JOURNAL OF VIROLOGY, Nov. 1991, p. 6024–6030
0022-538X/91/116024-07$02.00/0

Vol. 65, No. 11

Phosphorylation in the Carboxyl-Terminal Domain of the Capsid Protein of Hepatitis B Virus: Evaluation with a Monoclonal Antibody

A. MACHIDA,[1] H. OHNUMA,[1] F. TSUDA,[1] A. YOSHIKAWA,[1,2] Y. HOSHI,[1,2] T. TANAKA,[2] S. KISHIMOTO,[3] Y. AKAHANE,[4] Y. MIYAKAWA,[5] AND M. MAYUMI[6]*

Department of Immunology, the Kitasato Institute, Tokyo 108,[1] Japanese Red Cross Blood Center, Saitama-ken 338,[2] Department of Public Health, Hamamatsu University School of Medicine, Shizuoka-ken 431-31,[3] the First Department of Internal Medicine, Yamanashi Medical College, Yamanashi-ken 409-38,[4] Mita Institute, Tokyo 108,[5] and Immunology Division, Jichi Medical School, Minamikawachi-machi, Tochigi-ken 329-04,[6] Japan

Received 1 April 1991/Accepted 15 August 1991

The capsid protein of hepatitis B virus ($p21^c$) is made of 183 amino acids coded for by the *C* gene. By using $p21^c$ isolated from Dane particles (hepatitis B virus) as an immunogen, a monoclonal antibody (no. 2212) which recognized an epitope dependent on the phosphorylation of $p21^c$ was raised. The binding of no. 2212 antibody to authentic $p21^c$ was completely inhibited by a synthetic undecapeptide with a sequence of RRRSQSPRRRR, representing amino acids 165 to 175 of $p21^c$, only when the peptide was phosphorylated. Either or both of Ser-168 and Ser-170 were phosphorylated in $p21^c$ in vivo, therefore, and contributed to the manifestation of the epitope. No. 2212 antibody bound to $p21^c$ from core particles derived from Dane particles or hepatocellular carcinoma tissues (PLC/342) propagated in nude mice but did not bind to $p21^c$ from core particles expressed in *Escherichia coli* or yeast cells, indicating different states of phosphorylation in them. Nonphosphorylated $p21^c$ showed a higher affinity for the viral DNA than did phosphorylated $p21^c$. Since the serum from an asymptomatic carrier, with a high titer for antibody to hepatitis B core antigen, specifically bound to phosphorylated undecapeptide (amino acids 165 to 175), the epitope would stimulate humoral antibody responses in the human host.

Hepatitis B virus (HBV) is a small DNA virus, in the family *Hepadnaviridae*, made of a nucleocapsid covered with a lipoprotein coat. The nucleocapsid is composed of unit protein ($p21^c$), encoded by the *C* gene, and contains viral DNA with a protein covalently bound to the 5′ terminus of minus strand DNA as well as to DNA polymerase (28).

$p21^c$ is coded for by the *C* gene and made of 183 amino acid residues. The carboxyl-terminal domain of $p21^c$, spanning 34 residues, is rich in arginine and can bind with nucleic acids (5, 17). Antigenic sites on the carboxyl-terminal domain of $p21^c$ have been identified, one of which is mimicked by a synthetic decapeptide (amino acids 150 to 159) and is immunogenic in human hosts, stimulating humoral antibody responses (11).

$p21^c$ can be phosphorylated both in vivo (20) and in vitro (1, 3, 6), with sites of phosphorylation in the carboxyl-terminal domain (21). Seven serine residues are present in the carboxyl-terminal domain, irrespective of HBV subtypes (4, 15, 16, 29), some of which are phosphorylated by endogenous protein kinase (3, 6, 20). It is not clear which serine residues are phosphorylated, however, nor is the effect of phosphorylation on the expression of epitopes borne by the carboxyl-terminal domain known.

A monoclonal antibody was raised against authentic $p21^c$ purified from cores of Dane particles (HBV virions), which recognized an epitope dependent on phosphorylation of the carboxyl-terminal domain. By using this monoclonal antibody as a probe, the state of phosphorylation was evaluated in core particles from Dane particles or those produced by primary hepatocellular carcinoma tissues propagated in nude mice (12), as well as in core particles produced by *Escherichia coli* or yeast cells harboring the recombinant *C* gene. The epitope, generated by phosphorylation of serine residues, was narrowed down on a synthetic undecapeptide representing amino acids 165 to 175 of the *C* gene product. Furthermore, antibodies reactive with this epitope were tested for in sera from persistent carriers of HBV with high titers of antibody to hepatitis B core antigen (anti-HBc).

MATERIALS AND METHODS

Core particles and HBV capsid proteins. Four preparations of core particles were used. Authentic core particles were isolated from Dane particles derived from plasma of asymptomatic carriers by the previously described method (27). Core particles were obtained also from nude mouse tumor cells (PLC/342); the tumor had been propagated from liver tissues from a patient with primary hepatocellular carcinoma and positive for hepatitis B surface antigen in serum and serially transplanted in nude mice (12). Core particles were expressed in *E. coli* harboring plasmids to which the *C* gene sequence was ligated downstream of the alkaline phosphatase gene promoter (14). Core particles expressed in yeast cells were obtained from Kaketsu-Ken (Tokyo, Japan).

$p21^c$ was liberated from each preparation of core particles by treatment with 1% (wt/vol) sodium dodecyl sulfate (SDS) and 5% (vol/vol) 2-mercaptoethanol at 100°C for 5 min and purified by electrophoresis on 13% (wt/vol) polyacrylamide gel in the presence of SDS (SDS-PAGE). p15.5 and p16.5 were obtained from Dane particle cores that had been prepared in the presence of pronase E and isolated on

* Corresponding author.

SDS-PAGE (25); p15.5 represents amino acids 1 to 147 of the *C* gene product (26).

Phosphorylation and dephosphorylation of core proteins and peptides. Core particles were phosphorylated with ^{32}P from [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham, Buckinghamshire, England) by means of endogenous protein kinase after the method of Albin and Robinson (1). They were then broken into constituent polypeptides to obtain p21^c radiolabeled with ^{32}P. Phosphorylation of synthetic oligopeptides was performed after they had been immobilized on a solid support. Wells of an immunoplate (Nunc, Roskilde, Denmark) received 50 μl of phosphate buffer (10 mM, pH 8.0) containing 10 μg of peptides per ml and were left at 4°C overnight. The plate was washed with distilled water, and then each well received 50 μl of potassium phosphate buffer (50 mM, pH 7.5) containing 20 mM dithiothreitol, 20 mM $MgSO_4$, 40 μM cyclic AMP (cAMP), 300 μg of ATP per ml, and 250 U of bovine heart protein kinase (Sigma Chemicals, St. Louis, Mo.) per ml.

Phosphorylation of oligopeptides in solution was carried out by incubating peptide (15 mg/ml) in potassium phosphate buffer (50 mM, pH 7.5) at room temperature overnight in the presence of 20 mM dithiothreitol–20 mM $MgSO_4$–40 μM cAMP–10 mg of ATP per ml–1,250 U of bovine heart protein kinase per ml.

For dephosphorylation, p21^c in glycine-HCl buffer (0.1 M, pH 10) containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$ was incubated in the presence of alkaline phosphatase from bovine intestinal mucosa (35 U/ml; Sigma) at room temperature overnight. Peptides immobilized onto wells were dephosphorylated in situ by incubation with the above solution.

Monoclonal antibodies. Each of two female BALB/c mice, 6 weeks of age, received intraperitoneally 100 μl of a solution of p21^c emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). They were given the same inoculum 3 weeks later and an inoculation with 10 μg of p21^c in 100 μl of saline intravenously after an additional 4 weeks. Spleen cells were harvested 3 days after the final inoculation and fused with NS-1 cells by means of polyethylene glycol.

Hybridomas producing antibodies that recognized the phosphorylation in the carboxyl-terminal domain of p21^c were selected by the following procedure. Wells of a microtiter plate received a solution of authentic p21^c (150 ng/ml) in phosphate buffer (10 mM, pH 8.0) and then were saturated with buffer supplemented with 40% (vol/vol) fetal calf serum. In half of the wells, immobilized p21^c was dephosphorylated in situ. Culture medium (50 μl) was delivered to a well coated with either untreated (phosphorylated) p21^c or dephosphorylated p21^c, and captured antibodies were detected by rabbit anti-mouse immunoglobulin labeled with horseradish peroxidase. Hybridomas that produced antibodies capable of binding to untreated p21^c but not to dephosphorylated p21^c were cloned. A clone producing desired antibodies (no. 2212) was propagated in the peritoneal cavities of mice pretreated with pristane (Wako Pure Chemicals, Osaka, Japan). Ascites fluid was harvested 7 to 10 days after inoculation, and antibodies were precipitated with 2 M ammonium sulfate.

Another monoclonal antibody (no. 2221) was raised against reduced and alkylated p21^c and recognized the determinant b of hepatitis B e antigen (anti-HBe/b [8]).

Splitting of p21^c with formic acid. p21^c was incubated in the presence of 70% (vol/vol) formic acid and 0.1% (wt/vol) SDS at 37°C for 48 h in order to cleave it at the peptide bond between Asp-4 and Pro-5, as well as between Asp-78 and Pro-79 (18). The obtained molecular fragments were separated by SDS-PAGE for staining proteins and immunoblotting.

Synthetic p21^c peptides. Four synthetic peptides were prepared by the solid-phase method of Merrifield (13), which copied parts of the arginine-rich carboxyl-terminal domain of p21^c (amino acids 150 to 183) as deduced from an HBV DNA clone of subtype *adr* (16). They were a 23-mer oligopeptide with a sequence of PSPRRRRSQSPRRRRSQSRESQC representing amino acids 161 to 183, a dodecapeptide (PSPRR RRSQSPR, representing amino acids 161 to 172), an undecapeptide (RRRSQSPRRRR, representing amino acids 165 to 175), and another undecapeptide (RRRSQSRESQC, representing amino acids 173 to 183).

Solid-phase enzyme immunoassays. Antigenic sites of p21^c and synthetic oligopeptides were determined after they had been immobilized on a solid support. Wells of an immunoplate were coated with authentic p21^c (150 ng/ml) in phosphate buffer, and some of the wells were dephosphorylated in situ. Wells of another immunoplate were coated with oligopeptides (amino acids 161 to 183; 10 μg/ml), and some of them were phosphorylated. Unsaturated binding sites on plates were quenched with 40% fetal calf serum in buffer. Wells then received 50 μl of buffer supplemented with 30% fetal calf serum and containing 10 ng of no. 2212 antibody labeled with horseradish peroxidase. The plate was incubated at room temperature for 1 h and washed. Thereafter, antibodies bound to a well were determined by colorimetry.

Hepatitis B core antigen (HBcAg) determinants on core particles and p21^c in solution were detected by sandwiching them between monoclonal anti-HBc antibody with a specificity of α (anti-HBc/α, monoclonal antibody no. 3105 [26]) fixed on a solid support and either monoclonal antibody no. 2212 or monoclonal anti-HBc antibody with a specificity of β (anti-HBc/β, no. 3120 [26]) labeled with horseradish peroxidase.

Immunoblotting. Polypeptides separated by SDS-PAGE were transferred to a nitrocellulose membrane. The membrane was immersed in Tris-HCl buffer (10 mM, pH 7.5) containing 0.15 M NaCl and 0.5% (vol/vol) Tween 80 and then incubated at room temperature for 30 min with monoclonal antibody labeled with horseradish peroxidase (8 μg/ml). The membrane was immersed in the buffer at room temperature for 30 min, and bound antibodies were visualized by a Konica immunostain kit (Konica Corp., Tokyo, Japan).

Binding with DNA. The nitrocellulose membrane with transferred polypeptides was washed with Tris-HCl buffer (10 mM, pH 7.0) containing 1 mM EDTA, 0.02% bovine serum albumin, 0.02% (vol/vol) Ficoll, 0.02% (wt/vol) polyvinylpyrrolidone, and 50 mM NaCl. It was then incubated with the *Bam*HI digest of HBV DNA labeled with [α-^{32}P]dCTP. After incubation, the membrane was washed in the buffer, dried, and subjected to autoradiography.

Serological tests for markers of HBV infection. Hepatitis B surface antigen was determined by reversed passive hemagglutination with a commercial assay kit (Mycell; Institute of Immunology Co., Ltd., Tokyo, Japan). Hepatitis B e antigen and anti-HBe were determined by enzyme immunoassay kits (IMMUNIS HBeAg/Ab EIA; Institute of Immunology). Anti-HBc was determined by hemagglutination inhibition (23). The results of hemagglutination tests were expressed by the highest twofold dilution (2^N) of serum that induced a positive reaction.

Antibodies directed to the phosphorylated carboxyl-termi-

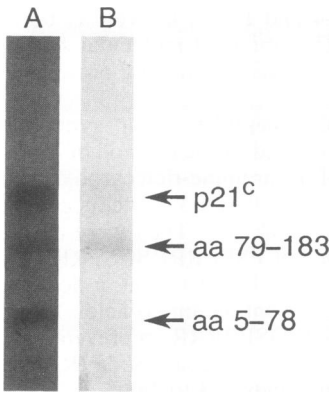

FIG. 1. Reactivity of amino-terminal and carboxyl-terminal fragments of p21c with no. 2212 antibody. Two fragments of p21c representing amino acids 5 to 78 and 79 to 183 were obtained by treatment with formic acid. They were separated by SDS-PAGE, stained for protein with silver (A), and immunoblotted for reactivity with no. 2212 antibody (B). aa, amino acids.

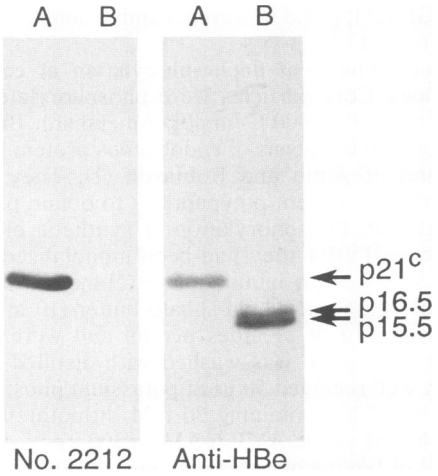

FIG. 2. Failure of p16.5 and p15.5 to bind with no. 2212 antibody. p21c (A) and p16.5 and p15.5 (B) were run on SDS-PAGE and evaluated for reactivity with no. 2212 antibody or monoclonal anti-HBe antibody of specificity b by immunoblotting.

nal domain of p21c. Five human serum samples from asymptomatic carriers, which contained anti-HBc in high titers ($>2^{10}$) and were positive for anti-HBe, but which did not contain detectable antibodies against the epitope expressed by amino acids 150 to 159 in the carboxyl-terminal domain of p21c (11), were selected. They were tested for binding with immobilized 23-mer peptide (amino acids 161 to 183), either untreated (nonphosphorylated) or phosphorylated, by the solid-phase enzyme immunoassay.

The specificity for the binding was confirmed by an inhibition test. A 25-μl portion of diluted sera was mixed with an equal volume of phosphate buffer supplemented with 30% fetal calf serum and containing synthetic undecapeptide (amino acids 165 to 175, 1 mg/ml), either nonphosphorylated or phosphorylated, and incubated at 4°C overnight. The mixture was then delivered to a well of the immunoplate coated with 150 ng of p21c per ml, and the plate was incubated at room temperature for 1 h. Another 25-μl portion of diluted sera was mixed with 25 μl of buffer supplemented with 30% fetal calf serum and processed similarly. Thereafter, bound antibodies were determined by colorimetry, and the percent inhibition was obtained by the following equation: (absorbance of buffer) − (absorbance of inhibitor)/(absorbance of buffer) × 100.

RESULTS

Localization of antigenic epitope recognizable by a monoclonal antibody (no. 2212) in the carboxyl-terminal domain of p21c. Monoclonal antibody (no. 2212) that bound to the authentic p21c molecules as they were phosphorylated, but not to them after they had been dephosphorylated, was produced. The epitope recognized by no. 2212 antibody was searched for in p21c obtained from Dane particles.

p21c was cut at peptide bonds between Asp and Pro by treatment with formic acid (18). The two fragments obtained, representing amino acids 5 to 78 and 79 to 183, were separated by SDS-PAGE and then evaluated for the binding with no. 2212 antibody (Fig. 1). Only the carboxyl-terminal fragment (amino acids 79 to 183) bound with the antibody. p15.5 or p16.5, representing amino acids 1 to 147 of p21c (25, 26), did not bind with no. 2212 antibody in immunoblotting (Fig. 2). On the basis of these results, the epitope recognized by no. 2212 antibody was deduced to localize in the carboxyl-terminal domain of p21c.

The antigenic determinant dependent on the phosphorylation of p21c and located in the carboxyl-terminal domain of p21c, which was recognized by no. 2212 monoclonal antibody, will henceforth be referred to as the 2212 epitope.

Generation of the 2212 epitope on synthesized and phosphorylated oligopeptides. A 23-mer peptide with a sequence of PSPRRRRSQSPRRRRSQSRESQC, representing amino acids 161 to 183 of p21c, was chemically synthesized and fixed on a solid support. The immobilized peptide, either as it was (nonphosphorylated) or after phosphorylation, was tested for binding with no. 2212 antibody. The phosphorylated peptide strongly bound to no. 2212 antibody with an A_{492} value of 1.2 in enzyme immunoassay, while nonphosphorylated peptide did not ($A_{492} < 0.2$). When the phosphorylated peptide was dephosphorylated in situ, however, it lost the activity to bind with no. 2212 antibody ($A_{492} < 0.2$).

For the purpose of narrowing down the 2212 epitope, three oligopeptides representing parts of the peptide (amino acids 161 to 183) were synthesized. They were tested, before or after phosphorylation, for the ability to interfere with the binding of no. 2212 antibody to immobilized p21c (Table 1). The undecapeptide with a sequence of RRRSQSPRRRR (amino acids 165 to 175 of p21c), when phosphorylated,

TABLE 1. Inhibition on the binding of authentic p21c to no. 2212 antibody by synthetic oligopeptides[a]

Position in p21c	Amino acid sequences of oligopeptides	Inhibition (%) by oligopeptides	
		Untreated	Phosphorylated
161–172	PSPRRRRSQSPR	4	5
165–175	RRRSQSPRRRR	5	100
173–183	RRRSQSRESQC	4	0

[a] p21c isolated from Dane particle cores was immobilized on a solid support and incubated with monoclonal antibody no. 2212 in the presence of various synthetic oligopeptide, either untreated or after phosphorylation. The bound antibody was determined by colorimetry, and the extent of inhibition is expressed in percent.

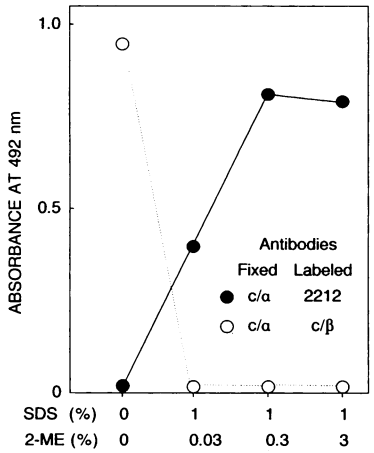

FIG. 3. Binding of monoclonal antibody (no. 2212) with p21^c. Core particles from Dane particles, before or after treatment with SDS and 2-mercaptoethanol, were sandwiched between monoclonal anti-HBc antibody of specificity α (anti-HBc/α) and either monoclonal anti-HBc antibody of specificity β (anti-HBc/β) or no. 2212 antibody labeled with horseradish peroxidase.

completely inhibited the binding; nonphosphorylated undecapeptide did not show any appreciable inhibitory activity. The other two oligopeptides, with sequences of PSPRRRR SQSPR (amino acids 161 to 172) and RRRSQSRESQC (amino acids 173 to 183), did not show any inhibitory activity before or after they had been phosphorylated.

Unavailability of the 2212 epitope on the surface of Dane particle cores. Core particles derived from Dane particles were treated with 1% SDS and 0.03 to 3% 2-mercaptoethanol and, along with untreated core particles, tested for antigenic determinants by being sandwiched between monoclonal anti-HBc antibody of specificity α and either monoclonal antibody no. 2212 or monoclonal anti-HBc antibody of specificity β (Fig. 3). Intact core particles, with both HBcAg/α and HBcAg/β determinants, did not exhibit the ability to bind with no. 2212 antibody, thereby indicating that no antigenic sites for the antibody were available on their surface. The ability to bind with no. 2212 antibody emerged when core particles were disrupted into p21^c. Since p21^c possesses the HBcAg/α but not the HBcAg/β determinant (26), it was not sandwiched between anti-HBc/α and anti-HBc/β. These results indicated that the 2212 epitope on p21^c would be buried inside the core particle.

Binding of no. 2212 antibody to p21^c preparations of various origins. Core particles derived from one or another of Dane particles, nude mouse tumor cells, *E. coli*, and yeast cells were split into p21^c by treatment with SDS and 2-mercaptoethanol and run on SDS-PAGE. Proteins were eluted from gel slices, immobilized on a solid support, and then evaluated for the binding with no. 2212 antibody (Fig. 4). Dane particle p21^c and nude mouse p21^c bound with no. 2212 antibody, while *E. coli* p21^c or yeast p21^c did not. The availability of p21^c on a solid support was ascertained by the binding with monoclonal anti-HBe antibody of specificity b for all four p21^c preparations.

In vitro phosphorylation of HBV core particles of various origins. Four different preparations of core particles obtained from various sources were phosphorylated with ^{32}P. They were then broken into p21^c and subjected to SDS-PAGE, and phosphate acceptors were visualized by autoradiography. Core particles isolated from Dane particles in

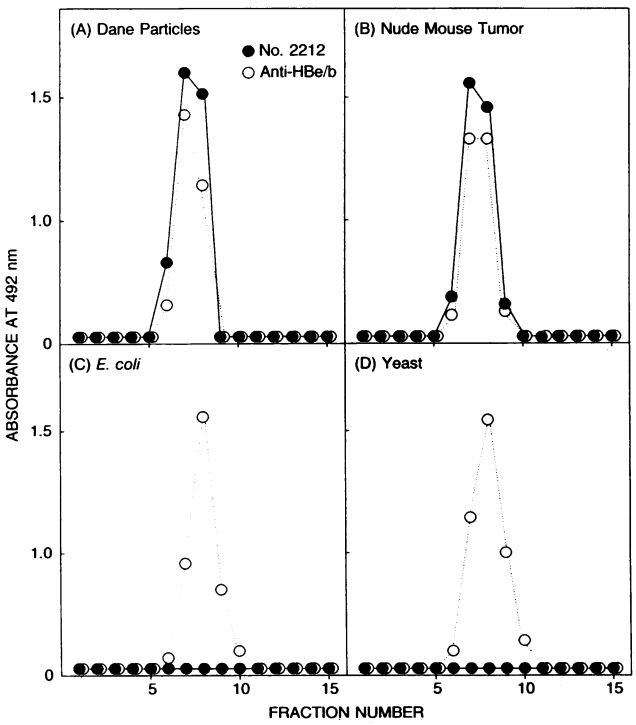

FIG. 4. Binding of p21^c from various sources with no. 2212 antibody. Core particles from Dane particles (A), nude mouse tumor cells (B), *E. coli* (C), and yeast cells (D) were broken into p21^c and subjected to SDS-PAGE. p21^c was eluted from gel slices, fixed on a solid support, and then tested for binding with no. 2212 antibody or monoclonal anti-HBe antibody of specificity b labeled with horseradish peroxidase.

plasma from asymptomatic carriers and those obtained from nude mouse tumor cells were phosphorylated, while recombinant core particles expressed in *E. coli* or yeast cells were not (Fig. 5). These results were in agreement with the failure

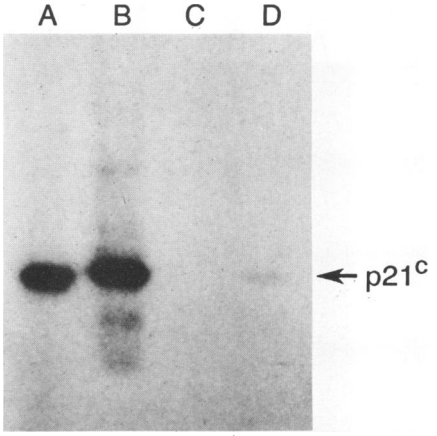

FIG. 5. In vitro phosphorylation of core particles. Core particles obtained from Dane particles (A), liver tissues from a patient with hepatocellular carcinoma propagated in nude mice (B), an expression system for recombinant *C* gene in *E. coli* (C), or yeast cells (D) were phosphorylated with ^{32}P. They were broken into p21^c and subjected to SDS-PAGE, and the state of phosphorylation was evaluated by autoradiography.

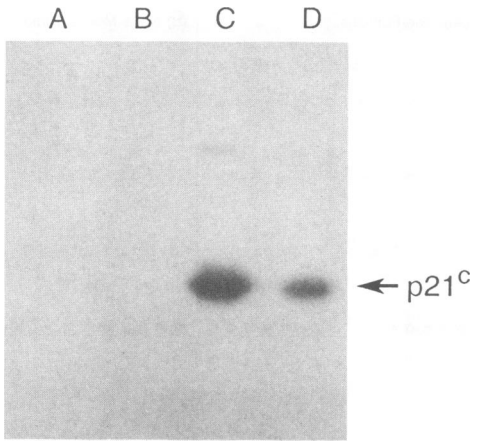

FIG. 6. Binding of p21c of various origins with radiolabeled HBV DNA. p21c from Dane particles (A), nude mouse tumor cells (B), *E. coli* (C), or yeast cells (D) harboring the *C* gene were run on SDS-PAGE, transferred to a nitrocellulose membrane, and tested for binding with radiolabeled HBV DNA.

of p21c from *E. coli* or yeast cells to bind with no. 2212 antibody, which recognized the phosphorylated state of the carboxyl-terminal domain.

Binding of p21c preparations of various origins with radiolabeled HBV DNA. Four p21c preparations derived from core particles of various origins were run on SDS-PAGE, transferred to a nitrocellulose membrane, and then tested for the capacity to bind radiolabeled HBV DNA by autoradiography (Fig. 6). *E. coli* and yeast p21c bound radiolabeled HBV DNA, whereas Dane particle or nude mouse p21c did not show any appreciable binding.

Dane particle p21c was dephosphorylated and, along with untreated p21c, tested for the binding with radiolabeled HBV DNA (Fig. 7). The capacity of Dane particle p21c to bind radiolabeled HBV DNA was markedly increased after dephosphorylation.

Test for antibodies against the 2212 epitope in human sera. The carboxyl-terminal domain of p21c contains antigenic

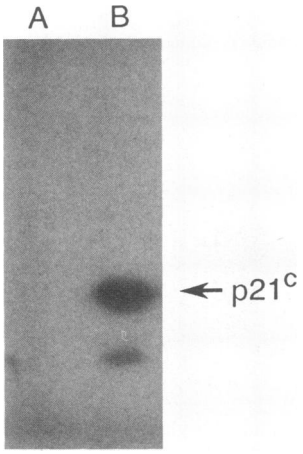

FIG. 7. Binding of the authentic p21c with radiolabeled HBV DNA. p21c obtained from Dane particle cores, either untreated (A) or after dephosphorylation (B), was run on SDS-PAGE, transferred to a nitrocellulose membrane, and tested for binding with radiolabeled HBV DNA.

TABLE 2. Binding of human antibodies with the 2212 epitope[a]

Serum sample no.	Binding with 23-mer peptide[b]		Inhibition (%) of binding with p21c by undecapeptide[c]	
	Untreated	Phosphorylated	Untreated	Phosphorylated
1	14	38	−2	−10
2	10	17	1	−6
3	14	126	0	42
4	9	13	4	6
5	7	9	3	6

[a] Five human serum samples with antibodies against the carboxyl-terminal domain of p21c, which were not absorbed with the decapeptide representing amino acids 150 to 159 (11), were tested for the ability to bind with the epitope dependent on phosphorylation of p21c (the 2212 epitope).

[b] Untreated or phosphorylated 23-mer peptide (amino acids 161 to 183) was immobilized on a solid support and tested for binding with human antibodies. A_{492} readings (10^3) are shown for visual clarity.

[c] Binding of human antibodies to Dane particle p21c (phosphorylated), fixed on a solid support at a concentration of 150 ng/ml, was inhibited by untreated or phosphorylated undecapeptide (amino acids 165 to 175).

epitopes to which human hosts raise humoral antibodies (12), and one of them is identified on a synthetic decapeptide with a sequence of RRRGRSPRRR representing amino acids 150 to 159 of the *C* gene product. Five human serum samples, which contained antibodies directed to the arginine-rich carboxyl-terminal domain of p21c that were not absorbed with the decapeptide (amino acids 150 to 159), were evaluated for the binding with phosphorylated undecapeptide (amino acids 165 to 175) bearing the 2212 epitope.

Antibodies in one of the five serum samples (no. 3 in Table 2) bound to phosphorylated 23-mer peptide (amino acids 161 to 183) but not to its nonphosphorylated form. Furthermore, the binding to p21c of antibodies in this serum was inhibited by phosphorylated undecapeptide (amino acids 165 to 175) but not by its nonphosphorylated form. These results indicated that the 2212 epitope was presented to the immune system of the human host with the stimulation of humoral antibody responses.

DISCUSSION

The core of HBV is associated with protein kinase activity (1, 3, 6), capable of phosphorylating the arginine-rich, carboxyl-terminal domain of p21c (20, 21). The carboxyl-terminal domain of p21c represents amino acids 150 to 183 of the *C* gene product and contains seven serine residues (4, 15, 16, 29), some of which are phosphorylated by the protein kinase (3, 6, 20). Whether the protein kinase is coded for by HBV or host cells, however, is not established yet. Nor is it known which serine residues in the carboxyl-terminal domain of p21c are phosphorylated.

A monoclonal antibody (no. 2212) raised against authentic p21c was instrumental in analyzing the phosphorylation of the HBV capsid, because it recognized an epitope in the carboxyl-terminal domain of p21c that was dependent on the phosphorylation of serine residues. Dane particle cores did not bind with no. 2212 antibody, indicating the location of the epitope inside the viral capsid.

On the basis of a three-dimensional model for the molecular configuration of p21c, Argos and Fuller (2) predicted the four serine residues at positions 155, 162, 168, and 170 as candidates for phosphate acceptors. The binding of no. 2212 antibody to the undecapeptide with a sequence of RRRSQS PRRRR (amino acids 165 to 175 of p21c), only when it was phosphorylated, indicated that either or both of serine resi-

dues at positions 168 and 170 would be phosphorylated in the HBV capsid.

When four preparations of core particles of various origins were incubated with [γ-^{32}P]ATP, those from Dane particles and hepatocellular carcinoma tissues (PLC/342) propagated in nude mice incorporated radioactivity. In contrast, core particles expressed in *E. coli* or yeast cells harboring the recombinant *C* gene did not. From these results it was not determined whether p21^c molecules in core particles produced by expression systems in microbial cells were not phosphorylated or were phosphorylated to the full extent so that any additional phosphorus atoms were hardly accepted. The binding of no. 2212 antibody to p21^c from cores of Dane particles and those from the nude mouse tumor cells, but not to p21^c from cores expressed in *E. coli* or yeast cells, supported a nonphosphorylated state of p21^c produced in single-celled organisms.

The origin of the protein kinase, which copurifies with core particles (1, 3, 6) and which is responsible for phosphorylation of p21^c, is not clear. Implicated in the protein kinase activity are the *P* gene product as a hypothetical multifunctional precursor (6) and the *X* gene product (30), which is known to have a transcriptional *trans*-activating function (24); they both lack structural motifs common to protein kinases (7).

Core particles expressed in mammalian or insect cells are phosphorylated (9, 20). We could not find phosphorylated p21^c in core particles expressed in *E. coli*, however. Core particles of duck hepadnavirus expressed in *E. coli* are not phosphorylated, either (21). These observations are relevant to the fact that, unlike eukaryotes, prokaryotes are not dependent on phosphorylation for regulating protein synthesis (19).

These lines of evidence would favor a host origin of the protein kinase associated with hepadnavirus cores, like most protein kinases associated with viruses which are derived from host cells (10). The protein kinase associated with HBV core, however, would not be acquired by random incorporation of a cellular kinase. p21^c from core particles produced by the expression system in yeast cells was not phosphorylated, despite the availability of many serine/threonine kinases in yeast cells (7).

The carboxyl-terminal domain of p21^c binds HBV RNA and DNA (17, 20). The capacity of p21^c to bind HBV DNA was inversely related to the extent of phosphorylation. p21^c from *E. coli* or yeast cells had a high affinity for radiolabeled HBV DNA; its carboxyl-terminal domain was not phosphorylated as judged by the lack of reactivity with no. 2212 antibody. On the contrary, p21^c from Dane particles or nude mouse tumor cells bound to no. 2212 antibody but hardly with HBV DNA. This view is reinforced by the fact that dephosphorylation of p21^c from Dane particles restored the affinity with HBV DNA. Phosphorylation of capsid protein would affect its affinity with the viral genome for HBV (3, 21), as is proposed for Rausher murine leukemia virus (22), thereby regulating viral replication and transcription.

An antigen-antibody system involving the epitope generated by the phosphorylation of the carboxyl-terminal domain of p21^c may have applications in the immunology of HBV infection. We have described an antigenic determinant expressed by amino acids 150 to 159 of p21^c, to which persistently infected hosts raise humoral antibodies (11). One of five carriers with antibodies to the arginine-rich carboxyl-terminal domain of p21^c, but without antibodies directed to the epitope present on amino acids 150 to 159, disclosed antibodies against the phosphorylated undecapeptide (amino acids 165 to 175). The 2212 epitope, therefore, would be able to stimulate humoral antibody responses in the human host.

REFERENCES

1. **Albin, C., and W. S. Robinson.** 1980. Protein kinase activity in hepatitis B virus. J. Virol. **34**:297–302.
2. **Argos, P., and S. D. Fuller.** 1988. A model for the hepatitis B virus core protein: prediction of antigenic sites and relationship to RNA virus capsid proteins. EMBO J. **7**:819–824.
3. **Feitelson, M. A., P. L. Marion, and W. S. Robinson.** 1982. Core particles of hepatitis B virus and ground squirrel hepatitis virus. II. Characterization of the protein kinase reaction associated with ground squirrel hepatitis virus and hepatitis B virus. J. Virol. **43**:741–748.
4. **Galibert, F., E. Mandart, F. 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.
5. **Gallina, A., F. Bonelli, L. Zentilin, G. Rindi, M. Muttini, and G. Milanesi.** 1989. A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids. J. Virol. **63**:4645–4652.
6. **Gerlich, W. H., U. Goldmann, R. Müller, W. Stibbe, and W. Wolff.** 1982. Specificity and localization of the hepatitis B virus-associated protein kinase. J. Virol. **42**:761–766.
7. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241**:42–52.
8. **Imai, M., M. Nomura, T. Gotanda, T. Sano, K. Tachibana, H. Miyamoto, K. Takahashi, S. Toyama, Y. Miyakawa, and M. Mayumi.** 1982. Demonstration of two distinct antigenic determinants on hepatitis B e antigen by monoclonal antibodies. J. Immunol. **128**:69–72.
9. **Lanford, R. E., and L. Notvall.** 1990. Expression of hepatitis B virus core and precore antigens in insect cells and characterization of a core-associated kinase activity. Virology **176**:222–233.
10. **Leader, D. P., and M. Katan.** 1988. Viral aspects of protein phosphorylation. J. Gen. Virol. **69**:1441–1464.
11. **Machida, A., H. Ohnuma, E. Takai, F. Tsuda, T. Tanaka, M. Naito, E. Munekata, Y. Miyakawa, and M. Mayumi.** 1989. Antigenic sites on the arginine-rich carboxyl-terminal domain of the capsid protein of hepatitis B virus distinct from hepatitis B core or e antigen. Mol. Immunol. **26**:413–421.
12. **Matsui, T., M. Takano, K. Miyamoto, Y. Itoh, H. Yoshizawa, M. Koike, T. Mochizuki, E. Tanaka, H. Okamoto, M. Imai, S. Mishiro, Y. Miyakawa, and M. Mayumi.** 1986. Nude mice bearing human primary hepatocellular carcinoma that produces hepatitis B surface, core, and e antigens, as well as deoxyribonucleic acid polymerase. Gastroenterology **90**:135–142.
13. **Merrifield, R. B.** 1969. Solid-phase peptide synthesis. Adv. Enzymol. **32**:221–296.
14. **Oka, T., S. Sakamoto, K. Miyoshi, T. Fuwa, K. Yoda, M. Yamasaki, G. Tamura, and T. Miyake.** 1985. Synthesis and secretion of human epidermal growth factors by *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82**:7212–7216.
15. **Okamoto, H., M. Imai, M. Shimozaki, Y. Hoshi, H. Iizuka, T. Gotanda, F. Tsuda, Y. Miyakawa, and M. Mayumi.** 1986. Nucleotide sequence of a cloned hepatitis B virus genome, subtype *ayr*: comparison with genomes of the other three subtypes. J. Gen. Virol. **67**:2305–2314.
16. **Ono, Y., H. Onda, R. Sasada, K. Igarashi, Y. Sugino, and K. Nishioka.** 1983. The complete nucleotide sequences of the cloned hepatitis B virus DNA; subtype adr and adw. Nucleic Acids Res. **11**:1747–1757.
17. **Petit, M.-A., and J. Pillot.** 1985. HBc and HBe antigenicity and DNA-binding activity of major core protein P22 in hepatitis B virus core particles isolated from the cytoplasm of human liver cells. J. Virol. **53**:543–551.
18. **Piszkiewicz, D., M. Landon, and E. L. Smith.** 1970. Anomalous cleavage of aspartyl-proline peptide bonds during amino acid sequence determinations. Biochem. Biophys. Res. Commun. **40**:1173–1178.

19. **Rickenberg, H. V., and B. H. Leichtling.** 1987. Protein phosphorylation in prokaryotes and single-celled eukaryotes, p. 419–445. *In* P. D. Boyer and E. G. Krebs (ed.), The enzymes, vol. XVIII. Academic Press, Inc., Orlando, Fla.
20. **Roossinck, M. J., and A. Siddiqui.** 1987. In vivo phosphorylation and protein analysis of hepatitis B virus core antigen. J. Virol. **61**:955–961.
21. **Schlicht, H.-J., R. Bartenschlager, and H. Schaller.** 1989. The duck hepatitis B virus core protein contains a highly phosphorylated C terminus that is essential for replication but not for RNA packaging. J. Virol. **63**:2995–3000.
22. **Sen, A., C. J. Sherr, and G. J. Todaro.** 1977. Phosphorylation of murine type C viral p12 proteins regulates their extent of binding to the homologous viral RNA. Cell **10**:489–496.
23. **Shimizu, M., M. Ohyama, Y. Takahashi, K. Udo, M. Kojima, M. Kametani, F. Tsuda, E. Takai, Y. Miyakawa, and M. Mayumi.** 1983. Immunoglobulin M antibody against hepatitis B core antigen for the diagnosis of fulminant type B hepatitis. Gastroenterology **84**:604–610.
24. **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.
25. **Takahashi, K., M. Imai, T. Gotanda, T. Sano, A. Oinuma, S. Mishiro, Y. Miyakawa, and M. Mayumi.** 1980. Hepatitis B e antigen polypeptides isolated from sera of individuals infected with hepatitis B virus: comparison with HBeAg polypeptide derived from Dane particles. J. Gen. Virol. **50**:49–57.
26. **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.** 1983. Immunochemical structure of hepatitis B e antigen in the serum. J. Immunol. **130**:2903–2907.
27. **Takahashi, T., S. Nakagawa, T. Hashimoto, K. Takahashi, M. Imai, Y. Miyakawa, and M. Mayumi.** 1976. Large-scale isolation of Dane particles from plasma containing hepatitis B antigen and demonstration of a circular double-stranded DNA molecule extruding directly from their cores. J. Immunol. **117**:1392–1397.
28. **Tiollais, P., C. Pourcel, and A. Dejean.** 1985. The hepatitis B virus. Nature (London) **317**:489–495.
29. **Valenzuela, P., M. Quiroga, J. Zaldivar, P. Gray, and W. J. Rutter.** 1980. The nucleotide sequence of the hepatitis B viral genome and the identification of the major viral genes, p. 57–70. *In* B. N. Fields, R. Jaenisch, and C. F. Fox (ed.), Animal virus genetics. Academic Press, Inc., New York.
30. **Wu, J. Y., Z.-Y. Zhou, A. Judd, C. A. Cartwright, and W. S. Robinson.** 1990. The hepatitis B virus-encoded transcriptional *trans*-activator hbx appears to be a novel protein serine/threonine kinase. Cell **63**:687–695.