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Hepatitis B Virus Polypeptide X: Expression in *Escherichia coli* and Identification of Specific Antibodies in Sera from Hepatitis B Virus-Infected Humans

MICHAEL L. MEYERS,[1] LUDMILLA VITVITSKI TREPO,[2] NRAPENDRA NATH,[3] AND JOHN J. SNINSKY[1,4]†*

Department of Molecular Biology[1] and Department of Microbiology and Immunology,[4] Albert Einstein College of Medicine, Bronx, New York 10461; Institut National de la Santé et de la Recherche Medicale, Unite de Recherche fur les Hepatites, Faculté de Medècine Alexis Carrel, 69372 Lyon, France[2]; and American Red Cross, Biomedical Research and Development Laboratories, Bethesda, Maryland 20014[3]

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Sequence analysis of the hepatitis B virus (HBV) genome revealed the presence of an open reading frame (ORF X) which has the potential to encode a 154-amino acid polypeptide. A fusion protein containing 145 of the amino acids encoded by ORF X and 8 amino acids of β-galactosidase was expressed and characterized in bacterial extracts. Immunoprecipitations with the ORF X fusion protein as a radioactively labeled antigen were performed to screen sera of humans infected with HBV for the presence of antibodies against ORF X-encoded determinants (anti-X). Such antibodies were identified in 9 samples from a set of 26 sera characterized as positive for HBV surface antigen but were not found in 16 normal human sera. The data reported here demonstrate that sera from some patients with markers of HBV infection contain antibodies directed against the polypeptide encoded by ORF X. As such, these findings represent evidence that ORF X constitutes a gene, or a portion of a gene, which is expressed during HBV infection. Although there does not appear to be a direct relationship between anti-X and any individual markers of HBV infection, our data suggest that anti-X is more prevalent in HBV-positive sera containing antibodies to HBe_3 antigen (anti-HBe_3).

The study of hepatitis B virus (HBV) has proven uniquely difficult because of the inability to propagate HBV in tissue culture or in convenient laboratory animals. Molecular cloning of fully double-stranded HBV DNA in *Escherichia coli* has allowed the determination of the total nucleotide sequence of several viral genomes (14, 32, 54). Although the DNA sequences of HBV genomes isolated from different patients exhibit marked heterogeneity (40), DNA sequence analysis has consistently identified four open reading frames (ORFs). One of these regions codes for the HBV surface antigen (HBsAg) (5, 53) and another for core antigen (HBcAg) (33). The ORF which codes for HBcAg also codes for the determinants of e antigen (HBeAg) (23, 45, 46). Proteins encoded by the remaining two ORFs have not been identified.

The smallest of the four ORFs was designated initially as region 5 by Galibert et al. (14) and more recently as ORF X (48) and ORF B (54). This ORF could code for a polypeptide composed of 154 amino acids. Comparison of this region among the HBV DNAs isolated from different patients demonstrates significant conservation of nucleotide sequence. Pairwise comparisons of this ORF indicate that divergence between the encoded amino acid sequences is less than 4% (48). More recently, cloning and nucleotide sequence analysis of the genomes of other HBV subtypes has confirmed that the genetic organization of HBV, and specifically the presence of ORF X, is conserved (12, 20, 32). However, a cloned viral genome (*adr* subtype) has been reported to contain a 27-base-pair deletion in ORF X, resulting in a predicted polypeptide nine amino acids shorter (32). The significance of this observation is unclear.

HBV serves as the prototype for a new class of viruses termed hepadnaviruses (34). Other members of this class, which are similar to HBV with respect to primary DNA sequence, physical structure of encapsidated DNA, and endogenous DNA polymerase activity, have been isolated from woodchucks (44, 56), Beechey ground squirrels (26), and Pekin ducks (27). The regions of the genomes of the woodchuck and ground squirrel hepatitis viruses that correspond to ORF X could encode polypeptides similar in size to that encoded by the HBV ORF X (13, 38). The N and C termini of the predicted polypeptides are strictly conserved among HBV ORF X and the corresponding ORFs of the other mammalian hepadnaviruses. Although the amino acid sequences of the internal regions of the polypeptides differ considerably, computer-assisted analysis indicates significant homology at the level of secondary structure, particularly in terms of β-pleated-sheet-forming potential (36). In addition, the nucleotide sequences of the ground squirrel and woodchuck viral ORFs corresponding to ORF X are highly homologous (38). In the case of duck hepatitis virus, a discrete ORF corresponding to ORF X does not exist. However, the regions of the duck hepatitis viral genome which correspond in both size and position to ORF X and ORF C in HBV appear to be fused to form a larger ORF (24). The 35-kilodalton (kDa) molecular mass of a polypeptide purified from duck HBV nucleocapsids is consistent with the expression of this entire extended ORF (W. Mason and J. Newbold, personal communication).

The production of putative viral polypeptides or portions thereof in *E. coli* with expression vectors can facilitate attempts to define the proteins synthesized during viral infection. We report here the construction of a plasmid encoding a fusion protein containing the 8 N-terminal amino acids of β-galactosidase and 145 amino acids from the C terminus of ORF X. This fusion protein, designated $\beta\text{-gal}_8\text{:X}_{145}$, is expressed and characterized by in vitro tran-

* Corresponding author.
† Present address: Cetus Corporation, Emeryville, CA 94608.

scription and translation. Further, in sera from HBV-infected humans, we detected antibodies which specifically recognize the ORF X-encoded sequence of β-gal$_8$:X$_{145}$.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* C600 (*hsdR hsdM*$^+$) (2) was used for all transformations. Luria broth (29) was used in the cultivation of bacteria except for the induction of the β-galactosidase fusion protein encoded by pHBV-X700 (see below). Selection for ampicillin (25 μg/ml) resistance was on Penassay base agar (antibiotic medium 2; Difco Laboratories, Detroit, Mich.).

DNA manipulation and analysis. Plasmid DNA was isolated from CsCl-ethidium bromide gradients (47) or by a mini-lysate procedure (3). Plasmid DNAs to be used as templates for in vitro transcription and translation were then phenol extracted and fractionated by gel filtration with Bio-Gel A-5M (Bio-Rad Laboratories, Richmond, Calif.) before concentration to 0.5 to 1 mg/ml in 10 mM Tris hydrochloride–1 mM EDTA (pH 8.0). Restriction endonucleases and bacteriophage T4 DNA ligase were used as recommended by the supplier (New England Biolabs Inc., Beverly, Mass.) Linearized plasmid DNA was treated with calf intestine alkaline phosphatase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to prevent recircularization and optimize insertion of DNA fragments (51). For subcloning, 5 to 25 μg of plasmid DNA was cleaved with restriction enzymes. The recovery of specific DNA fragments from 5% polyacrylamide gels was performed by electroelution (37). The DNA fragment (5 to 20 μg/ml) in a 2- to 10-fold molar excess was ligated to the vector DNA, and the products of this reaction were used to transform bacteria (7). Agarose gel electrophoresis was performed with horizontal slab gels in Loening buffer (16) (36 mM Tris hydrochloride, 30 mM NaH_2PO_4, 1 mM EDTA; pH 7.8). Polyacrylamide gels were prepared as described by Maniatas et al. (25).

Protein analysis. Proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (22). An acrylamide-to-bisacrylamide ratio of 29:1 was used for 12% gels as noted in the figure legends. Protein bands were visualized by staining with Coomassie brilliant blue R-250. Protein concentrations were determined by Protein Assays (Bio-Rad). The Prokaryotic DNA-Directed Translation Kit, L-[^{35}S]methionine, and L-[4,5-^{3}H]leucine were purchased from Amersham Corp. (Arlington Heights, Ill.), and the reactions were performed as specified by the vendor. Each reaction contained 2.5 to 10 μg of template DNA, which was prepared as described above. Isopropyl-β-D-thiogalactopyranoside (IPTG) (5×10^{-4} M) was included in initial reactions but was later omitted, since a comparison between the products of reactions with or without IPTG showed little difference. The extent of incorporation of radioactive amino acids into polypeptides was determined by trichloroacetic acid precipitation. Radioactively labeled proteins were identified by fluorography of gels treated with En^{3}Hance as suggested by the vendor (New England Nuclear Corp., Boston, Mass.).

Expression and purification of β-gal$_{1007}$:X$_{145}$. The strategy for the identification of β-gal$_8$:X$_{145}$ utilized antisera generated with another ORF X-encoded fusion protein, which in contrast to β-gal$_8$X:$_{145}$ can be overproduced in bacteria. The construction of the recombinant plasmid, designated pHBV-X700, that directs the synthesis of a hybrid protein containing 1,007 N-terminal amino acids of β-galactosidase and 145 C-terminal amino acids encoded by HBV ORF X (β-gal$_{1007}$:X$_{145}$) has been previously described (43). Expression of the β-gal$_{1007}$:X$_{145}$ fusion protein was maximized when the host bacterial strain contained a chromosomal *lacZ* (C600) rather than a *lacZ* deletion (MC1000). Experiments using various combinations of media (Luria broth or minimal), carbon source (lactose, glucose, or glycerol), cyclic AMP, and IPTG indicated that the culture conditions described below resulted in maximal expression of the fusion protein in C600 cells as determined by SDS-PAGE. Bacterial strain C600 harboring pHBV-X700 was inoculated at a 1:100 dilution from a frozen culture into minimal medium-glucose (M9 salts, 1% Difco Casamino Acids, 2 mM $MgCl_2$, 50 μg of thiamine per ml, 0.2% glucose) (29) with ampicillin (25 μg/ml) and incubated with shaking at 37°C overnight. At stationary phase, the culture was diluted 1:500 into minimal medium-glycerol (0.8% glycerol), and growth was allowed to continue with shaking at 37°C. After 2 h, IPTG was added to a concentration of 0.5 mM. Incubation was continued, and the cells were harvested at an A_{650} of 1.0 to 1.5 by centrifugation at 8,000 × *g* for 20 min. The fusion protein was purified from total cell protein by a method described previously (6, 9, 15, 17) which exploits the insolubility of fusion polypeptides containing portions of various eucaryotic proteins substituted for the 16 C-terminal amino acids of β-galactosidase. Briefly, harvested cells were resuspended in 1/100th the culture volume of 10 mM Tris hydrochloride–1 mM EDTA (pH 7.5). The suspension was sonicated with a Heat Systems cell disrupter in a rosette flask on ice at setting 8 and 50% duty cycle for six 5-min intervals with intervening periods for cooling. The sonicate was spun at 10,000 × *g* for 10 min, and the supernatant was decanted. The pellet contained the large fusion protein and was resuspended at 1/100th the culture volume in a solution containing 15 mM sodium phosphate and 150 mM NaCl.

Immunization. The partially purified β-gal$_{1007}$:X$_{145}$ fusion protein (200 μg) solubilized with 0.1% SDS (0.5 ml) was emulsified with an equal volume of complete Freund adjuvant for both primary injections and boosts. Complete Freund adjuvant was prepared by suspending nonviable, desiccated *Mycobacterium tuberculosis* H37RA (Difco) in incomplete Freund adjuvant (Difco) at a concentration of 2 mg/ml. Female New Zealand White rabbits were injected intradermally at multiple sites along the back. Beginning 1 month after the primary injection, boost injections were given at intervals of 1 week to 1 month. Blood was drawn from the ear 1 month after the primary injection and 1 week to 1 month after subsequent injections. Sera that contained antibodies which recognized the β-gal$_{1007}$:X$_{145}$ fusion protein were identified by immunodiffusion and Western blotting (49, 52). These sera were designated anti-β-gal$_{1007}$:X$_{145}$.

Human sera. Sera negative for HBsAg, anti-HBs, and anti-HBc were collected from humans with no signs of clinical illness and characterized by the Clinical Virology Laboratory of the Montefiore Medical Center of the Albert Einstein College of Medicine. Sera from humans infected with HBV were identified as positive for antibody to HBe$_3$Ag (anti-HBe$_3$) as described previously (55). Briefly, double immunodiffusion was carried out with 0.9% (wt/vol) agarose with 2% polyethylene glycol in 0.03 M veronal buffer (pH 8.6). Only those sera reacting against all reference reagents and providing clear lines of identity were considered positive. All sera characterized as positive for anti-HBe$_3$ were from asymptomatic HBV carriers.

All other sera screened were obtained from the American Red Cross Blood Services Laboratories and had been char-

acterized with respect to the presence of HBsAg, HBeAg, their respective antibodies (anti-HBs and anti-HBe), and antibodies to core antigen (anti-HBc) by procedures suggested by the supplier of the diagnostic kits (Abbott Laboratories, North Chicago, Ill.). For clarity, the antigen detected in the commercially available diagnostic kits is referred to as HBe_1Ag in this report. Sera positive for anti-delta were identified by an enzyme-linked immunoassay for which the reagents were provided by Abbott.

Immunoprecipitations. Immunoprecipitations were performed as described by Oliver and Beckwith (31) with some modifications. Portions (1 to 5 μl corresponding to 1×10^5 to 5×10^5 cpm) of the in vitro coupled transcription-translation reaction mixtures containing labeled proteins were suspended in 1% SDS–1 mM EDTA–10 mM Tris hydrochloride (pH 8.0) (20 μl) and allowed to stand for 5 min. The denatured proteins were then added to 200 μl of 2% Triton X-100–150 mM NaCl–1 mM EDTA–50 mM Tris hydrochloride (pH 8.0) containing 1 mg of ovalbumin per ml. Human or rabbit sera (5 μl) were added and incubated overnight at 4°C. IgGSORB (The Enzyme Center, Inc.) (100 μl), resuspended as described by the supplier and prepared by the method of Kessler (19), was then added, and incubation at 4°C continued for 30 min. The IgGSORB was pelleted with a Fisher microcentrifuge for 1 min, and the pellet was washed three times with 1 ml of 1% Triton X-100–1 M NaCl–50 mM Tris hydrochloride (pH 7.5) and once with 1 ml of 10 mM Tris hydrochloride (pH 8.0). Pellets were suspended in 60 μl of sample buffer (22), boiled for 10 min, and centrifuged with a Fisher microcentrifuge for 5 min before analysis by SDS-PAGE. In competition experiments, the sera were incubated with various concentrations (0, 10, 50, and 250 μg/ml) of unlabeled, partially purified $\beta\text{-gal}_{1007}\text{:X}_{145}$ or β-galactosidase (Boehringer Mannheim) overnight at 4°C before the addition of labeled antigen.

RESULTS

Construction of a recombinant plasmid that encodes $\beta\text{-gal}_8\text{:X}_{145}$. Synthesis of a fusion protein containing primarily determinants predicted by the HBV ORF X was desired to minimize the effect of heterologous determinants on immunoprecipitations performed with sera from HBV-infected humans. The addition of as few extraneous amino acids as possible was also expected to increase the probability of yielding an enzymatically active polypeptide for studies not reported here. We accomplished expression of the chosen fusion protein by constructing a hybrid gene that encodes the N-terminal eight amino acids of β-galactosidase and most of polypeptide X. A 584-bp *Bam*HI-*Bgl*II fragment spanning 145 of the 154 codons predicted by HBV ORF X (Fig. 1) was purified from pHBV1, a recombinant plasmid that contains a fully double-stranded HBV genome inserted at the *Eco*RI site of pACYC184 (42). The HBV sequence in pHBV1 corresponds to that reported by Valenzuela et al. (54). Plasmid pZL801 is a pBR322 derivative which contains the efficient *trp-lac* (TAC) hybrid promoter (1, 10, 35) and a sequence encoding eight N-terminal amino acids of β-galactosidase (18). The *lac* sequences are followed by a polylinker devoid of translational stop codons with a *Bam*HI site located so as to allow in-frame translation of a *lacZ*:ORF X hybrid gene (41). To decrease the frequency of recircularization of the vector and optimize for recombinant plasmids with inserts, *Bam*HI-linearized pZL801 DNA was treated with alkaline phosphatase and then ligated with a five-fold molar excess of the HBV *Bam*HI-*Bgl*II DNA fragment. The cohesive termini resulting from *Bam*HI and *Bgl*II digestion are identical, thereby simplifying the construction. However, since the ligation of a *Bam*HI and a *Bgl*II terminus does not regenerate either endonuclease site, a *Bam*HI endonuclease site is only regenerated at one of the two junctions between the inserted HBV DNA fragment and the expression vector. Plasmid DNAs from ampicillin-resistant transformants were screened by *Sau*3A digestion. Endonuclease *Sau*3A recognizes both of the viral-vector junction sequences, as well as a site found within the *Bam*HI-*Bgl*II HBV DNA fragment (54). As expected, plasmid DNAs containing the HBV insert exhibited two additional fragments (351 and 233-base pairs in size) not observed with the parent vector (data not shown). Double digestion of plasmid DNA with *Eco*RI and *Bam*HI allowed the determination of the orientation of the inserted HBV DNA fragment in the candidate plasmids (Fig. 1) because a *Bam*HI site is regenerated at only one junction. A representative plasmid containing the HBV DNA fragment inserted in the orientation which would allow the synthesis of a β-gal:X hybrid protein was designated pHBV-X500 and used in all subsequent studies.

Expression of $\beta\text{-gal}_8\text{:X}_{145}$ by in vitro coupled transcription-translation. Protein extracts of bacteria harboring pHBV-X500 did not contain a prominent protein of the molecular weight expected for $\beta\text{-gal}_8\text{:X}_{145}$ (M_r ca. 17 kDa). However, upon comparison with protein extracts of bacteria containing the parent plasmid pZL801 by SDS-PAGE and Coomassie brilliant blue staining, an additional protein band (ca. 17 kDa) was observed from bacteria harboring pHBV-X500 (data not shown).

Several procedures have been developed to characterize polypeptides specifically encoded by plasmids in bacteria. A simple and particularly sensitive procedure employs plasmid DNA as a template for in vitro transcription and translation (8, 57). A comparison of [^{35}S]methionine-labeled polypeptides directed by pZL801 (Fig. 2, lane A) and pHBV-X500 (Fig. 2, lane D) indicated that the reaction containing pHBV-X500 generated a prominent, additional 17-kDa polypeptide (solid arrow). The prominent high-molecular-weight band seen in lanes C through F is located at the interface between the stacking and resolving gels. This band appears consistently in reactions that use pHBV-X500 as the template for coupled transcription-translation but not when other plasmid DNA are used. The nature of this material is unclear. As another control, plasmid pZL811, which encodes a $\beta\text{-gal}_8\text{:CAT}_{219}$ fusion polypeptide, was used as a template for the [^{35}S]methionine-labeled in vitro polypeptide synthesis reaction. When the products were fractionated, a prominent band of the size expected for the $\beta\text{-gal}_8\text{:CAT}_{219}$ fusion polypeptide (ca. 27 kDa) was observed (Fig. 2, lane F). The protein band with a molecular mass of 30 kDa represents the β-lactamase encoded by each of the three plasmids.

Since the $\beta\text{-gal}_8\text{:X}_{145}$ fusion protein contains 19 leucine residues versus only 3 methionine residues, we performed in vitro transcription and translation reactions with [^{3}H]leucine as the labeled amino acid. Again, when pHBV-X500 (Fig. 2, lane C) was used as the template, a 17-kDa protein band (solid arrow) was easily discerned relative to the parent plasmid pZL801 (Fig. 2, lane B). The virtual absence of background protein bands when [^{3}H]leucine was used as the labeled amino acid presumably occurred because leucine constitutes a greater proportion of the polypeptide encoded by ORF X than of β-lactamase or other plasmid-encoded proteins.

Because the bacterial strain used to produce the extracts

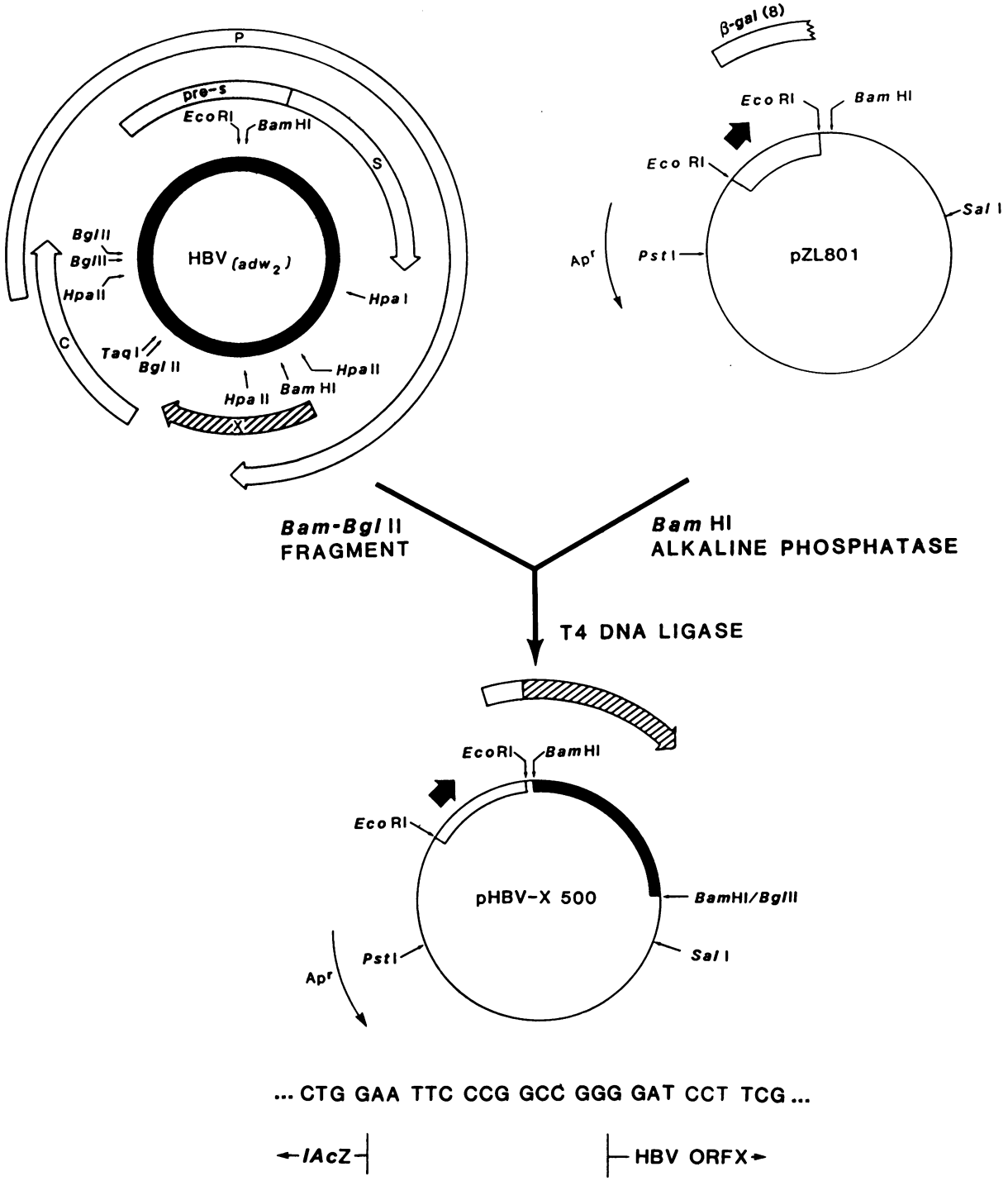

FIG. 1. Schematic of the method used and construction of the plasmid vector for the expression of ORF X. Top left: Representation of the genetic organization of the HBV genome. Broad arrows represent the polypeptide products of the ORFs, which are oriented relative to the specific endonuclease sites of the HBV subtype adw_2 (42). In each case, the ORF would be translated from N to C terminus in a clockwise direction. Regions of arrows designated S and C represent the major coat and nucleocapsid proteins, respectively. Gene S is preceded by a region designated pre-S. Open arrow designated as P represents the polypeptide predicted by the largest ORF. Hatched arrow represents the ORF X-encoded polypeptide. For simplicity, the HBV genome has been represented as the source of the *Bam*HI-*Bgl*II fragment carrying the ORF X sequences. In the actual construction, this fragment was obtained from pHBV1, which contains the fully double-stranded HBV genome inserted at the unique *Eco*RI site of pACYC184 (42). Top right: Expression vector pZL801. Thin line represents pBR322 sequences, and thin arrow labeled Ap^r represents β-lactamase. Open block segment represents an *Eco*RI fragment that contains the hybrid *trp-lac* (TAC) promoter and *lac* operator sequences and encodes eight N-terminal amino acids of β-galactosidase (open block with jagged end). Solid bold arrow identifies site of initiation and direction of transcription from the TAC promoter. Bottom: Recombinant plasmid pHBV-X500 that directs synthesis of the β-gal:X fusion polypeptide. Solid block represents *Bam*HI-*Bgl*II DNA fragment from HBV genome. *Bam*HI/*Bgl*II

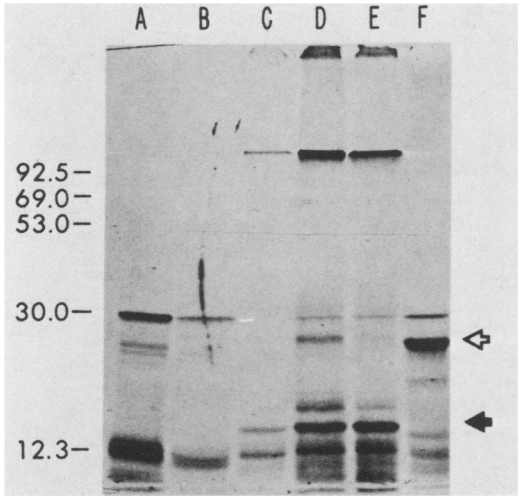

FIG. 2. In vitro coupled transcription-translation of plasmid DNA. Fluorograph of 12% SDS-PAGE. After in vitro coupled transcription-translation, samples of reaction mixtures containing 1×10^5 to 2×10^5 cpm of labeled proteins were fractionated. Reactions were directed by plasmid DNA noted as template and utilized L-[^{35}S]methionine or L-[4,5-^{3}H]leucine label as noted. Lanes A and B, pZL801 template labeled with methionine and leucine, respectively; C, pHBV-X500 labeled with leucine; D and E, pHBV-X500 labeled with methionine, without and with IPTG (5×10^{-4} M), respectively; F, pZL811, which encodes a β-gal$_8$:CAT$_{219}$ fusion protein, labeled with methionine. Numbers at left represent molecular mass of protein standards in kilodaltons. At right, open arrow indicates β-gal$_8$:CAT$_{219}$ fusion protein (27 kDa), and solid arrow indicates the β-gal$_8$:X$_{145}$ fusion protein (17 kDa).

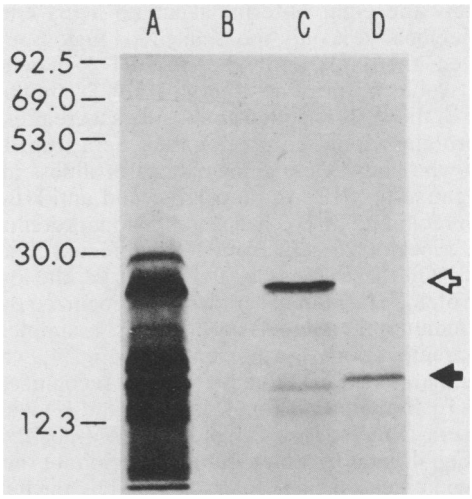

FIG. 3. Identification of β-gal$_8$:X$_{145}$ by immunoprecipitation with rabbit anti-β-gal$_{1007}$:X$_{145}$. Fluorograph of 12% SDS-PAGE. After in vitro coupled transcription-translation reactions directed by pHBV-X500 and pZL811 as described in the legend to Fig. 3, a mixture of the L-[^{35}S]methionine-labeled polypeptides was fractionated before and after immunoprecipitation. Lane A, Before immunoprecipitation. Lanes B through D contained the labeled polypeptides after immunoprecipitation with the antisera noted. Lane B, Normal rabbit serum. Lane C, Rabbit anti-β-gal$_8$:CAT$_{219}$. Lane D, Rabbit anti-β-gal$_{1007}$:X$_{145}$. Numbers at left represent molecular mass of protein standards in kilodaltons. At right, open arrow indicates the β-gal$_8$:CAT$_{219}$ fusion protein (27 kDa) and solid arrow indicates the β-gal$_8$:X$_{145}$ fusion protein (17 kDa).

for the in vitro coupled transcription-translation reaction contains the gene for the *lac* repressor, the addition of IPTG, which prevents binding of the *lac* repressor to the *lac* operator, was expected to result in increased synthesis of β-gal$_8$:X$_{145}$. However, a comparison of in vitro reactions with pHBV-X500 as the template DNA carried out with (Fig. 2, lane E) or without (Fig. 2, lane D) IPTG showed little or no difference in the quantity of labeled 17-kDa fusion protein. This suggests that the TAC promoter is expressed constitutively in the bacterial extracts.

Taken together, these data suggested that the 17-kDa polypeptide represents the translation product of the *lacZ*:ORF X hybrid gene carried by pHBV-X500.

Characterization of β-gal$_8$:X$_{145}$. To further characterize the 17-kDa polypeptide, immunoprecipitations of the products of the in vitro coupled transcription-translation reaction were performed with various antisera. To unambiguously demonstrate the specificity of the antigen-antibody interactions, we used a mixture of ^{35}S-labeled polypeptides synthesized by in vitro coupled transcription-translation reactions directed by pZL811 (the recombinant plasmid which contains a β-gal$_8$:CAT$_{219}$ gene) and pHBV-X500 as DNA templates (Fig. 3, lane A). None of the polypeptides in this mixture was immunoprecipitated by normal rabbit serum (Fig. 3, lane B). In lane D (Fig. 3), the 17-kDa polypeptide alone was recognized by antisera to β-gal$_{1007}$:X$_{145}$. This previously described fusion protein (43) contains 1,007 N-terminal amino acids encoded by *lacZ* and the identical viral component as β-gal$_8$:X$_{145}$. As a control, only the 27-kDa β-gal$_8$:CAT$_{219}$ fusion protein was precipitated by antisera generated to the β-gal$_8$:CAT$_{219}$ fusion protein (Fig. 3, lane C). The inability of the polyclonal serum directed against β-gal$_{1007}$:X$_{145}$ to recognize labeled β-gal$_8$:CAT$_{219}$ indicates that this serum does not contain detectable titers of antibodies which recognize the N-terminal eight amino acids of β-galactosidase. The recognition of the 17-kDa polypeptide by the polyclonal anti-β-gal$_{1007}$:X$_{145}$ further supports the contention that the 17-kDa polypeptide is the product of the *lacZ*:ORF X hybrid gene of recombinant plasmid pHBV-X500.

Characterization of antibodies to X antigen in human sera. The experiments described above suggested that immunoprecipitations utilizing the β-gal$_8$:X$_{145}$ fusion protein labeled in vitro could be used effectively to probe human sera for antibodies to viral protein encoded by HBV ORF X. The use of a mixture of ^{35}S-labeled polypeptides containing β-gal$_8$:X$_{145}$ as well as the β-gal$_8$:CAT$_{219}$ fusion protein allowed us to ensure that the antisera specifically recognized the virally encoded determinants of the fusion protein and

signifies junction of *Bgl*II cohesive end of HBV DNA fragment and *Bam*HI cohesive end of pZL801; neither site is regenerated at this junction. Broad arrow represents fusion polypeptide containing eight amino acids of β-galactosidase (open region) and 145 amino acids encoded by HBV ORF X (hatched region). Representations of polypeptides and plasmids are not to scale. The sequences below pHBV-X500 represent the junction of *lacZ*, the polylinker, and ORF X. The translational reading frame of the sequence is denoted by the separation of codons with a space. The triplet CTG encodes the eighth N-terminal amino acid of β-galactosidase, the polylinker encodes five amino acids, and GAT encodes the 10th amino acid of the X polypeptide.

not merely the eight N-terminal amino acids encoded by *lacZ*. Specifically, if only the β-gal$_8$:X$_{145}$ fusion protein was recognized, then only antibodies to ORF X-encoded determinants would be present; alternatively, if antibodies that recognized the *lacZ*-encoded amino acids were present, both fusion proteins would be precipitated. Sera from 16 human subjects who showed no clinical signs of illness and which were negative for HBsAg, anti-HBs, and anti-HBc did not immunoprecipitate the β-gal$_8$:X$_{145}$ fusion protein. By this method, a set of 26 sera from HBV-infected humans was screened. Of these 26 sera, 9 recognized the β-gal$_8$:X$_{145}$ fusion protein. The human sera which recognized β-gal$_8$:X$_{145}$ did not immunoprecipitate β-gal$_8$:CAT$_{219}$. Examples of these immunoprecipitations are presented in Fig. 4, which contrasts human HBV-positive sera that recognized the β-gal$_8$:X$_{145}$ fusion protein (Fig. 4, lanes G and H) with normal human sera (Fig. 4, lane C) and an HBV-positive human serum (Fig. 4, lane F) which did not precipitate the antigen. The polypeptides specifically precipitated by the human sera (Fig. 4, lanes G and H) and the rabbit anti-β-gal$_{1007}$:X$_{145}$ (Fig. 4, lane E) are identical in molecular weight. Further, the addition of unlabeled, partially purified β-gal$_{1007}$:X$_{145}$ fusion protein to the immunoprecipitation reactions prevented this recognition for both the rabbit and human sera (Fig. 5, lanes D and H, respectively). On the other hand, the addition of unlabeled β-galactosidase to the immunoprecipitation reactions did not prevent recognition of β-gal$_8$:X$_{145}$ by the rabbit or human sera (data not shown). These results demonstrate that antibodies to determinants encoded by HBV ORF X can be identified in human sera and strongly suggest that a polypeptide encoded by this ORF is produced during viral infection.

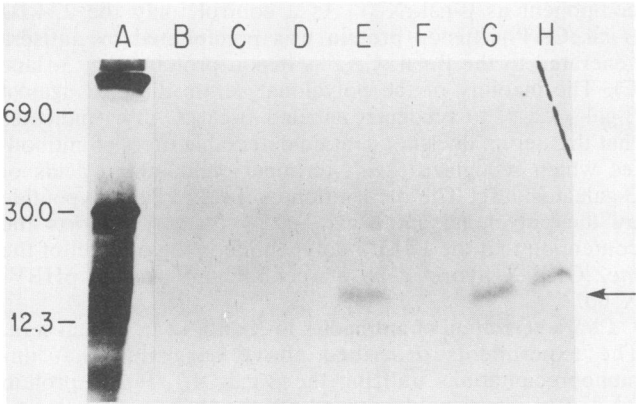

FIG. 4. Characterization of representative human sera by immunoprecipitation of fusion polypeptides synthesized in vitro. Fluorograph of 12% SDS-PAGE. After in vitro coupled transcription-translation of pHBV-X500 (which encodes β-gal$_8$:X$_{145}$) and pZL811 (which encodes β-gal$_8$:CAT$_{219}$), L-[^{35}S]methionine-labeled polypeptides were fractionated before and after immunoprecipitation. Lane A, Before immunoprecipitation. Lane B, Precipitation with IgGSORB but without antisera. Lanes C through H contained the labeled proteins after immunoprecipitation with the sera noted. Lane C, Normal human sera. Lane D, Normal rabbit serum. Lane E, Rabbit anti-β-gal$_{1007}$:X$_{145}$. HBsAg-positive human sera: lane F, no. 334; lane G, Br; lane H, no. 191. Numbers at left represent molecular mass of protein standards in kilodaltons. At right, arrow indicates β-gal$_8$:X$_{145}$ fusion protein (17 kDa).

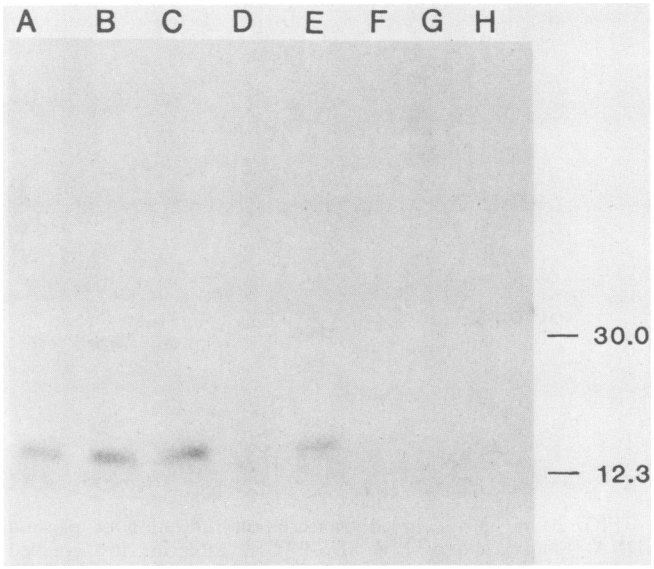

FIG. 5. Characterization of specificity of antisera for ORF X-encoded determinants. Fluorograph of 12% SDS-PAGE of immunoprecipitation competition. Immunoprecipitations with either anti-β-gal$_{1007}$:X$_{145}$ (lanes A through D) or HBsAg-positive human sera (Br) (lanes E through H) were performed on a mixture of labeled polypeptides synthesized by in vitro coupled transcription-translation of pHBV-X500 and pZL811. Before addition of the labeled polypeptides, the sera were incubated with the noted concentrations of unlabeled partially purified β-gal$_{1007}$:X$_{145}$. Lanes: A and E, no β-gal$_{1007}$:X$_{145}$; B and F, 1 μg/ml; C and G, 10 μg/ml; D and E, 50 μg/ml. Numbers at right represent molecular masses of protein standards in kilodaltons.

DISCUSSION

We constructed two recombinant plasmids, each with the capacity to direct the synthesis of a fusion polypeptide containing 145 of the 154 amino acids predicted from the DNA sequence of the HBV ORF X. The plasmid reported here, pHBV-X500, encodes a polypeptide composed of only eight amino acids of the N terminus of β-galactosidase followed by the viral sequence. The other plasmid, pHBV-X700, reported previously (43), encodes a polypeptide which consists of the N-terminal 1,007 amino acids of β-galactosidase followed by the amino acids encoded by ORF X. We adopted the two-plasmid strategy because it was thought that each construction would afford unique advantages. The small fusion protein, because of its predominantly viral composition, was expected to closely approximate the structure, antigenic properties, and enzymatic activities of a native protein encoded by ORF X. However, this fusion protein was not expressed efficiently in bacteria. On the other hand, the large fusion protein, while lacking the above advantages, was more stable in the bacterial host presumably due to the greater structural contribution of the procaryotic segment. In addition, the enzymatic, antigenic, and biochemical properties of the β-galactosidase facilitated characterization and purification of the large fusion polypeptide, thereby enabling specific antisera to be generated.

We describe here the synthesis of a labeled polypeptide of the size predicted for the small ORF X fusion protein. This polypeptide was expressed by in vitro coupled transcription-translation reactions employing plasmid pHBV-X500 as the template. Further, we verified that the polypeptide synthesized in vitro represented the small fusion protein, β-

gal_8:X_{145}, by demonstrating that it was specifically recognized in immunoprecipitations by antisera generated to the large ORF X fusion protein (β-gal_{1007}:X_{145}).

Immunoprecipitations utilizing β-gal_8:X_{145} as antigen have been applied to the screening of human sera to investigate whether antibodies to the undefined ORF X protein are produced as a result of HBV infection. Sera lacking diagnostic markers for HBV infection do not contain such antibodies. We screened a set of 26 HBsAg-positive sera and found 9 samples to contain antibodies to an ORF X-encoded polypeptide (anti-X). These findings demonstrate that sera from HBV-infected humans contain antibodies directed against the protein encoded by ORF X. Thus, they represent evidence that ORF X constitutes a gene, or part of a gene, which is expressed during the course of HBV infection. Coincident with our studies, A. Kay, E. Mandart, C. Trepo, and F. Galibert (personal communication), using immunoprecipitation of *E. coli*-synthesized fusion proteins containing ORF X-encoded sequences in a procedure similar to the one described here, have also found antibodies to this predicted viral antigen. Two of the sera found to contain anti-X antibodies in our study (no. 191 and Br) were also found to be positive for such antibodies by these investigators.

All the above 26 sera had been characterized as containing anti-HBe_3. HBe_3Ag was the third specificity characterized for the heterogeneous HBeAg system, which consists of a complex family of soluble proteins. Because the HBe_3 specificity has been reported to be associated with increased numbers of circulating Dane particles and higher titers of HBsAg (50), this set of sera was chosen for our initial attempts to detect antibodies to the undefined viral protein predicted by ORF X.

To compare the prevalence of anti-X in the sera containing anti-HBe_3 with that in a more representative sampling, we examined a second set of sera containing HBV markers. These samples contained various well-characterized combinations of HBV antigens (HBsAg, HBe_1Ag) and antibodies (anti-HBs, anti-HBe_1, anti-HBc) as well as an HBV-associated marker (anti-delta). This second set, which contained 49 sera, was distinct from the first because the samples were chosen independent of the presence of anti-HBe_3. Moreover, 23 of the samples contained markers of prior HBV infection but were characterized as negative for HBsAg; none of these sera exhibited anti-X antibodies. In addition, of the HBsAg-positive sera in the second set, only one contained anti-X antibodies. Although the relationship between anti-X and anti-HBe_3 is tenuous, our data suggest that the anti-HBe_3 marker identified a particular subset of HBV-positive sera in which the prevalence of anti-X is higher than usual. At this time the biological significance of the association between anti-HBe_3 and anti-X remains unclear, but the observation that anti-X is prevalent in anti-HBe_3 serum merits further investigation. We are also investigating presently whether any other HBV markers will allow for the identification of a larger number of sera positive for anti-X.

Antibodies in humans to the ORF X-encoded polypeptide may represent an additional diagnostic marker for HBV. The significance of this marker in terms of its relationship to the stage of infection or course of disease or both will be addressed in future experiments. During the preparation of this manuscript, Moriarity et al. (30) reported the identification of antibodies to two synthetic oligopeptides predicted from the C terminus of this ORF in patients infected with HBV. Their data suggest a prevalence of such antibodies in patients with cirrhosis and hepatocellular carcinoma. Additional studies will be required to compare the antibodies identified in our report and those identified by Moriarity et al. (30).

Future studies will also utilize the anti-β-gal_{1007}:X_{145} antibodies generated in rabbits and affinity-purified anti-X antibodies from human sera to detect and identify the ORF X-encoded antigen in virus particles, infected hepatocytes, and infectious sera. The goal of our studies is to determine the role of X in the life cycle of the virus.

Analysis of integrated HBV sequences obtained from human primary liver carcinoma tissue and cell lines, including nucleotide sequencing of the junctions between the HBV and human sequences, indicates that some sites of integration are within ORF X (11, 21, 39). In such cases, expression of ORF X may be manifest as a fusion protein containing amino acids encoded by both human and viral genes. These fusion proteins would be analogous to those produced by the hybrid *gag-onc* genes of certain retroviruses (4, 28). Antibodies generated to two predicted synthetic oligopeptides identified a 28-kDa polypeptide in a hepatoma cell line that contains multiple copies of integrated HBV DNA and in extracts of liver tissue from infected humans (30). The rabbit antibodies we generated may be able to recognize an ORF X-encoded polypeptide present in the form of a fusion in hepatocellular carcinoma and other cells with integrated HBV DNA.

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