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Trans-activation of HLA-DR gene by hepatitis B virus X gene product

(hepatitis B virus X protein/HLA-DR gene expression)

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ABSTRACT **Hepatocellular injury during hepatitis B virus (HBV) infection has been postulated to result from a human leukocyte antigen (HLA)-restricted T-lymphocyte host immune response against HBV antigens. Although HLA expression is enhanced in the presence of hepatic inflammation, whether HBV itself can induce HLA expression on infected hepatocytes is unknown. In this study, we demonstrate the induction of HLA-DR expression on human hepatoma cell lines transfected with HBV DNA sequences. The HBV X gene alone was capable of inducing HLA-DR expression. This induction correlated with elevated HLA-DR RNA, and this resulted directly from transcriptional trans-activation of the HLA-DR gene by the HBV X protein. These studies suggest that the HBV X protein can regulate the expression of HLA-DR and thus raise the possibility of participation by the X gene in the immunopathogenesis of HBV infection.**

Hepatitis B virus (HBV) infection is a worldwide health problem because of its prevalence and association with chronic liver disease and hepatocellular carcinoma (1, 2). HBV is a small partially double-stranded DNA virus. Sequence analysis of cloned HBV DNA has identified at least four open reading frames (ORFs): pre-S/S, core/e, X, and Pol (Fig. 1*A*, also see ref. 1). Pre-S/S and core/e genes encode envelope and nucleocapsid proteins of HBV, respectively. Pol ORF encodes the viral DNA polymerase, which is essential for HBV replication. The X ORF codes for a polypeptide of 16,500 Da, and antibody reactive to this polypeptide has been found in HBV-infected individuals (3, 4). Although the X gene product has been defined as a transcriptional trans-activator (5–9), its role in the viral life cycle is not understood.

Although significant advances have been made in the molecular biology of HBV, an understanding of the pathogenesis of HBV infection has been severely limited by its narrow host range and lack of an efficient system for propagation *in vitro*. Evidence that HBV is not directly cytopathic (10, 11) suggests that hepatocellular necrosis during acute or chronic HBV infection may be mediated by the host immune response against one or more HBV peptides expressed on the surface of infected hepatocytes (10–16). Recognition of foreign antigens in the context of self human leukocyte antigen (HLA) class I and II is known to be a prerequisite for an antigen-specific immunopathogenic mechanism (17). Furthermore, infections with certain viruses can induce or enhance the expression of HLA antigens (18–20).

HLA class II molecules, including DR, DP, and DQ, are cell surface glycoproteins that play a key role in controlling the level of immune response to certain antigens by presenting the antigen to helper lymphocytes (21, 22). HLA-DR is primarily expressed on the surface of certain cells of the immune system (23) and is not expressed on normal human hepatocytes (24). Although aberrant expression of HLA-DR on hepatocytes observed during HBV infection has been suggested to be due to cytokines produced by the adjacent inflammatory cells (24, 25), it is not known whether HBV itself is involved in the induction process. This study demonstrates that transfection of cultured human hepatoma cell lines with genomic HBV sequences can induce HLA-DR expression *in vitro* in the absence of inflammation. We further show here that the X gene product of HBV alone is capable of inducing transcription of the HLA-DR gene.

MATERIALS AND METHODS

Construction of Plasmid Vectors. Recombinant plasmids (Fig. 1) were constructed from a cloned *adw* strain (26). The previously constructed plasmids, pNER and pMNX, contain the entire HBV DNA and the X gene sequences, respectively (4, 27). Plasmid pUCSL contains the HBV pre-S/S and X genes cloned as a *Bgl* II fragment (2432/1987, 2.8 kb) in *Bam*HI-digested plasmid pUC13, whereas plasmid pUCC contains the HBV core/e (C) and X genes cloned as a *Hpa* I/*Eco*RI (966/3221, 2.3 kb) fragment in *Hin*cII/*Eco*RI-digested plasmid pUC13. Plasmid pHLA-DRα contains the cDNA sequence of HLA-DRα chain (28). pD164-2, the parental plasmid, contains a promoterless chloramphenicol acetyltransferase (CAT) gene. Plasmid pDR1000 contains about 1 kb of upstream sequences of the HLA-DR gene cloned in front of the CAT gene (22).

Cell Lines and Transfection. HepG2 and HuH-7 cell lines derived from human hepatoblastoma and hepatocellular carcinoma, respectively, are negative for HBV DNA and retain several hepatocyte-specific markers (29, 30). Both cell lines were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum. DNA transfections were carried out by using the calcium phosphate precipitation method. Transfection with whole HBV genome was performed by removing the plasmid sequences and subsequently recircularizing the whole genome (31, 32). A stably transformed cell line, MNX, which constitutively expresses X protein, was established by transfecting HepG2 cells with pMNX followed by selection with antibiotic G418. The 2.2.15 cell line is a stably transformed derivative of HepG2 cells developed by transfection with a dimerized HBV genome (32).

Assays for HBV Antigens and HLA-DR. Samples of the culture media were collected 48 hr after transfection and centrifuged at 10,000 × *g* for 15 min. The clarified supernatants were assayed for HBsAg and HBc/eAg by using commercial radioimmunoassay kits (Abbott). Both pre-S1 and pre-S2 proteins were detected by a modified radioimmu-

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Abbreviations: HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBc/eAg, hepatitis B core/e antigen; HLA-DR, human leukocyte antigen DR; ORF, open reading frame; CAT, chloramphenicol acetyltransferase.

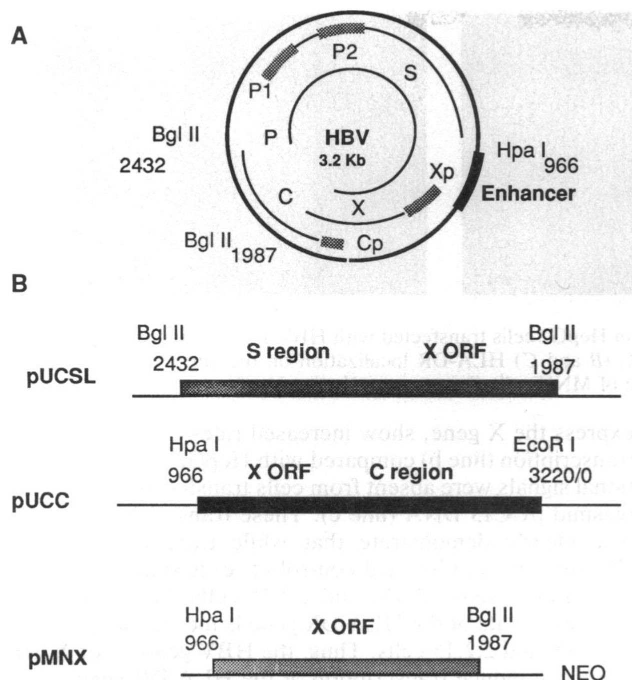

FIG. 1. Construction of recombinant vectors. (*A*) Genetic map of HBV DNA sequences, showing the four ORFs. S, surface antigen (HBsAg) gene; C, core/e antigen (HBc/eAg) gene; X, X gene; P, DNA polymerase ORF; P1, pre-S1 promoter; P2, pre-S2/S promoter; Xp, X promoter; and Cp, core/pregenomic promoter. The nucleotides are numbered as indicated. (*B*) Recombinant plasmid vectors. pUCSL contains a 2.8-kilobase (kb) *Bgl* II fragment including the transcription unit for the biosynthesis of surface antigen and X genes. pUCC contains an *Hpa* I/*Eco*RI fragment including the core/e and X genes. The *Bgl* II fragment was inserted at the *Bam*HI site, and the *Hpa* I/*Eco*RI fragment at the *Hin*cII/*Eco*RI site of the plasmid pUC13. pMNX contains only the X gene (*Nco* I/*Bgl* II, 1375/1997) as previously described (4).

noassay as previously described (33). The supernatants from nontransfected HepG2 and HuH-7 cells were used as negative controls.

For immunocytochemical alalysis, transfected cells were transferred to Lab-Tek Chamber Slides (Nunc) 36-48 hr after transfection and incubated overnight. Cells were fixed with cold acetone for 10 min; the presence and distribution of HBV peptide antigens and HLA-DR were assessed by either indirect immunoperoxidase or immunofluorescence techniques, as previously reported (16, 25). The monoclonal antibody TAL-1B5 against HLA-DRα chain was employed in this analysis (34). Briefly, cells were incubated with primary antibody at 4°C overnight, followed by a horseradish peroxidase of fluorescein isothiocyanate-conjugated secondary antibody for 1 hr at 37°C. For immunoperoxidase staining, color development was at room temperature for 10 min in a substrate solution containing 0.03% diaminobenzidine and 0.02% hydrogen peroxide. HepG2 and HuH-7 cells, transfected with pUC13 and nontransfected, served as negative controls. Similar immunoprocedures were employed.

RNA Analyses. Total cytoplasmic RNA was isolated from cells 48 hr after transfection. For RNA slot hybridization, 20 μg of total cytoplasmic RNA was loaded onto nitrocellulose filters and probed with ^{32}P-labeled HLA-DR cDNA. Nuclear run-on assays were carried out according to the method of Clayton *et al.* (35) with modifications (36). For run-on analysis the HepG2, MNX, and 2.2.15 cells were maintained in Ham's medium plus 5% fetal calf serum plus 2× MEM amino acids (GIBCO) until fully confluent.

CAT Assays. CAT was assayed 48 hr after transfection according to the method of Gorman *et al.* (37).

RESULTS

Expression of HBV Genes. HBV peptide antigens were detected in the culture medium of HepG2 cells transfected with recircularized HBV genome or vectors containing subgenomic HBV DNA sequences (Table 1). Transfection with whole HBV genome produced viral particles as evidenced by endogenous DNA polymerase activity (data not shown) as well as viral antigens. Transfection with vectors containing one or more HBV genes: S and X (pUCSL), C and X (pUCC), and X (pMNX), resulted in the production of the respective polypeptides.

Indirect immunofluorescence and immunoperoxidase techniques were used to detect the expression of pre-S1, pre-S2, HBsAg, HBc/eAg, and X protein in the transfected HepG2 cells (Table 1). When the immunoperoxidase technique was used, HBV envelope proteins (pre-S1, pre-S2, and HBsAg) were localized as fine granules in the cytoplasm of cells transfected with HBV genome and pUCSL. HBc/eAg was detected in both the cytoplasm and nuclei of the cells transfected with HBV genome and pUCC (data not shown). By using indirect immunofluorescence, X protein was localized in both the cytoplasm and the nuclei of HepG2 cells transfected with HBV genome as well as in 2.2.15 and MNX cells (Fig. 2*A*). The expression of X protein in cells transiently transfected with pUCSL or pUCC was weaker than in stably transformed 2.2.15 cells or MNX cells (Table 1). Results obtained with HuH-7 cells transfected with HBV DNA (genomic and subgenomic) sequences were identical (data not shown). As previously described by others (32, 38) and shown here, the 2.2.15 cell line produced viral particles and expressed all viral proteins, including X (Table 1).

HLA-DR Expression in Cells Transfected with HBV DNA Sequences. Examination of cells expressing one or more HBV genes revealed the coexpression of HLA-DR (Table 2 and Fig. 2). HepG2 or HuH-7 cells and those transfected with the plasmid pUC13 did not show expression of HLA-DR. How-

Table 1. Expression of HBV antigens by HepG2 cells transfected with HBV DNA sequences

	Antigen expression in culture medium*				Antigen expression in HepG2 cells†				
HBV DNA	S1	S2	sAg	cAg	S1	S2	sAg	cAg	X
HBV dimer (2.2.15)	9.3	6.4	33.3	6.8	+	+	+	+	+
HBV genome	5.4	4.6	20.8	5.4	+	+	+	+	+
S/X (pUCSL)	4.3	5.2	19.5	0.8	+	+	+	−	+
C/X (pUCC)	1.1	0.7	0.8	4.2	−	−	−	+	+
X gene (pMNX)	ND	ND	ND	ND	−	−	−	−	+

S1, pre-S1; S2, pre-S2; sAg, HBsAg; cAg, HBc/eAg; and X, X protein.
*Culture medium was collected after transfection and radioimmunoassayed for HBV antigens. Positive-to-negative ratio (P/N) was used to evaluate the relative amount of HBV antigens. P/N > 2 is considered positive. ND, not determined.
†Transfected HepG2 cells were examined for HBV antigens by immunohistochemical methods.

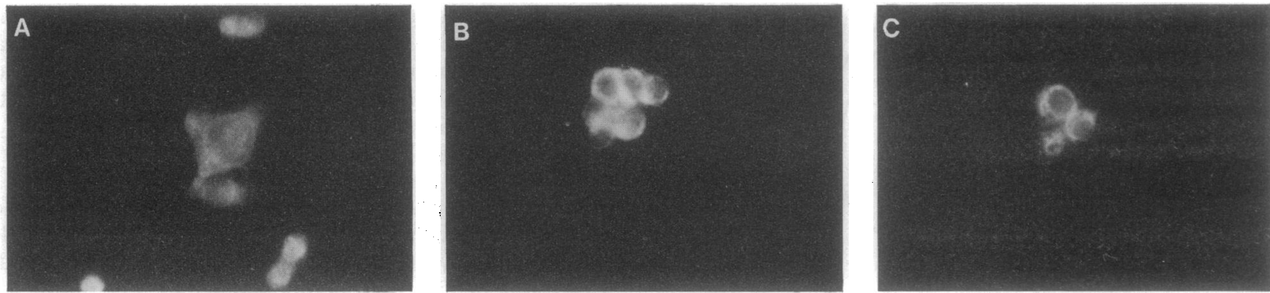

FIG. 2. Indirect immunofluorescence of HBV X protein and HLA-DR in HepG2 cells transfected with HBV DNA sequences. (×400.) (*A*) Cellular distribution of X protein in MNX cells transformed with pMNX. (*B* and *C*) HLA-DR localization on the surface of HepG2 cells transfected with recircularized genomic HBV DNA (*B*) and on the surface of MNX cells that express X protein (*C*).

ever, transfection of these cells with cloned recircularized HBV genome resulted in expression of HLA-DR on the cell surface (Fig. 2*B*). Similar expression of HLA-DR was also observed on the surface of the stably transformed cell line 2.2.15 positive for HBV.

To assess the individual HBV gene or combinations of HBV genes responsible for this induction of HLA-DR expression, transfections with vectors containing subgenomic fragments were performed. Transfection with pUCSL (containing pre-S/S and X genes) or pUCC (containing C and X genes) resulted in the induction of HLA-DR (Table 2). Since the X gene is known to trans-activate HBV and other viral cis-acting sequences (5–9) and was expressed in the transfected cells, we asked whether the X gene alone could induce HLA-DR expression. To test this, the MNX cell line, which constitutively expresses the X protein, was used. Indirect immunofluorescence of these cells clearly demonstrated the expression of HLA-DR on the cell surface (Fig. 2*C*). These data indicate that the expression of HLA-DR in hepatoma cells can be induced by the HBV X protein.

RNA Analyses. Next, we analyzed the steady-state levels of HLA-DR RNA and its rate of transcription. RNA slot hybridization was performed to determine the presence of HLA-DR RNA in transfected cells with genomic or subgenomic HBV DNA sequences. The RNA hybridization data are shown in Fig. 3. Whereas HLA-DR RNA was absent from nontransfected HepG2 and HuH-7 cells, these cells transiently transfected with the HBV genome and the 2.2.15 stably transformed cells showed enhanced levels of HLA-DR RNA (Fig. 3*A*). This RNA was also detected in HepG2 cells transfected with pUCSL or pUCC, but the hybridization signals were weaker (Fig. 3*B*). In contrast, stably transformed MNX cells, which express only X protein, displayed substantial levels of HLA-DR RNA (Fig. 3*B*). This descrepancy in the RNA levels may be the result of two strategies of transfection used—i.e., transient vs. stable.

To examine whether the enhanced expression of HLA-DR RNA resulted from a transcriptional or post-transcriptional mechanism, we performed nuclear run-on assays on the nuclei from HepG2, MNX, and 2.2.15 cells. This technique permits the determination of relative RNA polymerase loading on a gene and hence its transcriptional activity (35). As can be seen in Fig. 4, 2.2.15 and MNX cells, both of which express the X gene, show increased rates of HLA-DR gene transcription (line b) compared with HepG2 cells. Transcriptional signals were absent from cells transfected with control plasmid pUC13 DNA (line c). These transcriptional run-on data clearly demonstrate that while transcription of the albumin gene (an internal control gene) is similar in HepG2 (non-transfected), MNX, and 2.2.15 cells (line a), the transcriptional rate of the HLA-DR gene is increased selectively in MNX and 2.2.15 cells. Thus, the HBV genome or X gene alone can induce transcription of the HLA-DR gene.

Trans-activation of HLA-DR Regulatory Sequences. We further examined if the HLA-DR transcriptional control elements are the target of trans-activation by the HBV X protein. For this, we used plasmid pDR1000, which contains the HLA-DR upstream sequences linked to the bacterial CAT reporter gene (22). The results of CAT assays are presented in Fig. 5. Transfection of HepG2, MNX, and 2.2.15 cells with p164-2, a promoterless CAT plasmid, did not result in any noticeable CAT expression (lanes 2–4). CAT activity was also not detectable in HepG2 cells transfected with pDR1000 (lane 5). However, transfection of MNX and 2.2.15 cells with pDR1000 resulted in an efficient expression of the CAT gene (lanes 6 and 7). This stimulation appears to be 4- to 5-fold. These data identify the upstream regulatory sequences of the HLA-DR gene as the target sequences for X gene trans-activation.

DISCUSSION

Since HBV infection does not produce any cytopathic effect (10, 11), hepatocellular necrosis during HBV infection is attributed to the host immune response against one or more HBV peptide antigens expressed on the surface of infected hepatocytes (10–16). Expression of both envelope (pre-S1, pre-S2, and S) and nucleocapsid (HBcAg) antigens has been reported on the membrane of HBV-infected hepatocytes in close proximity to infiltrates of mononuclear inflammatory cells containing T lymphocytes (15, 16). The immune response to HBV antigens has been shown to be major histocompatibility complex (MHC)-restricted in mice (39), and the human immune response to HBV envelope antigens is also HLA-restricted (40). Both HLA class I and II expression is observed during HBV infection in areas of inflammation and necrosis (24, 25). This aberrant HLA-DR expression has been

Table 2. Expression of HLA-DR in hepatoma cells transfected with HBV DNA sequences

Cells	DNA sequence	HLA-DR
HepG2 or HuH-7	None	−
HepG2 or HuH-7	pUC13 (control vector)	−
2.2.15 (HepG2 derivative)	HBV genome (dimer)	+
HepG2 or HuH-7 (transient)	HBV genome (recircularized)	+
HepG2 or HuH-7	S/X genes (pUCSL)	+
HepG2 or HuH-7	C/X genes (pUCC)	+
MNX (HepG2 derivative)	X gene (pMNX)	+

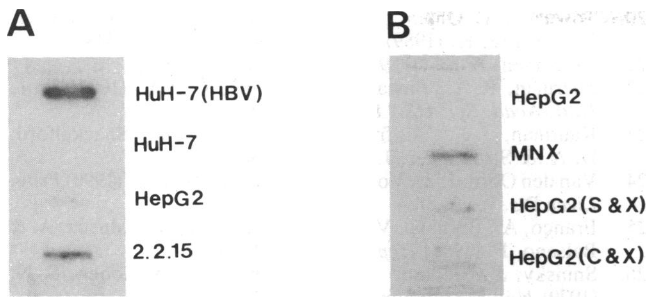

FIG. 3. RNA slot hybridization of hepatoma cells transfected with HBV DNA sequences. HLA-DR cDNA was radiolabeled and used as the probe. Approximately 20 μg of total cellular RNA was loaded on each nitrocellulose filter. (*A*) HepG2 and HuH-7 RNA, and the RNA from HuH-7 cells transfected with recircularized genomic HBV DNA and 2.2.15 cells (containing the integrated HBV sequences). (*B*) RNA from HepG2 cells and HepG2 cells transfected with pUCSL (S and X), pUCC (C and X), or pMNX (X gene alone).

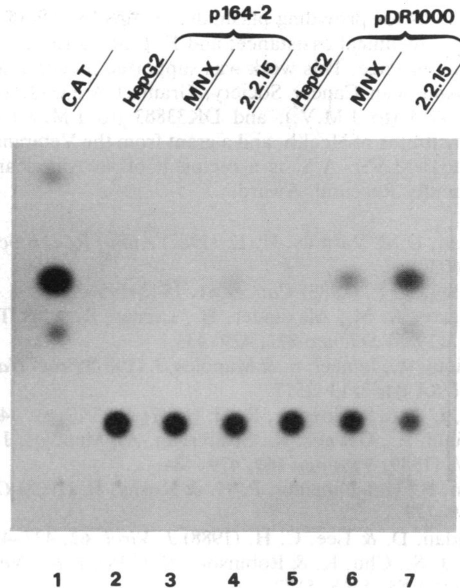

FIG. 5. CAT expression in transfected HepG2, MNX, and 2.2.15 cells with the indicated plasmid vectors. The spots on the TLC autoradiogram are from [^{14}C]chloramphenicol (bottom) and its acetylated derivatives. Lane 1, the CAT control. Lanes 2–4, cells transfected with promoterless CAT plasmid p164-2. Lanes 5–7, cells transfected with pDR1000, which contains upstream regulatory sequences of the HLA-DR gene (22).

postulated to be a consequence of cytokines, such as interferon-γ, secreted by adjacent inflammatory T lymphocytes. The possibility that HBV gene products participate in HLA-DR induction could not be evaluated in infected humans. *In vitro* expression systems for HBV (29, 30) provide an opportunity to study the role of HBV infection in the induction of HLA-DR.

By using an *in vitro* expression system for HBV, we show here the coexpression of HBV gene products and HLA-DR in the hepatoma cell lines transfected with HBV genome. Because these cells do not express HLA-DR normally, our studies suggest that induced expression of HLA-DR resulted from HBV gene expression. Among HBV genes, the X gene product, now acknowledged to be a trans-activator, stimulates expression of the HLA-DR gene by directly interacting with its upstream regulatory element(s). The X protein has been shown to trans-activate promoters/enhancers of several viral and cellular genes (5, 6, 40). Recent studies have demonstrated that a truncated sequence derived from S ORF displays trans-activation function (41). This function, however, could not be seen when intact S ORF was used in their studies. In light of these results it is unlikely that these sequences contributed to the transcriptional trans-activation of HLA-DR gene in our experiments.

Studies from this and other laboratories localized NF-κB and AP-2 sequence motifs as possible sites of trans-activation by the X gene product (6, 9, 42). HLA-DR regulatory sequences employed here (about 1000 bp) do not contain these motifs, thus implying the role of other sequence motifs as likely targets of trans-activation by the X gene product. Further studies are necessary to identify the responsive element(s).

As this *in vitro* system excluded the potential that immune cells and cytokines participate in the induction of HLA-DR, the induction observed in this study must be the direct consequence of HBV gene expression. These results clearly

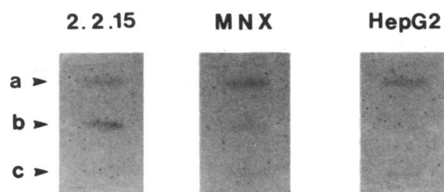

FIG. 4. Nuclear run-on analysis of HLA-DR gene. The DNA fragments (a, albumin cDNA; b, HLA-DR cDNA; c, plasmid pUC13) were loaded on the nitrocellulose filter and probed with radiolabeled run-on-generated RNA from the HepG2, MNX, and 2.2.15 cells as described previously (35, 36).

demonstrate that expression of HBV genomic sequences can induce expression of HLA-DR on human hepatoma cells in the absence of inflammation, active infection, or exogenous cytokines. Taking these findings together, it is tempting to speculate that natural HBV infection may lead to the transcription and activation of the HLA-DR gene during the course of the disease and that host immune response is initiated subsequently.

The potential of HBV to induce expression of HLA-DR in infected hepatocytes in the absence of inflammation and cytokines suggests that HBV may uniquely affect the immune response to viral infection. One obvious possibility is that infected hepatocytes become intrinsically capable of HBV antigen presentation of CD4$^+$ T lymphocytes that promote the development of CD8$^+$ T lymphocytes and antibody responses to infected cells. A vigorous immune response could eliminate infected cells from the liver. Conversely, expression of HLA-DR on the cell surface of infected hepatocytes, which are not normally antigen-presenting cells, might facilitate escape from immune hepatocytolysis by T-lymphocyte anergy. Indeed, it has been shown that presentation of antigen to T lymphocytes in the absence of appropriate "second signals" can produce clonal anergy (43). The clinical spectrum of HBV infection suggests that both these situations may exist. The majority of acute HBV infections can rapidly resolve. In chronic carriers of HBV, persistence of virus occurs without an apparent immune response or hepatocellular necrosis. In chronic active hepatitis B, a continuous immune response to the virus presumably causes necrosis culminating in cirrhosis. Our data raise the possibility that X protein may play a key role in the immunopathogenesis of HBV infection by regulating the magnitude of HLA class II-restricted immune responses of infected individuals. More work using hepatocyte cultures and animal model systems will be needed to explicate the role of X gene expression in the HBV infection and subsequent immunopathogenesis.

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