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Hepatitis B virus suppresses expression of human β-interferon

(interferon induction/trans-acting factor/core antigen/bovine papilloma virus vector)

JR-SHIN TWU*, CHAO-HUNG LEE†, PEI-MAO LIN†‡, AND ROBERT H. SCHLOEMER*

Departments of *Microbiology and Immunology and †Pathology, Indiana University School of Medicine, Indianapolis, IN 46223

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ABSTRACT **To determine whether hepatitis B virus (HBV) regulates the expression of the human β-interferon gene, a series of recombinant bovine papilloma virus plasmids containing the human interferon gene and various fragments of the HBV genome were constructed and used to transform C127 cells, a murine fibroblast line. Analysis of the DNA from transformed C127 cells indicated that the interferon gene was intact and that the plasmids replicated as stable multicopy elements. The 1828-base-pair *Bam*HI HBV DNA fragment containing the core antigen gene, but not the 2755-base-pair *Bgl* II HBV DNA fragment encoding both the surface antigen and the X antigen, suppressed the production of human β-interferon. No effect by any of the recombinant plasmids on the synthesis of murine interferon was detected. The suppression of human β-interferon by HBV occurs via a trans-acting factor. A frameshift mutation within the HBV core gene alleviates the inhibitory activity; thus we infer that the core protein is this factor or is crucially associated with this activity.**

Hepatitis B virus (HBV) is a double-stranded, circular DNA virus that is an etiological agent of acute and chronic hepatitis in man (1). HBV is also strongly implicated in the development of hepatocellular carcinoma, which has a predilection for males rather than females (2–4). Chronic hepatitis is considered to be due, in part, to an impaired immunological defense as indicated by decreased responses to phytohemagglutinin (5–7). Defects in other host defense mechanisms could also play a role in the development and/or maintenance of chronic HBV infections.

Interferons, a heterogeneous group of secreted cellular proteins, exhibit antiviral activity that protects homologous cells from viral infection and are induced by infection of cells with viruses (8–11). Interferon has been detected in serum of patients with a variety of viral illnesses but not in many individuals exhibiting chronic HBV infections (12–16). Evaluation of the interferon response by measuring the activity of the interferon-inducible enzyme (2′-5′)oligoadenylate synthetase also suggests that poor activation of the interferon system exists in chronic HBV carriers (17). Furthermore, a lack of production of interferon in peripheral blood mononuclear cells that were either stimulated *in vitro* with plant lectins or infected with viruses also indicates a defect in the expression of interferon in chronic HBV carriers (12).

To determine whether the decreased amounts of human interferon that are observed during HBV infection are due to a direct effect of a HBV moiety on the expression of the human interferon gene, we examined the effect of various fragments of HBV DNA on the expression of the cloned human β-interferon gene. In this study, we found that the production of human β-interferon was inhibited in cells containing the 1828-base-pair (bp) *Bam*HI HBV DNA fragment encoding the core antigen.

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MATERIALS AND METHODS

Plasmid DNA Constructions. The adw serotype of HBV was used in this study (18). The 1828-bp *Bam*HI HBV DNA fragment [map position (mp) 1400–28] or the 2755-bp *Bgl* II HBV DNA fragment (mp 2429–1984) was cloned into the same *Bam*HI site of the plasmid pdBPV-1, which had one of the two *Bam*HI sites destroyed (19). The resulting plasmids, pBHC and pBHS (Fig. 1), were used in further plasmid constructions. The plasmid pIF_R containing the human β-interferon gene as a 1.6-kilobase (kb) *Eco*RI–*Hin*dIII fragment (20) was linearized by digestion with *Eco*RI and the *Eco*RI ends were converted to *Hin*dIII ends. Following digestion with *Hin*dIII, the resulting 1.6-kb *Hin*dIII fragment containing the human β-interferon gene was isolated and inserted into the *Hin*dIII site of (*i*) pdBPV-1, (*ii*) pBHS, and (*iii*) pBHC, resulting in the formation of pBI, pBHSI, and pBHCI, respectively (Fig. 1).

An insertion mutation at the *Taq* I site (mp 2014) that is within the HBV core antigen gene was created by digesting circularized 1828-bp *Bam*HI HBV DNA fragments with *Taq* I. The linearized fragment was filled-in, ligated, and then cleaved with *Bam*HI. The resulting *Bam*HI HBV DNA fragment then was cloned into the *Bam*HI site of pBI, yielding pBIHCdTaqI. The insertion mutation resulted in the destruction of the *Taq* I site in the HBV core antigen gene and in a shift in the open reading frame.

Cell Cultures and DNA Transfections. Murine C127 fibroblasts were grown as described (20). One day before transfection, 8×10^4 cells were plated in each well of a 24-well plate; the cells were refed with fresh medium 4 hr before transfection. C127 cells were transfected by the calcium phosphate precipitation technique (21) with 0.2 μg of total plasmid DNA that included 50 ng of a plasmid containing the neomycin-resistance gene. Eight hours after transfection, cells were diluted and G418 (600 μg/ml) was added. After 14–21 days, well-separated foci were picked and stable transformants were grown to confluence in the presence of G418.

Interferon Induction and Assay. Induction of interferon by poly(I)·poly(C) in C127 cells that had been confluent for 4 days was performed as described (20). Uninduced cells were treated in the same manner except that poly(I)·poly(C) was omitted. Interferon was assayed by determining antiviral activity (inhibition of cytopathic effect) against vesicular stomatitis virus (11). Human interferon was titrated on human WISH cells, whereas murine interferon was titered on murine C127 cells.

Preparation and Analysis of Cellular DNA and RNA. Total cellular DNA was isolated from cells according to the method of Colbere-Garapin *et al.* (22), whereas total cellular RNA was prepared as described by Sackstein and Colten (23). Low molecular weight DNA was extracted by a modification of

Abbreviations: HBV, hepatitis B virus; BPV, bovine papilloma virus; mp, map position.

‡Present address: Department of Biology, Northwestern University, Xian, People's Republic of China.

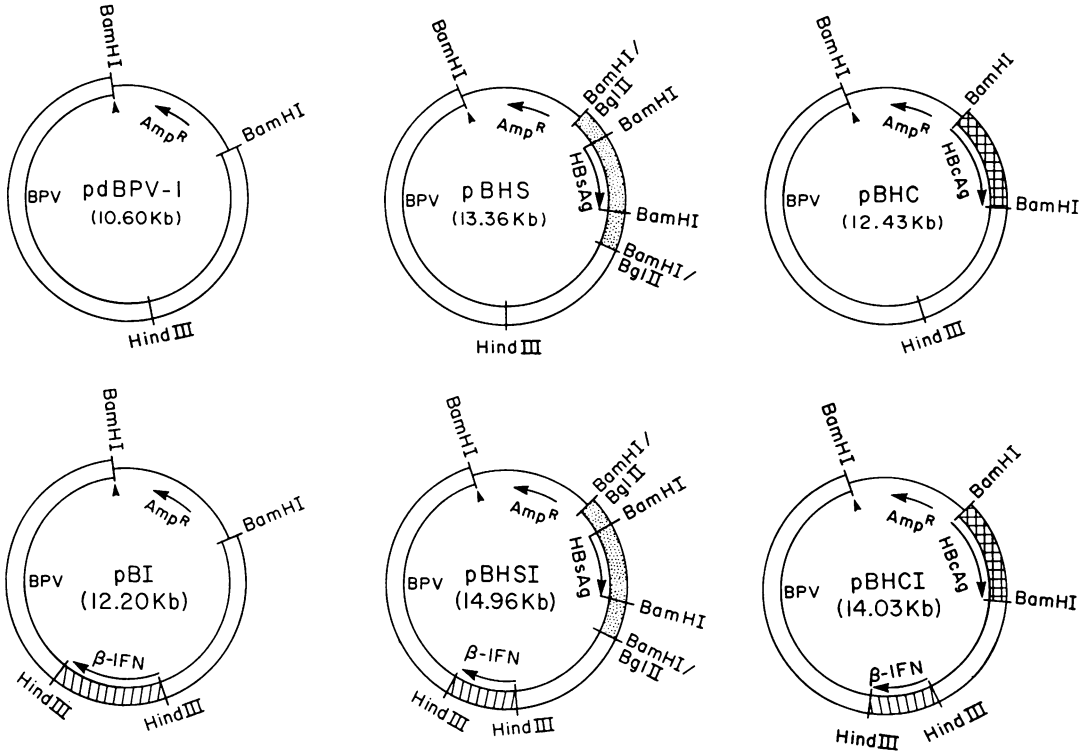

FIG. 1. Structure of bovine papilloma virus (BPV) plasmids containing inserted HBV DNA fragments and the human β-interferon gene. The HBV surface antigen (HBsAg) gene is represented by the stippled area, the HBV core antigen (HBcAg) gene by the crosshatched area, and the human β-interferon (β-IFN) gene by the slashed area. Open area indicates BPV DNA; the ampicillin-resistance gene (Amp^R) is shown as a line. Arrowhead indicates the destroyed *Bam*HI site of pdBPV-1. kb, Kilobases.

the procedure of Hirt (24). Agarose gel electrophoresis, Southern hybridization, and dot blot analyses were performed by standard methods (25).

RESULTS

Effect of HBV DNA on Expression of Human β-Interferon. To examine the effect of HBV on the production of human β-interferon, murine C127 cells were cotransfected with one of the recombinant BPV plasmids shown in Fig. 1 and a plasmid carrying the gene encoding neomycin resistance. Seven to 10 clones of cells from each transfection were isolated based upon the transformed phenotype and upon resistance to the drug G418. Each focus was expanded and analyzed for the production of human β-interferon after treatment of cells with poly(I)·poly(C). The presence of the interferon gene and the genes for the HBV core antigen and surface antigen was determined by dot blots of low molecular weight DNA from the transformants. Results for only three clones of each transfection are summarized in Table 1. Similar results were obtained when all the clones isolated from the same transfection were examined.

The low level of human β-interferon synthesized in cells transfected with pBI (C127/pBI cells) was increased 8- to

Table 1. Effect of HBV DNA on the induction of human and murine interferon activity

Cell line	Presence of gene			Interferon, units/ml		
				Human		Murine
	IFN	HBsAg	HBcAg	Uninduced	Induced	induced
C127	−	−	−	<40	<40	30,000
C127/pBI-1	+	−	−	640	5,120	30,000
C127/pBI-2	+	−	−	320	5,120	30,000
C127/pBI-3	+	−	−	320	5,120	30,000
C127/pBHSI-1	+	+	−	320	5,120	15,000
C127/pBHSI-2	+	+	−	320	10,240	30,000
C127/pBHSI-3	+	+	−	640	5,120	30,000
C127/pBHCI-1	+	−	+	<40	320	30,000
C127/pBHCI-2	+	−	+	<40	320	30,000
C127/pBHCI-3	+	−	+	<40	80	30,000
C127/pBHS-1	−	+	−	<40	<40	30,000
C127/pBHC-1	−	−	+	<40	<40	30,000

Interferon (IFN) activity values are from a single typical experiment. Presence of the gene segments was determined by dot blot analysis in which low molecular weight DNA prepared from each cell line was hybridized with nick-translated (*i*) 1.6-kb human β-interferon gene, (*ii*) 1372-bp *Bam*HI HBV DNA (mp 28–1400) to detect the HBV surface antigen (HBsAg) gene, and (*iii*) 421-bp *Bgl* II HBV DNA (mp 1984–2405) to detect the HBV core antigen (HBcAg) gene. Results of dot blots are expressed as whether the DNA fragment was present (+) or absent (−).

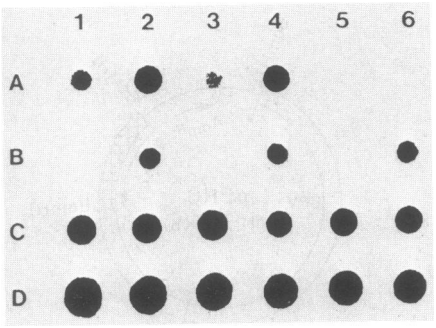

FIG. 2. Dot blot analysis of β-interferon RNA from transformed murine cells. Total cellular RNA was hybridized with nick-translated human interferon DNA (row A), murine β-interferon (26) (row B), tubulin cDNA (27) (row C), or actin cDNA (27) (row D). Columns 1 and 2, RNA isolated from C127/pBHSI cells; columns 3 and 4, RNA isolated from C127/pBI cells; columns 5 and 6, RNA from C127/pBHCI cells. Even-numbered columns contain RNA from cells treated with poly(I)·poly(C); odd-numbered columns contain RNA from mock-treated cells.

16-fold upon treatment with poly(I)·poly(C). In C127/pBHSI cells, the production of human β-interferon was not affected by the presence of the 2755-bp *Bgl* II fragment of HBV DNA on the BPV plasmid. In contrast, C127/pBHCI cells containing both the human β-interferon gene and the 1828-bp *Bam*HI fragment of HBV DNA produced much less human interferon in response to treatment with poly(I)·poly(C) than did C127/pBI cells. Furthermore, the reduction in the production of interferon also occurred in C127/pBHCI cells in the absence of treatment with poly(I)·poly(C).

The suppression in the production of interferon by the 1828-bp *Bam*HI fragment of HBV DNA was specific for human β-interferon. No inhibition in the production of murine interferon in C127 cells transformed with the various plasmids was detected (Table 1). Moreover, the levels of RNA specific for murine interferon, actin, or tubulin were unaffected by the presence of any of the HBV-containing plasmids (Fig. 2). The amount of human interferon-specific RNA was reduced only in C127/pBHCI cells.

Analysis of Human Interferon Gene in Transformed C127 Cells. As shown in Fig. 3, *Hin*dIII-digested total cellular

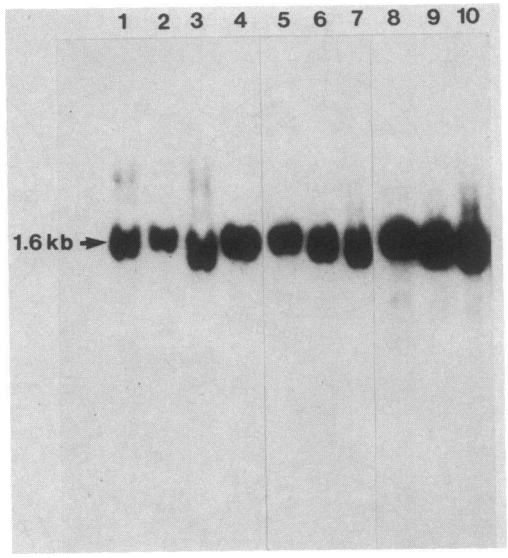

FIG. 3. Analysis of human β-interferon gene isolated from transformed murine cell lines. Total cellular DNA was digested with *Hin*dIII. The resulting digests were subjected to electrophoresis in an ethidium bromide/1% agarose gel, transferred to Nytran membrane, and hybridized to nick-translated 1.6-kb *Hin*dIII human β-interferon DNA. Lane 1, 1.6-kb *Hin*dIII interferon DNA; lanes 2–4, DNA of three clones of C127/pBI cells; lanes 5–7, DNA from three clones of C127/pBHSI cells; lanes 8–10, DNA from three clones of C127/pBHCI cells.

DNA extracted from all cell lines transfected with either pBI, pBHSI, or pBHCI plasmids contained a 1.6-kb *Hin*dIII DNA fragment that hybridized to the human interferon gene probe. Since the interferon gene was inserted into the BPV plasmids as a 1.6-kb *Hin*dIII DNA fragment, these results indicate that the interferon gene was not structurally altered as a result of the transfection. Furthermore, the lack of high molecular weight DNA that hybridized to the interferon gene probe suggests that integration of the recombinant BPV plasmids did not occur.

Total cellular DNA isolated from C127/pBI cells, C127/pBHSI cells, or C127/pBHCI cells was also digested with

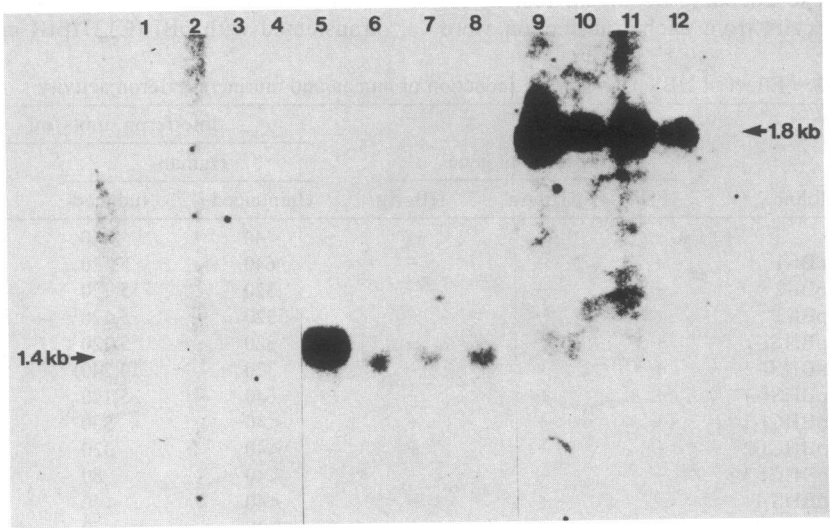

FIG. 4. Analysis of HBV DNA sequences isolated from transformed cell lines. Total cellular DNA isolated from transformed C127 cells was digested with *Bam*HI. The resulting digests were electrophoresed in 1% agarose gels, transferred to Nytran membranes, and hybridized to nick-translated 1372-bp *Bam*HI HBV DNA (mp 28-1400) to detect the HBV surface antigen gene (lanes 5–8), to nick-translated 1828-bp *Bam*HI HBV DNA to detect the HBV core antigen gene (lanes 9–12), or to both HBV DNA probes (lanes 1–4). Lane 1, DNA from C127 cells; lanes 2–4, DNA from three clones of C127/pBI cells; lane 5, *Bam*HI-digested pBHSI; lanes 6–8, DNA from three clones of C127/pBHSI cells; lane 9, *Bam*HI-digested pBHCI; lanes 9–12, DNA from three clones of C127/pBHCI cells.

Table 2. Trans-acting nature of the suppression of interferon by HBV DNA

Plasmids	Clone	Presence of gene		Human interferon, units/ml	
		IFN	HBcAg	Uninduced	Induced
None	1	−	−	<40	<40
pBI + pdBPV-1	1	+	−	<40	5120
	2	+	−	<40	5120
	3	+	−	<40	5120
pBI + pBHC	1	+	+	<40	160
	2	+	+	<40	160
	3	+	+	<40	160

C127 cells were cotransfected with the indicated plasmids in the presence of a third plasmid that carried the neomycin-resistance gene. Isolated foci of cells that were resistant to G418 were picked and expanded. Interferon assays and dot blot hybridization of low molecular weight DNA isolated from the cells were performed as described for Table 1.

*Bam*HI, electrophoresed, and probed with ^{32}P-labeled HBV DNA probes. Since the 2755-bp *Bgl* II HBV DNA fragment in pBHSI contains two *Bam*HI sites, a HBV DNA fragment ≈1.4 kb long should be generated by *Bam*HI digestion. Correspondingly, *Bam*HI digestion of pBHCI should result in the appearance of a 1.8-kbp HBV DNA fragment. Southern blot hybridization (Fig. 4) indicated that all C127/pBHSI clones contained the gene for the HBV surface antigen, whereas the gene encoding the core antigen was detected in extracts of all C127/pBHCI cells.

The copy number of plasmids in the various transformed cell lines was determined by dot blot hybridizations of cellular DNA, using both BPV DNA and the 1.6-kb *Hin*dIII interferon gene as probes. The results indicated that all of the transfected cell lines contained between 15 and 25 copies of the plasmid per cell (data not shown).

Suppression of Human β-Interferon Is Mediated by a Trans-Acting HBV Factor. To determine whether the observed suppression of the production of human β-interferon by the 1828-bp *Bam*HI HBV DNA fragment occurs via a trans-acting factor, a series of transfections was performed in which C127 cells were cotransfected with two plasmids, one containing the human β-interferon gene (pBI) and the other carrying the 1828-bp *Bam*HI HBV DNA fragment (pBHC). As controls, cells were cotransfected with pBI and pdBPV-1. All clones of cells stably transfected with pBI and pdBPV-1

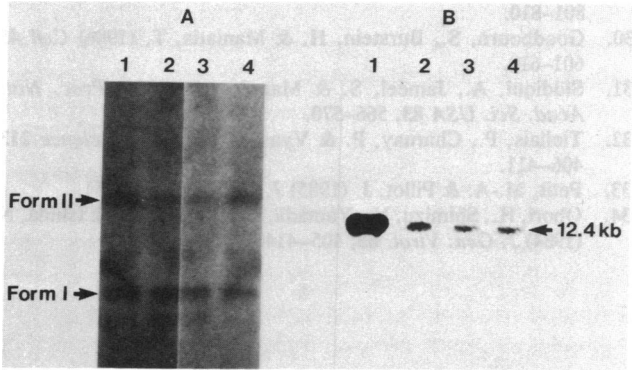

FIG. 5. Analysis of plasmids in C127 cells cotransfected with two recombinant BPV plasmids. Low molecular weight DNA prepared from three clones of C127 cells cotransfected with pBI and pBHC was digested with *Sac* II. After electrophoresis and transfer to Nytran membranes, DNA was hybridized either with labeled 1.6-kb *Hin*dIII interferon DNA (*A*) or with labeled 1828-bp *Bam*HI HBV DNA (*B*). Lane 1 in *A*, *Sac* II-digested pBI; lane 1 in *B*, *Sac* II-digested pBHC; lanes 2–4 in both *A* and *B*, DNA from cell clones 1–3, respectively.

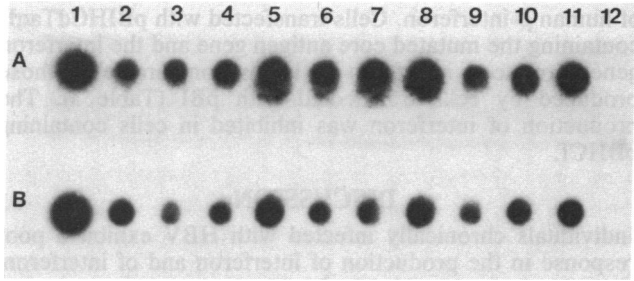

FIG. 6. Dot blot analysis of DNA from subclones of C127/(pBI+pBHC-1) cells. One clone of cells isolated after cotransfection of C127 cells with pBI and pBHC was subcloned. Low molecular weight DNA was prepared from nine subclones for dot blot hybridization using either 1.6-kb human interferon gene (row A) or 1828-bp *Bam*HI HBV DNA fragment (row B) as probe. Position 1, DNA from parental cell line C127/(pBI+pBHC-1); positions 2–10, DNA from the nine subclones; position 11, pBI (row A) or pBHC (row B); position 12, pdBPV-1.

contained the interferon gene and produced high levels of human β-interferon upon treatment with poly(I)·poly(C) (Table 2). However, all clones of cells stably transfected with both pBI and pBHC produced very low levels of human interferon although these cells contained both plasmids.

To demonstrate that the plasmids in cells transfected with pBI and pBHC remained as distinct entities, low molecular weight DNA was isolated from the cells and then digested with *Sac* II. The plasmid pBHC contains a single *Sac* II site, which lies within the HBV DNA fragment; pBI lacks a *Sac* II site. Therefore, only pBHC should be linearized by digestion with *Sac* II. Two bands, representing form I and form II of pBI, were detected when the 1.6-kb interferon gene was the probe (Fig. 5*A*). When the *Sac* II-digested DNA was hybridized with the HBV DNA probe, only one band, representing linearized plasmids, was observed (Fig. 5*B*). As further evidence that the two plasmids were in each cell of the focus, one clone of the C127 cells that contained pBI and pBHC was subcloned and each of the nine subclones assayed for the presence of pBI and pBHC. All nine subclones contained DNA that hybridized to the interferon gene probe and to the HBV core antigen gene probe (Fig. 6).

Frameshift Mutation in HBV Core Gene Abolishes Inhibitory Activity. A frameshift mutation within the gene encoding the HBV core antigen was created to determine whether the disruption of the open reading frame would abolish the ability of the 1828-bp *Bam*HI HBV DNA to suppress the production

Table 3. Frameshift mutation within the HBV core antigen gene eliminates suppression of interferon expression

Plasmid	Clone	Presence of gene		Human interferon, units/ml	
		IFN	HBcAg	Uninduced	Induced
None	1	−	−	<40	<40
pBI	1	+	−	<40	2,560
	2	+	−	<40	2,560
	3	+	−	<40	5,120
pBHCI	1	+	+	<40	160
	2	+	+	<40	80
	3	+	+	<40	160
pBIHCdTaqI	1	+	+	<40	5,120
	2	+	+	<40	2,560
	3	+	+	<40	10,240

C127 cells were cotransfected with the indicated plasmids in the presence of a third plasmid that carried the neomycin-resistance gene. Isolated foci of cells were picked and analyzed for DNA and for the production of interferon as described for Tables 1 and 2.

of human β-interferon. Cells transfected with pBIHCdTaqI, containing the mutated core antigen gene and the interferon gene, produced interferon at levels comparable to those produced by cells transfected with pBI (Table 3). The production of interferon was inhibited in cells containing pBHCI.

DISCUSSION

Individuals chronically infected with HBV exhibit a poor response in the production of interferon and of interferon-inducible enzymes (12–17). In this paper, evidence is provided that indicates a potential molecular basis for such responses. We have demonstrated that a fragment of HBV DNA containing the gene for the core antigen causes a drastic inhibition in the expression of human β-interferon.

The expression of human β-interferon is under complex regulation (20, 26, 28–30). The DNA at the 5′ end flanking the structural gene for β-interferon contains an enhancer element and interacts with cellular positive and negative regulatory trans-acting factors. The exact manner by which the putative HBV inhibitory factor suppresses human β-interferon synthesis remains to be investigated but involves a decrease in levels of RNA specific for human interferon. The observation that the synthesis of murine interferon is not inhibited by HBV DNA suggests that the mechanism of suppression of human interferon does not involve factors, such as the uptake of and induction with poly(I)·poly(C), that are common to the expression of both the human and the murine interferon genes.

Examination of the 1828-bp HBV DNA fragment indicates that the only intact gene present is that which encodes the core antigen. It is unlikely that the inhibitory factor is encoded by the partial X gene in the 1828-bp *Bam*HI fragment, since the 2755-bp *Bgl* II fragment, which contains the intact X gene and its promoter (31), does not inhibit the synthesis of human interferon. Furthermore, HBV DNA that contains a frameshift mutation within the gene for the core antigen does not suppress the production of human β-interferon. Thus, the inhibitory factor is encoded by the core antigen gene and could be the core antigen itself.

The carboxyl terminus of the core antigen contains a region rich in arginine, serine, and proline and contains sequences resembling protamine and other DNA-binding proteins (32). The core protein has been shown to bind to DNA, presumably via basic amino acids (33). Thus, a plausible hypothesis is that the HBV core antigen binds to the regulatory region of the interferon gene and prevents transcription of the gene. However, another HBV antigen, the e antigen, is also encoded by the core antigen gene region (34) and hence is another potential candidate as the inhibitory factor.

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