autoimmune phenomena which are occasionally associated with HBV infection (25).

The HBx protein is a transactivator of transcription in the cytoplasm and in the nucleus (4, 19, 20, 21a, 37, 43). It is not known how HBx is presented to the immune system of the host. It may be released from necrotic hepatocytes as a mono- or polymeric protein with or without associated cellular proteins, it may be secreted, or it may be processed and presented as peptides in free form or associated with cell membranes. The many epitopes scattered almost all over the HBx protein sequence as identified here suggest the involvement of processing of HBx as one driving force of the multispecific immune response.

Hybrid HBx-cell fusion proteins were speculated to be expressed from integrated HBV DNA, which is often found in liver tissue of chronic carriers and of patients with hepatocellular carcinomas, and from flanking cellular sequences (6, 11, 30, 32, 33). Such proteins could assume a different conformation and therefore exhibit different epitopes. In addition, because of the frequent loss of carboxy-terminal HBx sequences by preferential integration at the cohesive end region of the HBV genome (32), antibodies to carboxy-terminal HBx sequences might be underrepresented in the sera of these patients. We have addressed these questions by determining the anti-HBx antibody specificities in the few anti-HBx antibody-positive sera of HCC patients we had available. The results obtained do not indicate a different set of anti-HBx antibody specificities or an underrepresentation of antibodies to carboxy-terminal sequences in sera of HCC patients. Such changes in antibody specificities may, however, only become apparent long after HBV replication has ceased and after the disappearance of antibodies to the full-length wild-type HBx protein. Further studies are warranted to investigate this in more detail.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft.

We thank H. H. Guldner, F. Schödel, and G. Wildner for critical reading of the manuscript and G. Wildner for providing the monoclonal M52 antibody.

LITERATURE CITED

1. **Beasley, R. P., L.-Y. Hwang, C.-C. Lin, and C. S. Chien.** 1981. Hepatocellular carcinoma and hepatitis B virus. Lancet **ii:** 1129–1133.
2. **Chang, C., K.-S. Jeng, C.-P. Hu, S. J. Lo, T.-S. Su, L.-P. Ting, C.-K. Chou, S.-H. Han, E. Pfaff, J. Salfeld, and H. Schaller.** 1987. Production of hepatitis B virus in vitro by transient expression of cloned HBV DNA in a hepatoma cell line. EMBO J. **6:**675–680.
3. **Chang, L.-J., J. Dienstag, D. Ganem, and H. Varmus.** 1989. Detection of antibodies against hepatitis B virus polymerase antigen in hepatitis B virus infected patients. Hepatology **10:** 332–335.
4. **Chisaka, O., K. Araki, T. Ochiya, T. Tsurimoto, W. Hiranyawasitte-Attatippaholkun, N. Yanaihara, and K. Matsubara.** 1987. Purification of hepatitis B virus gene X product synthesized in Escherichia coli and its detection in human hepatoblastoma cell line producing hepatitis B virus. Gene **60:**183–189.
5. **Colgrove, R., G. Simon, and D. Ganem.** 1989. Transcriptional activation of homologous and heterologous genes by the hepatitis B virus X gene product in cells permissive for viral replication. J. Virol. **63:**4019–4026.
6. **Dejean, A., P. Sonigo, S. Wain-Hobson, and P. Tiollais.** 1984. Specific hepatitis B virus integration in hepatocellular carcinoma DNA through a viral 11-base-pair direct repeat. Proc. Natl. Acad. Sci. USA **81:**5350–5354.
7. **Desmet, V. J.** 1988. Liver lesions in hepatitis B viral infections. Yale J. Biol. Med. **61:**61–83.
8. **Dretzen, G., M. Ballard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112:**295–298.
9. **Elfassi, E., W. A. Haseltine, and J. L. Dienstag.** 1986. Detection of hepatitis B virus X product using an open reading frame Escherichia coli expression vector. Proc. Natl. Acad. Sci. USA **83:**2219–2222.
10. **Feitelson, M. A., I. Millman, G. D. Duncan, and B. S. Blumberg.** 1988. Presence of antibodies to the polymerase gene product(s) of hepatitis B and woodchuck hepatitis virus in natural and experimental infections. J. Med. Virol. **24:**121–136.
11. **Freytag von Loringhoven, A., S. Koch, P. H. Hofschneider, and R. Koshy.** 1985. Co-transcribed 3′ host sequences augment expression of integrated hepatitis virus DNA. EMBO J. **4:** 249–255.
12. **Fujinami, R. S., and M. B. A. Oldstone.** 1989. Molecular mimicry as a mechanism for virus-induced autoimmunity. Immunol. Res. **8:**3–15.
13. **Galibert, F., E. Mandart, E. Fitoussi, P. Tiollais, and P. Charnay.** 1979. Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in E. coli. Nature (London) **281:**646–650.
14. **Ganem, D., and H. E. Varmus.** 1987. The molecular biology of the hepatitis B virus. Annu. Rev. Biochem. **56:**651–693.
15. **Guldner, H. H., H. J. Netter, C. Szostecki, H. J. Lakomek, and H. Will.** 1988. Epitope mapping with a recombinant human p68-kDa (U1) ribonucleoprotein antigen reveals heterogeneous autoantibody profiles in human autoimmune sera. J. Immunol. **141:**469–475.
16. **Hess, J., M. Stemler, H. Will, C. H. Schröder, J. Kühn, and R. Braun.** 1988. Frequent detection of antibodies to hepatitis B virus X-protein in acute, chronic and resolved infections. Med. Microbiol. Immunol. **177:**195–205.
17. **Jean-Jean, O., M. Levrero, H. Will, M. Perricaudet, and J.-M. Rossignol.** 1989. Expression mechanism of the hepatitis B virus (HBV) C gene and biosynthesis of HBe antigen. Virology **170:**99–106.
18. **Kay, A., E. Mandart, C. Trepo, and F. Galibert.** 1985. The HBV HBx gene expressed in E. coli is recognised by sera from hepatitis patients. EMBO J. **4:**1287–1292.
19. **Ke-Qin, H., H. Lian-Jie, and H. Will.** 1988. A preliminary study on localization of HBxAg in liver tissue of patients with chronic liver disease and its significance. Chin. Med. J. **101:**671–674.
20. **Koike, K., T. Akatsuka, and T. Miyamura.** 1988. Characterization of hepatitis B virus X gene: in vitro translation of mRNA from COS-1 cells transfected with the X gene. Virology **163:** 233–235.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.

21a. **Levrero, M., O. Jean-Jean, C. Balsano, H. Will, and M. Perricaudet.** 1990. Hepatitis B virus (HBV) gene expression in human cells and anti-HBx antibody detection in chronic HBV infection. Virology **174:**299–304.

22. **Liang, X.-H., M. Stemler, H. Will, R. Braun, Z.-Y. Tang, and C. H. Schröder.** 1988. Low incidence and high titers of antibodies to hepatitis B virus X-protein in sera of Chinese patients with hepatocellular carcinoma. J. Med. Virol. **25:**329–337.

23. **Lo, S. J., M.-L. Chien, and Y.-H. W. Lee.** 1988. Characteristics of the X gene of hepatitis B virus. Virology **167**:289–292.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Manns, M.** 1989. Autoantibodies and antigens in liver diseases—updated. J. Hepatol. **9**:272–280.
26. **Meyers, M. L., L. Vitvitski Trepo, N. Nath, and J. J. Sninsky.** 1986. Hepatitis B virus polypeptide X: expression in *Escherichia coli* and identification of specific antibodies in sera from hepatitis B virus-infected humans. J. Virol. **57**:101–109.
27. **Milich, D. R.** 1987. Genetic and molecular basis for T- and B-cell recognition of hepatitis B viral antigens. Immunol. Rev. **99**: 71–103.
28. **Milich, D. R.** 1989. Synthetic T and B cell recognition sites: implications for vaccine development. Adv. Immunol. **45**:195–282.
29. **Miller, R. H., and W. S. Robinson.** 1986. Common evolutionary origin of hepatitis B virus and retroviruses. Proc. Natl. Acad. Sci. USA **83**:2531–2535.
30. **Miyaki, M., C. Sato, T. Gotanda, T. Matsui, S. Mishiro, M. Imai, and M. Mayumi.** 1986. Integration of region X of hepatitis B virus genome in human primary hepatocellular carcinoma propagated in nude mice. J. Gen. Virol. **67**:1449–1454.
31. **Moriarty, A. M., H. Alexander, and R. A. Lerner.** 1985. Antibodies to X-peptides detect new hepatitis B antigen: serological correlation with hepatocellular carcinoma. Science **227**:429–433.
32. **Nagaya, T., T. Nakamura, T. Tokino, T. Tsurimoto, M. Imai, T. Mayumi, K. Kamino, K. Yamamura, and K. Matsubara.** 1987. The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. Genes Dev. **1**:773–782.
33. **Ou, J.-H., and W. Rutter.** 1985. Hybrid hepatitis B virus-host transcripts in a human hepatoma cell line. Proc. Natl. Acad. Sci. USA **82**:83–87.
34. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in E. coli. Nature (London) **282**: 575–579.
35. **Persing, D. H., H. E. Varmus, and D. Ganem.** 1986. Antibodies to pre-S and X determinants arise during natural infection with ground squirrel hepatitis virus. J. Virol. **60**:177–184.
36. **Pfaff, E., J. Salfeld, K. Gmelin, H. Schaller, and L. Theilmann.** 1987. Synthesis of the X-protein of hepatitis B virus in vitro and detection of anti-X-antibodies in human sera. Virology **158**: 456–460.
37. **Pugh, J. C., C. Weber, H. Houston, and K. Murray.** 1986. Expression of the X gene of hepatitis B virus. J. Med. Virol. **20**:229–246.
38. **Remaut, E., P. Stannssens, and W. Fiers.** 1981. Plasmid vectors for high-efficiency expression controlled by the P_L promoter of coliphage no. 1. Gene **15**:81–93.
39. **Salfeld, J., E. Pfaff, M. Noah, and H. Schaller.** 1989. Antigenic determinants and functional domains in core antigen and e antigen from hepatitis B virus. J. Virol. **63**:798–808.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
41. **Schödel, F., R. Sprengel, T. Weimer, D. Fernholz, R. Schneider, and H. Will.** 1989. Animal hepatitis B viruses, p. 73–102. *In* G. Klein (ed.), Advances in viral oncology, vol. 8. Raven Press, Ltd., New York.
42. **Seto, E., T. S. B. Yen, B. M. Peterlin, and J.-H. Ou.** 1988. Trans-activation of the human immunodeficiency virus long terminal repeat by the hepatitis B virus X protein. Proc. Natl. Acad. Sci. USA **85**:8286–8290.
43. **Siddiqui, A., S. Jameel, and J. Mapoles.** 1987. Expression of the hepatitis B virus X gene in mammalian cells. Proc. Natl. Acad. Sci. USA **84**:2513–2517.
44. **Spandau, D. F., and C.-H. Lee.** 1988. *trans*-Activation of viral enhancers by the hepatitis B virus X protein. J. Virol. **62**: 427–434.
45. **Sprengel, R., E. F. Kaleta, and H. Will.** 1988. Isolation and characterization of a hepatitis B virus endemic in herons. J. Virol. **62**:3832–3839.
46. **Stemler, M., J. Hess, R. Braun, H. Will, and C. H. Schroeder.** 1988. Serological evidence for expression of the polymerase gene of human hepatitis B virus in vivo. J. Gen. Virol. **69**: 689–693.
47. **Twu, J.-S., and W. S. Robinson.** 1989. Hepatitis B virus X-gene can transactivate heterologous viral sequences. Proc. Natl. Acad. Sci. USA **86**:2046–2050.
48. **Twu, J.-S., and R. H. Schloemer.** 1987. Transcriptional *trans*-activating function of hepatitis B virus. J. Virol. **61**:3448–3453.
49. **Van Regenmortel, M. H. V.** 1989. Structural and functional approaches to the study of protein antigenicity. Immunol. Today **10**:266–272.
50. **Weimer, T., J. Salfeld, and H. Will.** 1987. Expression of the hepatitis B virus core gene in vitro and in vivo. J. Virol. **61**:3109–3113.
50a. **Weimer, T., K. Weimer, Z.-X. Tu, M.-C. Jung, G. R. Pape, and H. Will.** 1989. Immunogenicity of human hepatitis B virus P-gene derived proteins. J. Immunol. **143**:3750–3756.
51. **Wollersheim, M., U. Debelka, and P. H. Hofschneider.** 1988. A transactivating function encoded in the hepatitis B virus X gene is conserved in the integrated state. Oncogene **3**:545–552.
52. **Zahm, P., P. H. Hofschneider, and R. Koshy.** 1988. The HBV X-ORF encodes a transactivator: a potential factor in viral hepatocarcinogenesis. Oncogene **3**:169–177.