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The Amino Acid Residues at Positions 120 to 123 Are Crucial for the Antigenicity of Hepatitis B Surface Antigen▽

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The major hydrophilic region (MHR) of hepatitis B surface antigen (HBsAg) harbors conformational B-cell epitopes and is the major target of neutralizing antibodies to HBsAg (anti-HBs). Mutant HBsAg (mtHBsAg) with amino acid substitutions such as G145R is known to affect the binding of specific anti-HB antibodies and their detection by conventional diagnostic assays. In the present study, we focused on the role of the amino acid positions 120 to 123, which are around MHR 2 according to the spectrum of recently identified, naturally occurring mtHBsAg. Strikingly, the amino acid substitution K122I abolished the reactivity of HBsAg in all immunoassays tested so far. Also, mtHBsAg G145R could be clearly detected with four different enzyme-linked immunosorbent assays that were based on monoclonal anti-HB antibodies (MAbs) with high affinity. Positive immunofluorescence staining of mtHBsAg K122I was achieved only by polyclonal anti-HBs, while all MAbs tested failed. mtHBsAg T123N showed a low reactivity in immunoassays and appeared to be secretion defective. The amino acid substitution P120T reduced the binding of anti-HBs but did not completely prevent the detection of mtHBsAg by anti-HB MAbs. The testing of naturally occurring mtHBsAg confirmed that the presence of amino acid substitutions within the region of 120 to 123 is strongly associated with impaired detection in immunoassays. In conclusion, MHR 2 is essential for HBsAg antigenicity, a fact that has not been recognized before.

The hepatitis B surface antigens (HBsAg) are able to induce protection against hepatitis B virus (HBV) infection and therefore can be used as vaccines (37). The three surface antigens, large, middle, and small HBsAg (SHBsAg), share the C-terminal 226 amino acid (aa) residues. The major part of the viral envelope consists of SHBsAg, with 226 aa residues. The current model of SHBsAg predicts a protein with five transmembrane segments and three regions exposed to the outside (4, 10, 36). The major hydrophilic region (MHR) of HBsAg between aa 99 and 169 contains a highly conformational epitope cluster. This region was further divided into five different subregions: MHR 1, from aa 99 to 119; MHR 2, from aa 120 to 123; MHR 3, from aa 124 to 137; MHR 4, from aa 138 to 147; and MHR 5, from aa 148 to 169 (36). A large number of amino acid substitutions were found within the central region of aa 124 to 147, and some of the amino acid substitutions affect the antigenicity and immunogenicity of HBsAg (4–8, 13–18, 20, 21, 24–27, 30, 32, 34, 39, 40–42). In many cases, amino acid insertions were found in this region of HBsAg (9, 19, 42). These amino acid substitutions and insertions often were found in association with immune escape or diagnostic failure and were proven to impair the binding to anti-HB antibodies in some cases. For example, the amino acid substitution G145R within MHR 4 is known to change the antigenicity and immunogenicity of HBsAg (5, 11, 12, 32, 40, 44). In addition, HBsAg with amino acid substitutions may induce antibodies with changed specificity directed to the mutated HBsAg (mtHBsAg) (44). The cysteine residues 121, 124, 137, 139, and 147 are supposed to be important for the conformation of HBsAg MHR and therefore have a strong impact on antigenicity (2, 28, 29, 38). As these mutations occurred naturally in chronically infected patients and led to diagnostic failure and other problems, they are important for clinical diagnosis and prevention of HBV infections.

Previous reports described a number of naturally occurring mtHBsAg with amino acid substitutions within MHR 2 (8, 15, 20, 39, 41). To determine the impact of the amino acid substitutions within MHR 2 on HBsAg antigenicity, single-amino-acid substitutions were introduced into a defined HBsAg sequence by site-directed mutagenesis. A panel of newly generated anti-HB monoclonal antibodies (MAbs) were used to assess the antigenicity of mtHBsAg.

MATERIALS AND METHODS

Plasmids. Some plasmids used in this study were described previously (22, 35) and were provided by the authors of those reports (Table 1). The coding region for HBsAg (nucleotide [nt] 130 to 841 according to HBV genome sequence AF100308) was included. All vectors used the cytomegalovirus immediate-early promoter.

Construction of plasmids encoding mtHBsAg with single mutations within the type a determinant by site-directed mutagenesis. A series of expression vectors of mtHBsAg with amino acid substitutions at positions 120 to 123 was con-

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TABLE 1. Expression vectors for wild-type HBsAg and mtHBsAg used in this study

Plasmid	Subtype	Mutation(s) within MHR[a]	Vector	Source or reference
Gly Yw	ayw	Wild-type sequence	pJI	36
Arg145yw	ayw	G145R	pJI	36
1056sp	ayw	P120S/S143L	pJI	22
BA2.4	ayw	Y100C/P120T	pJI	22
BA3.2	ayw	T123N/C124R	pJI	22
BA3.4	ayw	T123N	pJI	22
SA7	ayw	M133T	pJI	22
Gly Dw	adw	Wild-type sequence	pJI	22
Arg145dw	adw	G145R	pJI	22
91-4696	adw	S113T/T143S	pJI	22
AP3.1	adw	D144A	pJI	22
SA4	adw	M133T/Y161F	pJI	22
SA6	adw	Q129R/G130N/A166V	pJI	22
HK188	adr	L98V/Q101R	pJI	22
Gly Dr	adr	Wild-type sequence	pJI	This work
Arg145dr	adr	G145R	pJI	This work
C126	adr	I126T/S143T	pJI	This work
pXF3HA-HBS	adw	Wild-type sequence	pXF3H	This work
P120T	adw	P120T	pXF3H	This work
C121S	adw	C121S	pXF3H	This work
K122I	adw	K122I	pXF3H	This work
T123N	adw	T123N	pXF3H	This work
pS2-HBsAg122I	adw	K122I	pcDNA3	This work

[a] The sequence variations given are according to the respective wild-type sequences.

structed based on pXF3H, a mammalian expression vector with the cytomegalovirus immediate-early promoter (kindly provided by Feng Xing-Hua). The coding region of HBsAg was amplified from a cloned *adw2* sequence, as described previously (43, 44). The primers used for PCR, SP1 and SP2, are listed in Table 2. The amplified fragment (nt 160 to 841 according to the HBV genome sequence AF100308) was digested with EcoRI and PstI and inserted into predigested pXF3H. This procedure resulted in a vector expressing HBsAg with an amino-terminal hemagglutinin (HA) tag. The amino acid sequence preceding the S protein is MYP YDV PDY ANS PYP YDV PDY AEF. Mutations were introduced into the wild-type HBsAg sequence by PCR-based mutagenesis using sense primers P120TL, C121SL, K122IL, and T123NL and an antisense primer designated T-primer, as listed in Table 2. PCR-based, site-directed mutagenesis was performed. Briefly, the cloned wild-type HBV S gene was used as a template to generate two DNA fragments using the primer pairs SP1/T-primer and either SP2/P120TL, SP2/C121SL, SP2/K122IL, or SP2/T123NL. The corresponding fragments were combined. Subsequently, the overlapping ends of the fragments were annealed, allowing each strand to serve as a primer for the extension of the complementary strand. The resulting fusion product was amplified further by PCR using the outer primers SP1 and SP2. The mutated sequences were cloned into pXF3H and were verified by sequence analysis.

An additional expression vector for the mtHBsAg with K/R122I was generated on the basis of a cloned HBV genome of the subtype adw2, pHBV991-12-1 (GenBank accession number X51970). The cloned DNA was extracted from *Escherichia coli* and was subjected to PCR amplification. The region encoding the HBsAg (nt 11 to 1027 according to the numbering of the pHBV991-12-1 sequence) of HBV was amplified using KS13 (nt 11 to 36; 5′-CCT TCC ACC AAA CTC TGC AAG ATC CC-3′) and KS14 (nt 1027 to 1005; 5′-GGA GCA GCA AAG CCC AAA AGA CC-3′) and was cloned into the pCR2.1 vector (41). The correctness of the sequence was verified by DNA sequencing analysis of the cloned fragments. Mutations were introduced by PCR-based, site-directed mutagenesis using specific primers. The wild-type and mutated HBV S genes were subcloned into the EcoRI site of the expression vector pcDNA3 and were tested for the expression of HBsAg by transient transfection in HepG2 cells. In addition, the coding sequence for the HBV pre-S2 region was added to the mutated S sequences to generate pS2-HBsAg122I by using a previously cloned sequence (43). This procedure resulted in a plasmid expressing the HB middle surface antigen with the amino acid substitution K122I within the S domain. The correctness of the sequence and the orientation of the insert in the pcDNA3 vector were verified by sequencing.

Amino acid substitution G145R was introduced into the wild-type HBV S gene by the same technique by using the primers MP-145-1 and MP-145-2 (Table 2).

Transient transfection with the expression plasmids. HepG2 and HeLa cells were maintained in appropriate media, supplemented with 10% fetal calf serum (FCS), in 8-well tissue culture chamber slides at 37°C and 5% CO_2 until the cells reached approximately 60 to 80% confluence. The transfection of cells was performed by using Lipofectamine (Invitrogen) according to a protocol described previously (44). To monitor the transfection efficiency, an expression vector for green fluorescent protein (GFP), pEGFP-N2, was cotransfected with expression vectors of HBsAg. One microgram of the expression vectors of HBsAg and 0.5 μg of pEGFP-N2 were mixed for transfection. Culture supernatants of transfected cells were collected after 72 h for the detection of HBsAg. At the same time, the expression of GFP in transfected cells was judged by fluorescence microscopy. Thirty to 40% of transfected cells regularly expressed GFP. Similar results were generated by using HepG2 and HeLa cells. For detection of HBsAg in enzyme-linked immunosorbent assays (ELISAs), results based on HeLa cells were used, since they usually give higher values due to a better transfection efficiency. The results of immunofluorescence (IF) were obtained by using HepG2 cells.

IF staining of transfected cells. Transfected cells were washed twice with phosphate-buffered saline (PBS) and were fixed with 50% methanol at 4°C for 30 min for IF staining. IF staining of transfected cells with polyclonal goat anti-HB antibodies to plasma-derived HBsAg of ad and ay types (Biotrend, Köln, Germany) (1:80 dilution with PBS) and monoclonal mouse anti-HBsAg type a antibodies (Dako, Hamburg, Germany; Roche, Penzberg, Germany; or our own preparations) (1:100 to 1:200 dilution with PBS) as primary antibodies was performed as described previously (44).

For confocal laser microscopy, transfected cells were fixed with 50% methanol, treated with 0.25% Triton X-100 for 5 min, and washed with PBS. After being stained with primary and secondary antibodies, the cells were incubated for 30 min with a staining solution containing 50 μg of propidium iodide per ml, 300 μg of RNase per ml, 1% Triton X-100, and 10 mM EDTA. After three washes, 30% glycerin was added to cells for confocal laser microscopy.

Preparation of cell lysates for detection of cell-associated HBsAg by Western blotting. To detect cell-associated HBsAg in transfected cells, cell lysates were prepared and subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Western blotting. Cells were transfected with expression vectors and were cultured for 48 h. Cells were washed three times with precooled PBS, detached from the plate with a cell scraper, and collected in Eppendorf tubes by centrifugation at 6,000 rpm. The cell pellets then were resuspended in

TABLE 2. Primers for the construction of the expression vectors[a]

Designation	Position (nt)	Sequence[b]	Polarity
SP1	160–176	5′-**ACGGAATTC**GAGAACATCGCATCAGG-3′	Sense
SP2	824–841	5′-**GCACTGCAG**CGGTTTAAATGTATACCC-3′	Antisense
P120TL	494–527	5′-CCTCAACAACCAGCACGGGA*ACA*TGCAAGACC-3′	Sense
C121SL	494–531	5′-CCTCAACAACCAGCACGGGACCA*AGC*AAGACCTGCA-3′	Sense
K122IL	494–537	5′-CCTCAACAACCAGCACGGGACCATGC*ATC*ACCTGCACGATTCC-3′	Sense
T123NL	494–537	5′-CCTCAACAACCAGCACGGGACCATGCAAG*AAC*TGCACGATTCC-3′	Sense
T-primer	490–513	5′-CCCGTGCTGGTTGTTGAGGATCC-3′	Antisense
MP-145-1	578–599	5′-AACCTACGGACAGAAACTGCAC-3′	Sense
MP-145-2	575–598	5′-TGCAGTTTCTGTCCGTAGGTTTTG-3′	Antisense

[a] The primers were designed according to the sequence of the HBV genome with accession number AF100308.
[b] The added sequences containing the restriction sites in SP1 and SP2 are in boldface. The restriction sites are underlined. The mutated codons in primers P120TL, C121SL, K122IL, and T123NL are italicized.

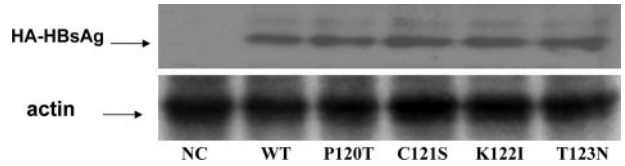

FIG. 1. Detection of wild-type HBsAg and mtHBsAg expressed in transiently transfected cells by Western blotting. Lysates of transfected cells containing 50 μg of proteins were loaded on a 12% SDS-polyacrylamide gel and were subjected to electrophoresis and Western blotting. The antibody to the HA tag was used for detection of expressed HBsAg. To compare the amounts of protein loaded on the SDS gel, β-actin was detected by using a specific antibody. NC, negative control, untransfected cells; WT, wild type.

100 μl of PBS and lysed by three freeze-thaw cycles. The cell lysates were cleared of cell debris by 2 min of centrifugation in an Eppendorf centrifuge at 12,000 rpm and were stored at −20°C. The protein concentrations in cell lysates were determined by a Bradford bicinchoninic acid assay (Bio-Rad). The lysates were mixed with twofold-concentrated protein sample buffer and were heated for 10 min for SDS-polyacrylamide gel electrophoresis. Fifty micrograms of proteins was loaded on a 12% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred from the SDS-polyacrylamide gel onto a nitrocellulose membrane. HBsAg with the HA tag was detected by polyclonal rabbit anti-HA antibodies at a dilution of 1:300 and horseradish peroxidase (HRP)-labeled goat anti-rabbit antibody at a dilution of 1:5,000. Polyclonal rabbit antibody to HA and HRP-conjugated goat antibody to rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology. The detected protein bands were visualized by chemiluminescence.

Determining the reactivity of wild-type HBsAg and mtHBsAg with eight commercial HBsAg ELISAs. Seven commercial HBsAg ELISA kits were purchased. All seven kits use monoclonal anti-HBsAg antibodies to coat the plate and use labeled polyclonal anti-HB antibodies for detection. The detection of wild-type HBsAg and mtHBsAg by the commercial HBsAg ELISA kits was performed according to the manufacturers' instructions. Fifty microliters of culture supernatants or cell lysates was used for the assays. The signal-to-noise (S/N) values were calculated as the optical density (OD) of the sample divided by the OD of the negative control, with S/N ≥ 2.1 as the cutoff. The S/N values of each sample were used to express the reactivity in seven commercial ELISA kits. For each wild-type HBsAg or mtHBsAg, the transfection of cells was repeated four times, and expressed HBsAg in culture supernatants and cells were detected by ELISA. The average values of four test runs were calculated and are given as the reactivity of wild-type HBsAg or mtHBsAg. In addition, the reactivity of wild-type HBsAg and mtHBsAg in supernatants was measured by using the ARCHITECT HBsAg assay kit (Abbott).

Determining the reactivity of wild-type HBsAg and mtHBsAg in ELISAs based on polyclonal anti-HBs and newly generated anti-HB MAbs. Fourteen new mouse anti-HB MAbs were generated by immunization of mice using purified HBV Dane particles of the subtype adr, genotype C. Among them, nine anti-HB MAbs were IgG1, and five were IgG2a. These anti-HB MAbs were purified and verified for their reactivity to HBsAg in commercial anti-HB ELISAs.

The anti-HB MAbs were used to coat microtiter plates at a concentration of 1 μg per ml. After incubation overnight at 4°C, plates were blocked with 5% FCS at 37°C for 2 h. Fifty microliters of each sample was added to wells, and the wells were incubated at 37°C for 1 h. The plates were washed five times with PBS. HRP-labeled polyclonal anti-HB antibodies (Ke Hua Biologicals, Shanghai, China) were dispensed into wells and incubated further at 37°C for 45 min. The plates were washed again five times, 1,3,5-trimethylbenzene was added, and then the plates were incubated for 15 min. The color development was measured at 450 nm. The S/N values were calculated as the OD of the sample divided by the OD of the negative control, with S/N ≥ 2.1 as the cutoff.

RESULTS

Construction of the expression vectors for mtHBsAg with amino acid substitutions at positions 120 to 123 of HBsAg. Previously published work suggested that the amino acid residues at positions 120 to 123 of HBsAg may be crucial for the recognition of anti-HB antibodies (1, 19, 22, 41). However, the natural isolates have multiple amino acid substitutions that may contribute synergistically to the change of HBsAg antigenicity. Thus, it is necessary to construct HBsAg with single-amino-acid substitutions for the assessment of the impact of individual amino acid substitutions on the antigenicity of HBsAg. For this purpose, we generated a series of mtHBsAg with the amino acid substitutions P120T, C121S, K122I, and T123N. The choice of the amino acid substitutions was made by analysis of naturally occurring HBsAg variants that have been found in patients so far (8, 15, 20, 39, 41). To assess the expression of HBsAg with amino acid substitutions, an HA tag was included so that the HBsAg could be detected by Western blotting using a specific antibody to the HA tag. It was found that the wild-type HBsAg and mtHBsAg were expressed at comparable levels after transient transfection of HepG2 cells (Fig. 1).

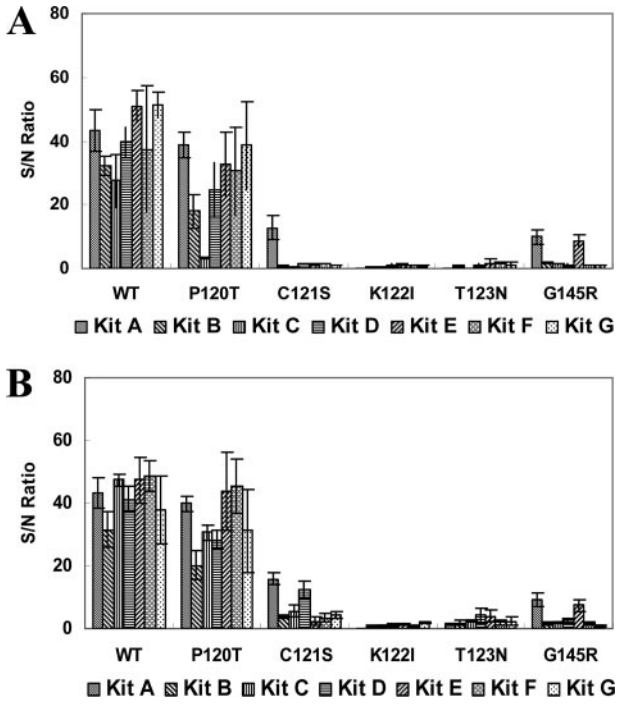

FIG. 2. Reactivity of expressed wild-type HBsAg and mtHBsAg in seven commercial ELISAs (kits A to G). The wild-type HBsAg and mtHBsAg were expressed by transient transfection. (A) The reactivity of HBsAg in culture supernatants. (B) The reactivity of HBsAg in cell lysates. The reactivity was expressed as the ratio of sample reactivity to that of the negative control (S/N ratio). The negative control was prepared using untransfected HepG2 cells. WT, wild type.

Reactivity of mtHBsAg in commercial ELISAs. The wild-type HBsAg and mtHBsAg with amino acid substitutions at positions 120 to 123 were expressed by transient expression in HepG2 cells. Wild-type HBsAg and mtHBsAg in culture supernatants and lysates of transfected cells were subjected to eight commercial diagnostic kits. The detection of HBsAg was performed by using an ARCHITECT HBsAg assay kit (Abbott) and detected 11.2, 8.3, 5.7, 0.27, and 1.8 IU/ml of wild-type HBsAg and mtHBsAg P120T, C121S, K122I, and T123N, respectively. The wild-type HBsAg with the HA tag was clearly detected by different ELISAs, indicating that it was properly assembled and secreted into the culture medium (Fig. 2). Dif-

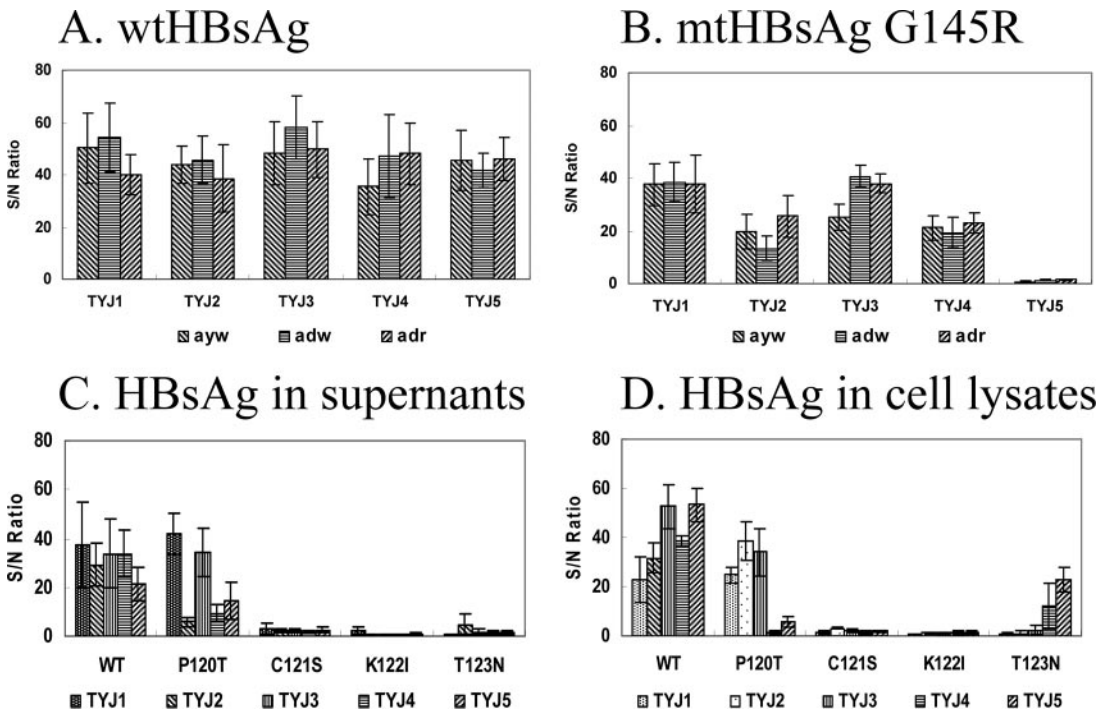

FIG. 3. Reactivity of expressed wild-type HBsAg (wtHBsAg) and mtHBsAg in ELISAs based on the five newly generated anti-HB MAbs TYJ1 to TYJ5. The wild-type HBsAg and mtHBsAg were expressed by transient transfection. Shown are the reactivities of wild-type HBsAg (A) and mtHBsAg G145R (B) of three HBsAg subtypes, adw, adr, and ayw, with the MAbs. The reactivities of wild-type HBsAg and mtHBsAg P120T, mtHBsAg C121S, mtHBsAg K122I, and mtHBsAg T123N in culture supernatants (C) and cell lysates (D) also are shown. The reactivity was expressed as the ratio of the reactivity of the samples to that of the negative control (S/N ratio). The negative control was prepared using untransfected HepG2 cells. WT, wild type.

ferent clones based on pJI expressed HBsAg at levels ranging from 7.3 to 12.1 IU/ml. However, the amino acid substitutions strongly affected the detection of HBsAg (Fig. 2). The mtHBsAg with P120T showed a reactivity like that of wild-type HBsAg in the majority of the commercial assays that have been tested so far (Fig. 2). The reactivity level of the mtHBsAg with P120T in one commercial assay (kit C) was very low. The other three amino acid substitutions, C121S, K122I, and T123N, appeared to impair strongly the detection of HBsAg, despite the normal expression level of these mtHBsAg. The mtHBsAg with C121S was detected by only one of seven commercial assays. All the other assays used did not detect the mtHBsAg with C121S, consistent with the previous finding that the loss of the cysteine residue may influence the antigenicity of HBsAg (2, 28, 29, 38). Both amino acid substitutions K122I and T123N abolished the reactivity of mtHBsAg in all ELISAs included in the study. Similarly, mtHBsAg G145 was not or was weakly recognized by these assays.

Reactivity of anti-HB MAbs to mtHBsAg with amino acid substitutions at positions 121 to 123 and comparison with mtHBsAg G145R. To further assess the impact of the amino acid substitutions on HBsAg, the reactivity of mtHBsAg with a panel of new anti-HB MAbs was determined. Among 14 newly generated anti-HB MAbs, four MAbs, TYJ1 to TYJ4, showed high affinity to HBsAg, as determined with serial dilutions in anti-HB ELISAs (data not shown). In particular, the anti-HB IgG2a MAb TYJ3 tested highly positive at the concentrations of 1 and 0.1 ng per ml. TYJ5 was determined to be less reactive and was representative of the other nine anti-HB MAbs prepared in this study. These results indicated that the anti-HB MAbs have great differences in their reactivities to HBsAg. The anti-HB MAbs TYJ1 to TYJ5 were used for the detection of HBsAg of different genotypes and serotypes and three different mtHBsAg with G145R. Three different HBsAg serotypes, adw2, adr, and ayr, were detected by the ELISAs with the anti-HB MAbs without a notable difference (Fig. 3A). Notably, mtHBsAg G145R, on the background of three different serotypes, was detected in ELISAs based on MAbs TYJ1 to TYJ4. The MAb TYJ5, representative of the other nine MAbs (TYJ6 to YYJ14), was not able to recognize mtHBsAg G145R (Fig. 3B).

The ELISAs based on the MAbs TYJ1 to TYJ5 were used to examine mtHBsAg P120T, C121S, K122I, and T123N. The mtHBsAg P120T was at least detected by some MAbs. Three mtHBsAg, C121S, K/R122I, and T123N, were not sufficiently recognized by any of these MAbs (Fig. 3C). Similar results were obtained with lysates of transfected cells (Fig. 3D). Only T123N in cell lysates was recognized by TYJ4 and TYJ5 to some extent. These findings indicated that anti-HB MAbs TYJ1 to TYJ4 are able to bind to mtHBsAg G145R with a sufficient affinity but still failed to recognize C121S, K122I, and T123N mtHBsAg.

IF staining of mtHBsAg with anti-HB MAbs. We examined whether mtHBsAg could be detected in IF with transfected cells, since only one MAb is needed for the assay (Fig. 4). Two anti-HB MAbs, TYJ1 and TYJ5, from this study and other commercially available anti-HBs were used for IF. Overall, the

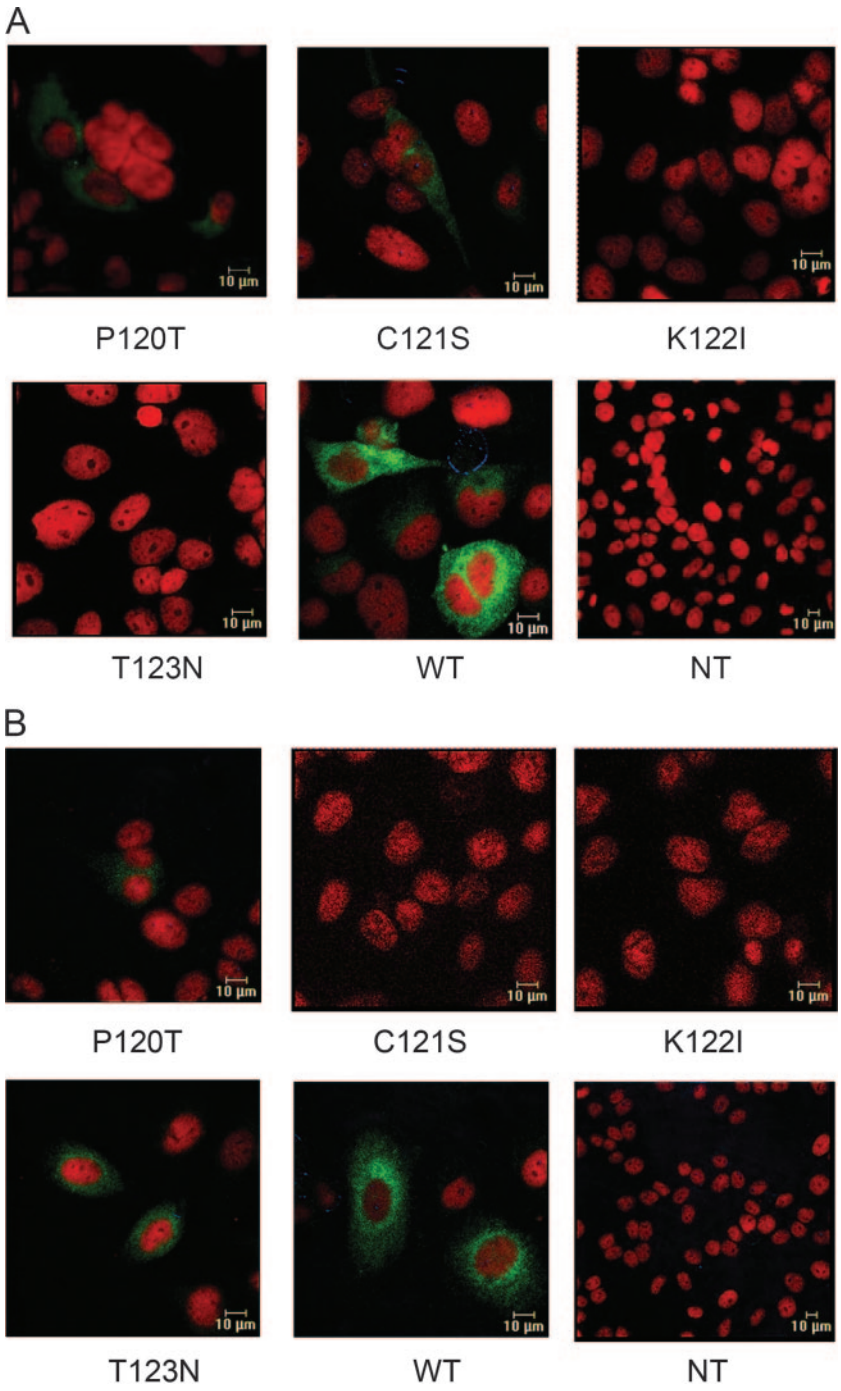

FIG. 4. IF staining of transfected cells expressing wild-type HBsAg and mtHBsAg with the MAbs TYJ1 (A) and TYJ5 (B). The cells were stained with propidium iodide for confocal laser microscopy. NT, negative control, untransfected cells; WT, wild type.

results were consistent with those generated by ELISAs. Figure 4 shows the IF staining of wild-type HBsAg and mtHBsAg with MAbs TYJ1 and TYJ5. Wild-type HBsAg was stained strongly by both MAbs. The mtHBsAg with P120T was positively stained by both anti-HB MAbs, but the intensity was reduced compared to that with the wild type. The mtHBsAg with C121S stained weakly positively by MAb TYJ1 but not by MAb TYJ5. Both MAbs failed to recognize the mtHBsAg with K122I. The mtHBsAg with T123N was not detected by IF staining using the MAb TYJ1. However, IF staining with TYJ5 showed a weak but clear perinuclear distribution of the mtHBsAg with T123N. Combined with the results generated by ELISAs for HBsAg detection, it could be assumed that the amino acid substitution T123N impaired the secretion of HBsAg, leading to an accumulation of mtHBsAg in cellular fractions.

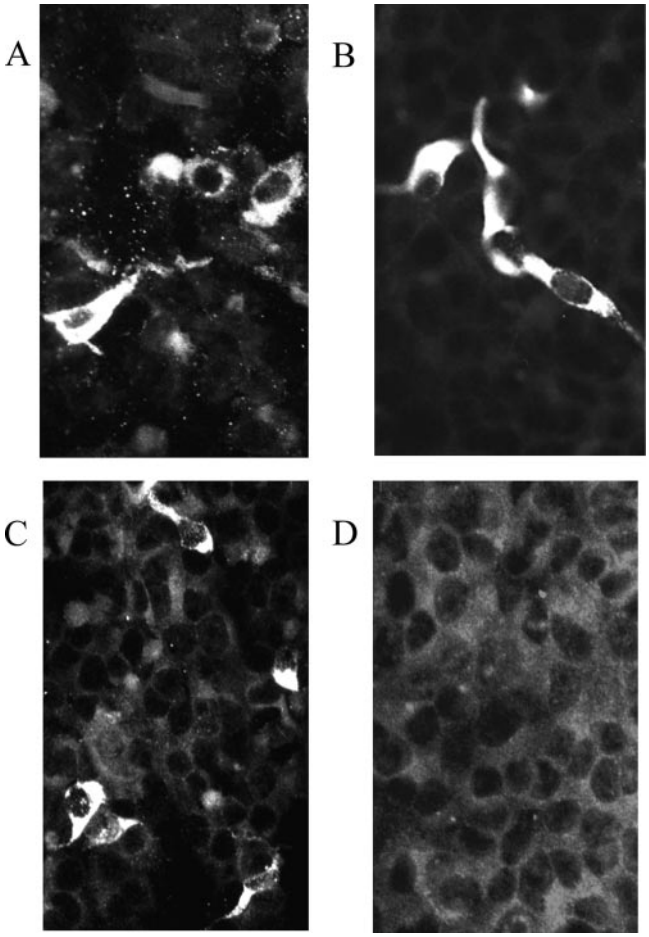

FIG. 5. IF staining of cells expressing mtHBsAg K122I in HepG2 cells. NC, negative control, untransfected cells. (A) Wild-type HBsAg, stained with polyclonal anti-HB antibodies; (B) wild-type HBsAg stained with MAb; (C) mtHBsAg K122I stained with polyclonal anti-HB antibodies; (D) mtHBsAg K122I stained with MAb.

The amino acid substitution K122I appeared to strongly affect the detectability of HBsAg. To verify the obtained results, we constructed an additional expression vector, pS2-HBsAg122I, for the mtHBsAg with the pre-S2 region based on another expression vector, pcDNA3. Again, the mtHBsAg were not detectable in culture supernatants of transfected cells (data not shown). IF staining with different MAbs failed to show the expression of the mtHBsAg with K122I in transfected cells. The presence of the mtHBsAg with K122I in transfected cells was demonstrated only by using a polyclonal anti-HB antibody (Fig. 5).

The naturally occurring mtHBsAg with amino acid substitutions within MHR 2 of HBsAg were not efficiently recognized by commercial ELISAs. A number of naturally occurring mtHBsAg were found in patients in different clinical settings (Table 1). Few of those mtHBsAg harbored amino acid substitutions at positions 120 and 123. Such amino acid substitutions frequently were accompanied by other amino acid substitutions. To verify the results obtained with the artificially constructed expression vectors for the mtHBsAg, a series of naturally occurring mtHBsAg were expressed by transient transfection, and their reactivity with anti-HB MAbs was investigated. The results are summarized in Table 3. The reactivity of a given mtHBsAg in ELISAs based on the anti-HB MAbs was expressed as the percentage of the reactivity of the wild-type HBsAg with the matched serotype. It could be seen that four MAbs, TYJ1 to TYJ4, were able to recognize the mtHBsAg with the amino acid substitution G145R, although the reactivity was reduced to a level of about 30% of that of the wild-type HBsAg control. Other MAbs showed a lower level of reactivity of less than 20% of that of the wild-type HBsAg control. The mtHBsAg HK188, with L98V and Q101R, was detected by all MAbs tested. In contrast, the mtHBsAg harboring amino acid substitutions within amino acids 120 to 123 were less efficiently recognized by MAbs. The mtHBsAg 1056sp, with P120S/S143L, was shown to be reactive with six MAbs with a level of reactivity that was 30% greater than that of the wild-type HBsAg control. BA2.4, with Y100C/P120T, was significantly less reactive. The mtHBsAg BA3.2 and BA 3.4 showed no or very low reactivity to all 14 MAbs, consistent with the previous results. Other mtHBsAg listed were effectively recognized by at least a few anti-HB MAbs, particularly by TYJ1 to TYJ4. Comparable results were obtained by testing mtHBsAg with the ARCHITECT HBsAg assay kit (data not shown). Taking these results together, the amino acid positions 120 to 123 of HBsAg are essential for HBsAg antigenicity, and the amino acid substitutions in this region may strongly reduce the recognition of HBsAg by anti-HB antibodies. Other mutations studied so far may have an impact on the HBsAg antigenicity, but the impact is less than those in the region of aa 120 to 123. As shown here, this is true for many anti-HB antibodies tested so far.

DISCUSSION

In the present work, we demonstrated that the amino acid substitutions within MHR 2 of HBsAg greatly impaired the antigenicity of HBsAg. Since many mutations found in HBV isolates occur in combinations, the influence of single mutations on HBsAg antigenicity could not be assessed properly. It is necessary to introduce single mutations into a common background to study their relevance. In the present work, we studied the antigenicity of HBsAg with single mutations and also that of the naturally occurring mtHBsAg harboring multiple mutations.

One surprising finding of our study is the essential role of R/K at position 122 for HBsAg antigenicity. Position 122 is known as an HBsAg subtype determinant (3, 31). The R/K substitutions at position 122 were identified in different chronically HBV-infected patients with antibody to HBV core protein as the only marker of infection (15, 20, 41). Another case was reported by Alexopoulou et al. (1). HBV variants with multiple amino acid substitutions, including K122I, emerged in a patient without detectable HBsAg, probably due to a reactivation of HBV under immunosuppressive treatment. However, the impact of the amino acid substitution K122I on the antigenicity of HBsAg was not clear in all cases, since other amino acid substitutions coexisted with K122I. Here, our data clearly indicated that mtHBsAg K122I was not detectable by a number of commercial ELISAs or anti-HB MAbs. While a few anti-HB MAbs with high affinity recognize mtHBsAg G145R,

TABLE 3. Reactivity of expressed wild-type HBsAg and mtHBsAg as determined by ELISAs based on anti-HB MAbs

HBsAg designation (subtype)	Amino acid substitution(s)	Reactivity to MAb[a]:													
		TYJ1	TYJ2	TYJ3	TYJ4	TYJ5	TYJ6	TYJ7	TYJ8	TYJ9	TYJ10	TYJ11	TYJ12	TYJ13	TYJ14
HK188 (adr)	L98V/Q101R	114.3	129.6	97.9	111.2	86.5	98.6	86.6	114.4	102.5	140	93.5	90.2	108.1	128.6
1056sp (ayw)	P120S/S143L	41.8	12.4	9.6	28.5	40.7	24.8	26.6	38.6	30.6	9.0	38.4	24.6	64.9	27.1
BA2.4 (ayw)	P120T	34.3	1.5	10.3	27.0	12.9	10.8	15.1	22.1	5.6	14.4	7.2	13.8	16.7	0.06
BA3.2 (ayw)	T123N/C124R	1.9	0.73	2.7	1.8	0.2	0.6	1.0	0.5	0.1	5.6	2.0	0.2	0.5	0.6
BA3.4 (ayw)	T123N	1.9	10.8	3.1	11.9	0.3	0.1	1.4	7.4	6.6	9.6	3.8	0.3	7.4	0.4
Ile122 (adw)[b]	R/K122I	0.3	0.2	1.3	0.6	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
C126 (adr)[b]	I126T/S143T	110.5	93.5	97.2	135.2	121.6	78.2	135.4	96.8	99.5	108.5	96.8	94.6	88.9	129.8
SA6 (ayw)	Q129R/G130N	69.5	98.8	60.8	103.5	45.4	39.3	71.4	87.8	63.6	50.4	61.2	69.6	87.1	0.1
SA4 (adw)	M133T	29.4	41.1	16.9	48.5	9.4	9.5	9.2	37.4	31.6	35.7	15.2	9.4	17.5	0.4
SA7 (ayw)	M133T	57.2	87.8	61.3	131.5	51.8	50.9	28.4	48.7	12.1	20.7	8.9	14.1	26.9	90.8
91-4696 (ayw)	T143S	93.5	92.6	78.9	119.7	100.5	70.4	68.9	108.1	55.1	48.7	110.9	84.6	76.8	97.1
AP3.1 (ayw)	D144A	104.4	116.1	100	86.3	83.1	98.5	92.9	50.4	77.2	26.3	90.0	27.8	42.3	1.2
Arg145yw[b]	G145R	63.0	52.0	42.4	40.7	20.2	10.4	0.68	3.9	3.8	4.6	1.0	0.5	3.2	0.06
Arg145dw[b]	G145R	50.7	34.9	38.8	50.2	12.0	14.2	1.4	1.2	2.9	7.1	1.0	0.6	2.9	7.4
Arg145dr[b]	G145R	94.9	67.2	45.9	34.2	7.9	10.4	1.52	1.9	1.2	9.1	4.5	3.5	3.6	8.3
Gly Dr	None	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Negative control		0.64	0.44	0.32	0.34	0.52	0.39	0.37	0.08	0.68	0.48	0.39	0.91	0.67	0.51

[a] Reactivity was measured as the OD of the mtHBsAg/the OD of the standard sequence of the same serotype × 100. NT, not tested.
[b] HBsAg sequences generated by in vitro site-directed mutagenesis.

none of the anti-HB MAbs showed a sufficient ability to detect mtHBsAg K122I. Thus, the amino acid substitution K122I has a major influence on HBsAg antigenicity.

Similar to the impact of the K122I substitution, the amino acid substitution T123N was shown to have a great impact on HBsAg antigenicity. However, our data suggested that the HBsAg harboring T123N also may be secretion defective, since two MAbs and polyclonal antibodies clearly detected mtHBsAg T123N in lysates of transfected cells. IF staining with a MAb indicated that mtHBsAg T123N was accumulated in the perinuclear region.

By using synthetic peptides, Qiu et al. showed that aa 121 to 124 is a critical site for binding of an anti-HB MAb (33). The amino acid substitutions at these positions strongly reduced the MAb binding, while the changes at aa 117 to 119 and aa 125 to 127 had no effect.

Previous studies suggested that other changes at aa 122 to 123 of HBsAg impair HBsAg antigenicity. HBV isolates with insertions at aa 122 and 123 were found by different groups (5, 20, 42). Such mtHBsAg were produced as recombinant proteins and show reduced reactivity in immunoassays (34).

The amino acid substitutions proline to threonine and proline to serine were found at position 120. These amino acid substitutions generally reduced the binding of mtHBsAg to anti-HB antibodies. However, mtHBsAg remained detectable by ELISAs or by IF staining. In agreement with this, the amino acid substitution P120T was found in the HBsAg sequence from patients positive for HBsAg (41).

The amino acid substitutions at C121 had been examined previously (2, 28, 29, 38). As shown here, mtHBsAg C121S was recognized by only one of seven commercial ELISAs and 1 of 14 MAbs tested in this study. These results confirmed the previous findings of other groups that C121 is required for the formation of intramolecular disulfide bonds and therefore is important for the conformation of HBsAg.

Taking these results together, MHR 2 harbors amino acid residues that are crucial for the antigenicity of HBsAg. None of the other regions was shown to have such significance, though single-amino-acid substitutions like G145R may strongly influence the antigenicity of HBsAg. It will be interesting to study whether the amino acid substitutions in this region will influence the biological functions of HBsAg. Jaoudé and Sureau showed that internal deletions within MHR 2 were tolerated for secretion of subviral HBV particles but lead to the production of defective hepatitis delta virus particles (23). Additionally, an artificial amino acid substitution of R122A in HBsAg affected the infectivity of hepatitis delta virus particles, indicating that this position may be important for binding of viral particles to hepatocytes. It is likely that different mutations reduce the viral replication fitness and need to be characterized for their influence on viral replication.

Our previous study demonstrated by DNA immunization that the immunogenicity of mtHBsAg may be changed (44). At present, the immunogenicity of mtHBsAg is under investigation based on DNA immunization. The preliminary results show that not all mutations prevent an induction of anti-HB antibodies, although they have influence on the antigenicity. Thus, a systematic and careful examination is needed to assess the immunogenicity of mtHBsAg in the future.

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