Journal of General Virology (1999), **80**, 1253–1262. Printed in Great Britain

Inhibition of interferon-inducible MxA protein expression by hepatitis B virus capsid protein

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Chronic hepatitis B treatment has been significantly improved by interferon (IFN) treatment. However, some studies have suggested that hepatitis B virus (HBV) might have a direct effect on the resistance to IFN. Defective particles, generated by spliced HBV RNA and associated with chronic hepatitis B, have been previously characterized; expression of these particles leads to cytoplasmic accumulation of the capsid protein. The aim of this study was to investigate the role of these defective genomes in IFN resistance. The global antiviral activity of IFN was studied by virus yield reduction assays, the expression of three IFN-induced antiviral proteins was analysed by Western blotting and confocal microscopy, and the regulation of MxA gene expression was studied by Northern blotting and the luciferase assay, in Huh7 cells transfected with a complete or the defective HBV genome. Results showed that the expression of the defective genome reduces the antiviral activity of IFN and that this modulation involves a selective inhibition of MxA protein induction by the HBV capsid protein. Our results also show the trans-suppressive effect of the HBV capsid on the MxA promoter, which might participate in this phenomenon. In conclusion, this study shows a direct interplay between the IFN-sensitive pathway and the capsid protein and might implicate this defective HBV genome in virus persistence.

Introduction

Hepatitis B virus (HBV) is a major cause of cirrhosis and hepatocarcinoma (Beasley, 1988; Bréchot, 1997). However, the incidence of chronic infection depends upon the time of exposure. Children born to infected mothers invariably become chronically infected, while the rate of development of chronic infection decreases to 20% for those infected between the first and second years, and to less than 5% for those in adulthood.

The treatment of chronic hepatitis B has been improved by the use of interferon (IFN)-α. IFN induces a number of different proteins which mediate antiviral, antiproliferative or other cellular effects (Pestka *et al.*, 1987). The antiviral action of IFN is indeed mediated by the induction and activation of at least three proteins, 2′,5′-oligoadenylate synthetase (OAS), p68 protein kinase (PK) and the MxA protein (Sen & Ransohoff, 1993). However, after IFN treatment, only about 30–40% of patients show clearance of HBV replication markers, normalization of transaminases and improvement in liver histology (Perrillo *et al.*, 1990). Several *in vivo* studies have shown a lack of activation of the IFN system in patients with acute or chronic hepatitis B (Ikeda *et al.*, 1986; Jakschies *et al.*, 1993; Nishiguchi *et al.*, 1989; Poitrine *et al.*, 1985). In addition, previous *in vitro* studies have suggested that HBV DNA might play a direct role in the development of resistance to endogenous or exogenous IFN. Expression of the HBV genome in a human amniotic cell line (FL5-1) has been shown to reduce sensitivity to IFN *in vivo* (Onji *et al.*, 1989). Several different mechanisms have been proposed to account for this effect (Sen & Ransohoff, 1993). HBV capsid protein (referred to as the HBc protein) has been shown to trans-suppress IFN-β gene expression (Whitten *et al.*, 1991); whilst in another study,

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the expression of HBV polymerase terminal protein was shown to inhibit IFN-induced 6-16 gene expression (Foster *et al.*, 1991). This latter result, however, is still debated (Foster *et al.*, 1995). In biopsy samples from chronically infected patients, HBV polymerase-expressing cells lacked expression of HLA class I antigens in response to IFN (Foster *et al.*, 1993). Finally, it has been shown recently that MxA responsiveness to IFN-α was diminished significantly in chronic hepatitis B patients (Fernandez *et al.*, 1997).

The mechanisms involved in the development of a chronic carrier state are still poorly understood, although they probably involve both viral and host factors. Recently, emphasis has been placed on the potential role of mutations in the HBc protein- and envelope-encoding regions (Gerken *et al.*, 1991; Tran *et al.*, 1991) and their interplay with the immune system. The role of defective particles in virus persistence has also been suggested for several viruses (Holland, 1990). In agreement with this, we have previously shown that the encapsidation of the singly spliced 2·2 kb HBV RNA leads to the secretion of circulating HBV defective particles (referred to as dHBV DNA) in patients who develop chronic hepatitis (Rosmorduc *et al.*, 1995; Terre *et al.*, 1991). These dHBV particles are detected at a higher level in the sera of patients with acute hepatitis progressing to chronic hepatitis, or with established chronic hepatitis, than in patients recovering from acute hepatitis (Rosmorduc *et al.*, 1995). This suggests that the presence of such dHBV particles might be associated with a chronic course of HBV infection and with virus replication. In addition, sequence analysis of the dHBV DNA shows a deletion from the last codon of the core gene to the middle of the S gene that creates new potential open reading frames (i.e. truncated S and P proteins and a small P fusion protein), in addition to the C and X open reading frames (Rosmorduc *et al.*, 1995). Finally, we have demonstrated that *in vitro* expression of dHBV DNA induces marked intracellular accumulation of the p21 HBc protein and increased secretion of HBeAg (Rosmorduc *et al.*, 1995).

The aim of our present study was to determine the potential role of this deregulated expression of HBV viral proteins on the antiviral action of IFN. With this goal in mind, we have analysed the global antiviral effect of IFN in cells that are stably transfected with dHBV DNA. We have also examined the expression profiles of cellular proteins induced by IFN and implicated in its antiviral effect in these cells. Our results demonstrate that accumulation of the HBc protein markedly reduces the antiviral effect of IFN. They also show that this is due to a direct inhibition of MxA expression on HBc protein accumulation.

Methods

■ **Plasmids.** The dHBV DNA corresponded to the 2·2 kb singly spliced HBV RNA inserted as a head-to-tail dimer in the Bluescript plasmid (Stratagene). HBV DNA, a wild-type complete HBV construct under the control of the C gene promoter, has been previously described (Rosmorduc *et al.*, 1995). The 550 bp minimal MxA gene promoter (−553, +10) (Horisberger *et al.*, 1990), was inserted in front of the luciferase gene of the pGL2 basic vector (Promega) at the *Bam*HI/*Kpn*I restriction sites (this plasmid was referred to as pMxA550-Luc). The p6-16 CAT plasmid corresponded to the CAT reporter gene under the control of the IFN-sensitive response element (ISRE) of the human 6-16 gene (Porter *et al.*, 1988). The preC/C coding region was amplified by PCR from a cloned wild-type HBV DNA plasmid using primers C/s (5′ GGATCCATGCAACTTTTTCACCTCTGCCTA) and C/as (5′ CTAC-TCGAGTTGGGAGCTGGAGATTGAGATCTT). This amplified pre-C/C region was inserted into *Hin*dIII/*Sal*I sites in the polylinker of an eukaryotic expression plasmid, under the control of the CMV promoter. This plasmid was referred to as pHBc. The complete sequence of this fragment showed a point-mutation at nt 1829, which prevented expression of HBeAg.

■ **Transfection experiments.** Briefly, $2{\cdot}5 \times 10^6$ hepatocarcinoma-derived Huh7 cells (Nakabayashi *et al.*, 1982) were transfected with 20 μg dHBV DNA, pHBc or HBV DNA, for transient experiments, using the calcium phosphate precipitation method, as described previously (Chang *et al.*, 1987). In order to obtain stable clones, 20 μg of either dHBV DNA or HBV DNA was co-transfected with 5 μg of a plasmid expressing the neomycin-resistance gene under the control of the SV40 early promoter. The clones were selected in medium containing 400 μg/ml geneticin (G418 sulphate; Gibco BRL).

■ **Virus yield reduction assays.** Cells (10^5) stably transfected with dHBV DNA or HBV DNA, and negative controls (cells transfected with the neomycin-resistance gene) were untreated or treated with 50, 100 or 500 IU/ml interferon-α-2a (referred to as 2aIFN; Produits Roche) for 20 h, and infected with vesicular stomatitis virus (VSV), or encephalomyocarditis virus (EMCV) at an m.o.i. of 0·1 for 1 h. After discarding the viral inoculum, fresh medium was added and the cells were incubated for a further 16 h at 37 °C. The supernatants were frozen at −80 °C. Vero cells were used for titration in plaque assays in 1% methylcellulose-containing medium, as described previously (Burleson *et al.*, 1991). The number of p.f.u./ml was counted for each point in duplicate, after fixation with formaldehyde 10% in PBS for 15 min and coloration with Crystal Violet. The results are presented on semi-logarithmic curves.

■ **Western blot analysis.** The HBc protein and three IFN-induced proteins involved in the antiviral effect of IFN (OAS, PK and the MxA protein) were detected by Western blot analysis and quantified in the stably transfected cells. The HBc protein was detected in dHBV DNA- and HBV DNA-transfected cells using a rabbit anti-HBc antibody (1 μg/μl) (1/100 dilution). After treatment of the cells with either 100 or 500 IU/ml 2aIFN for 20 h, the IFN-induced proteins were detected using three specific antibodies (1 μg/μl), treated for 16 h at 4 °C: a mouse polyclonal antibody against p100 OAS (1/300); a MAb against the MxA protein (1/300); and a MAb against p68 PK (1/300). Detection was carried out using peroxidase-conjugated secondary antibodies (1/2000 for 1 h) and a chemiluminescent assay (ECL; Amersham). The different proteins were semi-quantified by optical density (OD) scanning of the blot.

■ **Immunofluorescence and confocal microscopy analysis.** Cells either transiently or stably transfected with dHBV DNA, HBV DNA or pHBc were plated at low density onto sterile 10 mm round glass cover-slips and cultured for 24 h. The cells were then treated with 500 IU/ml 2aIFN for 20 h, fixed and permeabilized with cold acetone (−20 °C) for 10 min. Unspecific binding was blocked with 10% normal goat serum in PBS and, for immunofluorescence using the biotin–

streptavidin method, cells were incubated further with avidin followed by biotin, according to the manufacturer's instructions (Vector). Dilutions of primary antibodies were as follows: rabbit anti-HBc, 1/100; mouse anti-MxA, 1/200; and mouse anti-p100 OAS, 1/100.

For single labelling, either FITC-conjugated goat anti-rabbit or biotinylated donkey anti-rabbit, followed by incubation in streptavidin–FITC were used. Nuclear DNA was counter-stained with propidium iodide. HBc protein was semi-quantified in cells that were stably transfected with dHBV DNA and HBV DNA by measuring the mean fluorescence intensity of the whole cell and a longitudinal section, using confocal microscopy analysis.

For double labelling, the additional secondary antibodies used were Texas Red-conjugated sheep anti-mouse (MxA protein or p100 OAS detected as red fluorescence) and the streptavidin–FITC method as described in single labelling experiments (HBc protein detected as green fluorescence). Confocal microscopy of samples labelled with two fluorophores was performed with a confocal laser scanning microscope (Leica Instruments), which uses an argon/krypton laser operating in multi-line mode. Most of the cell preparations labelled with both fluorescein and Texas Red conjugates were sequentially analysed at wavelengths of 488 and 567 nm with filters that transmit light very selectively and optimally. Some cell preparations stained with a fluorescein conjugate and propidium iodide were also analysed with the less selective simultaneous mode of excitation and acquisition. Under our conditions, no overlap from one channel into the other was detected, even when using this last approach. FITC emission was detected through a narrow-band filter centred at 535 nm, while Texas Red was detected through a long-wave pass filter OG 590. For each selected field, four to eight sections, taken at steps of 0·5 μm, were recorded.

■ **Northern blot analysis.** Stably transfected cells and controls were treated with 500 IU/ml 2aIFN for 20 h. RNAs were extracted by a guanidinium thiocyanate-based method (Chomczynski & Sacchi, 1987) using a commercial kit (RNAβ; Bioprobe). RNAs (20 μg) were analysed by Northern blotting. The membranes were sequentially hybridized with probes specific for HBV DNA, OAS p40-46 and 6-16 genes and with the full-length human MxA cDNA (Horisberger *et al.*, 1990). Standardization was achieved with a probe corresponding to the 28S ribosomal gene.

■ **Luciferase and CAT assays.** Briefly, for the luciferase assay, Huh7 cells were transfected with 5 μg pMxA550-Luc alone, or co-transfected with 15 μg dHBV DNA, pHBc or HBV DNA. Two days after transfection, the cells were split into two identical aliquots by standard trypsinization. One aliquot was treated with 500 IU/ml 2aIFN for 20 h and the other served as a control sample. The cells were then lysed and the extracts were clarified by a short centrifugation. The standard luciferase assay was performed on 50 μl clarified supernatant, using a Lumat LB 9501 Berthold luminometer. For the CAT assay, Huh7 cells were transfected with 3 μg p6-16 CAT alone or co-transfected with 10 μg pHBc. The transfected cells were treated as mentioned above. CAT expression was quantified using the *Quan-T*-CAT assay system (Amersham). All experiments were done at least in duplicate.

Results

Characterization of clones stably transfected with dHBV DNA or HBV DNA

We have obtained stable clones expressing either dHBV DNA or HBV DNA. Northern blot analysis of cells stably transfected with dHBV DNA showed the expected 2·2 kb singly spliced HBV transcript and an additional band at 4·3 kb corresponded to readthrough events (Fig. 1*a*, lower panel), as previously described (Rosmorduc *et al.*, 1995). Northern blot analysis of the HBV DNA-transfected clone showed a band of 3·5 kb in size, corresponding to the pregenomic HBV RNA, a very faint band at 2·4 kb and a band at 2·1 kb, corresponding to the transcripts encoding the envelope proteins (Fig. 1*a*, upper panel), as previously described (Rosmorduc *et al.*, 1995).

Using immunofluorescence, we detected overexpression of the HBc protein in all cells transfected with dHBV DNA (data not shown). We have semi-quantified the HBc protein in the

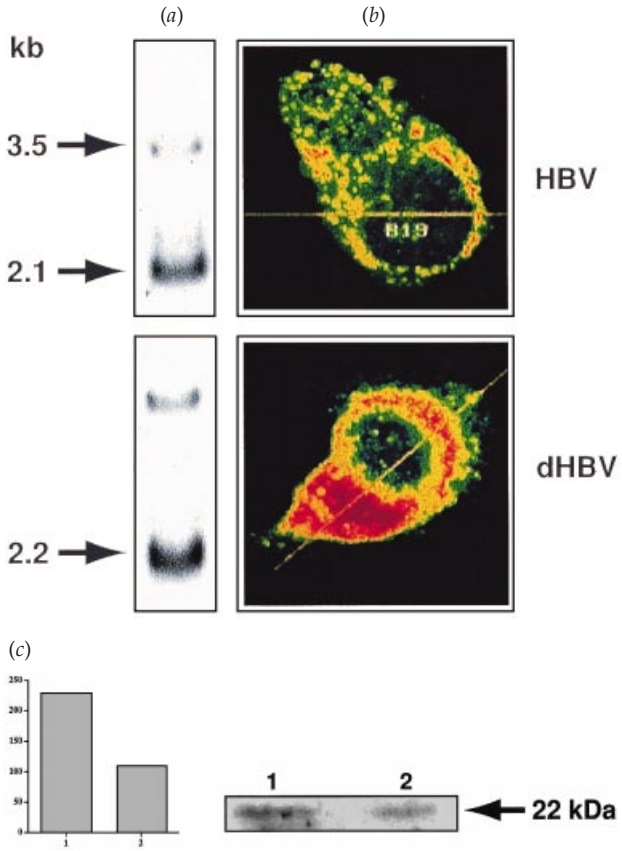

Fig. 1. Characterization of the viral transcripts and proteins in stably transfected clones. (*a*) Northern blot analysis of the HBV transcripts from Huh7 cells stably transfected with HBV DNA (upper panel) or dHBV DNA (lower panel). After Northern blotting, the membrane was hybridized with a full-length HBV DNA probe. The sizes corresponding to the specific HBV transcripts (3·5, 2·2 and 2·1 kb) are indicated. For the dHBV DNA-transfected cells, the additional band at 4·3 kb corresponds to readthrough events (data not shown). (*b*) Immunofluorescence detection, followed by semi-quantitative confocal microscopy analysis of HBc protein expression in cells stably transfected with HBV DNA (upper panel) and dHBV DNA (lower panel). The fluorescence intensity in these transfected cells is presented using an arbitrary coloured scale: blue to green (low, 0–120); yellow (intermediate, 120–200), and orange to red (high to saturated, > 200). (*c*) Western blot analysis of the HBc protein expression in cells stably expressing dHBV DNA (lane 1) and HBV DNA (lane 2). Quantification was performed by OD analysis and is represented as a histogram.

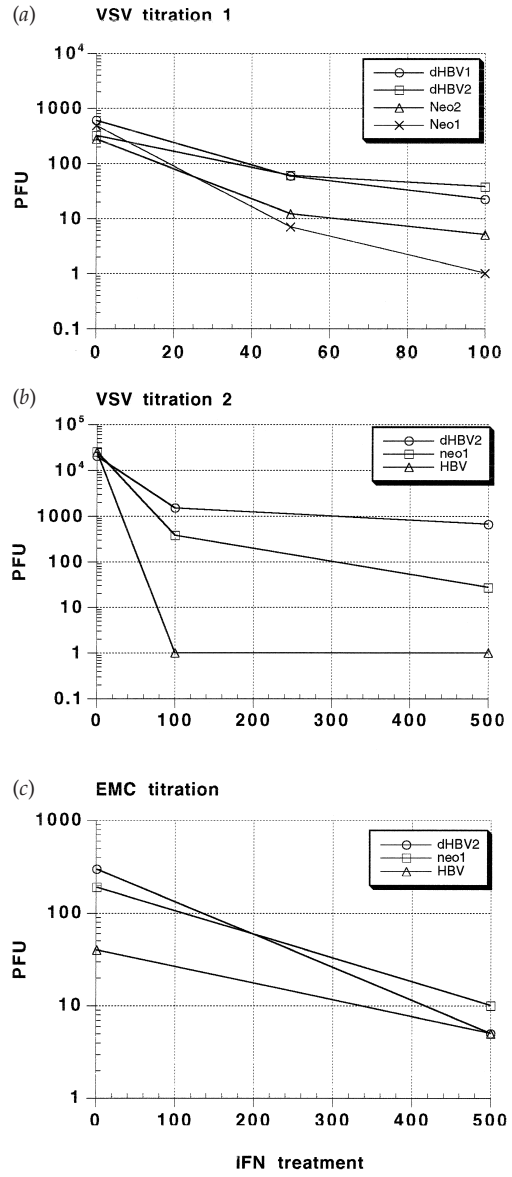

Fig. 2. Analysis of global antiviral effect of IFN in dHBV DNA- and HBV DNA-transfected cells. The global antiviral activity of IFN was investigated by virus yield reduction assays, after VSV (*a*) and (*b*), or EMCV (*c*) infection. Virus titration was performed in supernatants of cells transfected with dHBV DNA (dHBV1 and dHBV2), HBV DNA (HBV), or the neomycin-resistance gene (neo1 and neo2) as described in Methods. The figures represent the number of p.f.u./ml following 2alFN treatment (0, 50, 100 or 500 IU/ml for VSV and 0 or 500 IU/ml for EMCV) on semi-logarithmic curves.

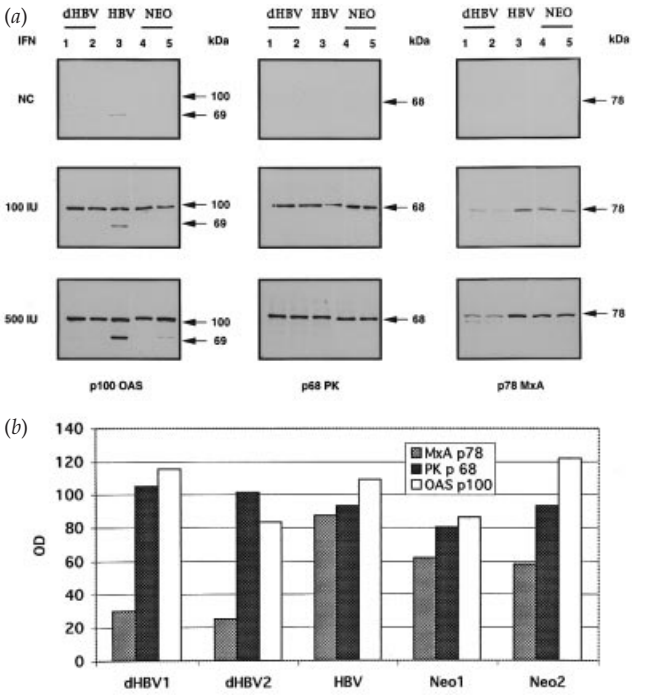

Fig. 3. Western blot analysis of the IFN-induced proteins in the stably transfected cells. (*a*) Proteins were extracted from two independent clones expressing dHBV DNA (lanes 1 and 2), one clone expressing HBV DNA (lane 3), and two clones only transfected with the neomycin-resistance gene (lanes 4 and 5). The cells were either untreated (NC), or treated with 100 or 500 IU/ml 2alFN. The different antibodies used for the Western blot were as follows: an anti-p100 OAS polyclonal antibody (left panels); an anti-p68 PK MAb (middle panels); and an anti-p78 MxA MAb (right panels). The sizes corresponding to the specific proteins are indicated. Semi-quantification of the different proteins is represented on a histogram (*b*) using OD scanning.

cells stably transfected with dHBV DNA and HBV DNA using immunofluorescence followed by confocal scanning microscopy. In brief, we have measured the fluorescence intensity in five independent cells either globally, using a coloured scale, or in a longitudinal section of the cells. In the latter case, the fluorescence intensity was represented graphically according to the cell length and estimated by measuring the area located under the curve (Fig. 1*b*). HBc protein expression was also quantified by Western blot analysis in both the dHBV DNA- and HBV DNA-transfected cells (Fig. 1*c*). Altogether, amounts of HBc protein in the dHBV DNA stably transfected clones were from 2·5- to 5-fold higher than those detected in HBV DNA-transfected cells. This is in agreement with our previous data determined using transient transfection experiments (Rosmorduc *et al.*, 1995).

Analysis of the global antiviral effect of IFN in cells stably transfected with dHBV DNA or HBV DNA

The IFN antiviral activity against VSV and EMCV was investigated by virus yield reduction assays with cells stably transfected with dHBV DNA or HBV DNA. The VSV and EMCV growth in dHBV DNA- or HBV DNA-transfected cells or the negative control was evaluated by determining the p.f.u./ml and is presented on semi-logarithmic curves (Fig. 2).

In a first set of experiments, the IFN antiviral activity against VSV was investigated on two dHBV stable clones and on two neo-stable clones, after treatment with 50 or 100 IU/ml IFN. In summary, IFN treatment induced an overall decrease in

the number of VSV p.f.u. of 18- and 200-fold for dHBV and neo-stable clones, respectively (Fig. 2*a*). This corresponded to a significant inhibition of the IFN antiviral activity against VSV in the dHBV DNA-expressing cells. Data from two additional independent experiments were consistent with this result (data not shown).

In a second set of experiments, IFN antiviral activity was compared between dHBV DNA- and HBV DNA-expressing cells (Fig. 2*b*). The decrease in the number of p.f.u. in cells expressing the dHBV DNA (clone dHBV2) was 13- and 30-fold, compared to untreated cells, after treatment with 100 and 500 IU/ml 2aIFN, respectively (Fig. 2*b*). Under the same conditions, the decrease in the number of p.f.u. was 65- and 900-fold in the cells expressing the neomycin-resistance gene (clone neo1), and approximately 20000-fold in HBV DNA-expressing cells (Fig. 2*b*). Thus, after treatment with 500 IU/ml 2aIFN, VSV replication increased 30- to 600-fold in dHBV DNA-transfected cells, in comparison to neomycin-resistance gene- and HBV DNA-transfected cells, respectively. Again, this indicated a lower protection of IFN against VSV infection in dHBV DNA-transfected cells. Data from two additional independent experiments were consistent with this result (data not shown).

The examination of the EMCV-infected cells, after treatment with 500 IU/ml 2aIFN, showed a mean decrease in the number of p.f.u. of 10-fold for the HBV DNA-transfected cells, 20-fold for untreated cells, and 80-fold for the dHBV DNA-transfected cells (Fig. 2*c*). Thus, in contrast to VSV infection experiments, we did not observe any significant inhibition of the 2aIFN antiviral effect on EMCV replication in the dHBV DNA-transfected cells.

In summary, these results demonstrate that expression of dHBV DNA modulates the 2aIFN-induced antiviral activity involved in the defence against VSV but not against EMCV.

Analysis of IFN-induced proteins in transfected cells

In order to determine whether expression of dHBV DNA could modulate the expression of IFN-induced proteins, we have analysed, by Western blotting, the three main proteins involved in the antiviral effect of IFN (namely OAS, PK and the MxA protein) in the different stably transfected cells.

Using an anti-p100 OAS polyclonal antibody, the amount of this protein was found to be comparable in dHBV DNA-transfected cells, HBV DNA-transfected cells and negative controls (OD scanning of 170–240). An additional 69 kDa band was also observed, more strongly (about 5-fold more intense) in cells expressing HBV DNA (Fig. 3*a*, left panels, and *b*).

Using an anti-p68 PK MAb, no significant difference in the amount of this protein was found in cells expressing dHBV DNA, HBV DNA and negative controls (OD scanning of 80–105) (Fig. 3*a*, middle panels, and *b*).

In contrast, using an anti-MxA polyclonal antibody, we observed, in three independent experiments, that the amount of this protein was on average 3-fold lower in two independent clones expressing dHBV DNA (OD of 25) compared to cells expressing HBV DNA and neomycin-resistance gene-expressing cells (OD of 60–90) (Fig. 3*a*, right panels, and *b*). As a control, we hybridized in parallel an independent blot with both anti-p68 PK and anti-MxA antibodies. OD scanning analysis of the result confirmed a decrease of the order of 3-fold in the level of MxA protein in dHBV DNA-transfected cells, after treatment with both 100 and 500 IU/ml 2aIFN, compared to HBV DNA-transfected cells and neomycin-resistance gene-expressing cells. In contrast, the amount of p68 PK was found to be consistent in all cells, irrespective of the treatment (data not shown).

Thus, after IFN treatment, we have obtained evidence for an overall decrease of 3-fold in the level of the MxA protein in cells stably expressing dHBV DNA, when compared to cells expressing HBV DNA and the neomycin-resistance gene. In contrast, the amount of p100 OAS and p68 PK remained unchanged in all stably transfected cells.

HBc protein accumulation in dHBV DNA- and pHBc-transfected cells reduces the MxA expression

Transient, as well as stable, transfection of Huh7 cells with dHBV DNA leads to intracellular accumulation of HBc protein (Rosmorduc *et al.*, 1995). In order to determine whether this HBc protein accumulation is involved in the regulation of the level of MxA protein, we have studied the expression of both proteins after 2aIFN treatment in the same cell using immunofluorescence double staining after transfection with dHBV DNA or pHBc (Fig. 4).

In all dHBV DNA-transfected cells, the presence of accumulated HBc protein (detected as green fluorescence), correlated with a significant decrease in the amount of MxA protein (fluorescence intensity was measured using an arbitrary coloured scale) as compared to the surrounding untransfected cells (Fig. 4, upper panels). The same result was also observed in all the pHBc-transfected cells examined (Fig. 4, middle panels), but not in HBV DNA-transfected cells (data not shown). In contrast, after transient transfection with pHBc, an independent immunofluorescence double-staining experiment with anti-p100 OAS and the same anti-HBc antibodies, did not show any reduction in the amount of p100 OAS in cells overexpressing the HBc protein, compared to the untransfected surrounding cells (Fig. 4, lower panels). These results were reproduced by studying more than 10 cells in at least three independent experiments. Thus, our results show an inverse correlation between the amount of intracellular HBc protein and the expression of MxA protein in transfected cells.

Northern blot analysis of MxA RNAs in transfected cells

To determine whether the modulation of the expression of the MxA protein was due to transcriptional downregulation,

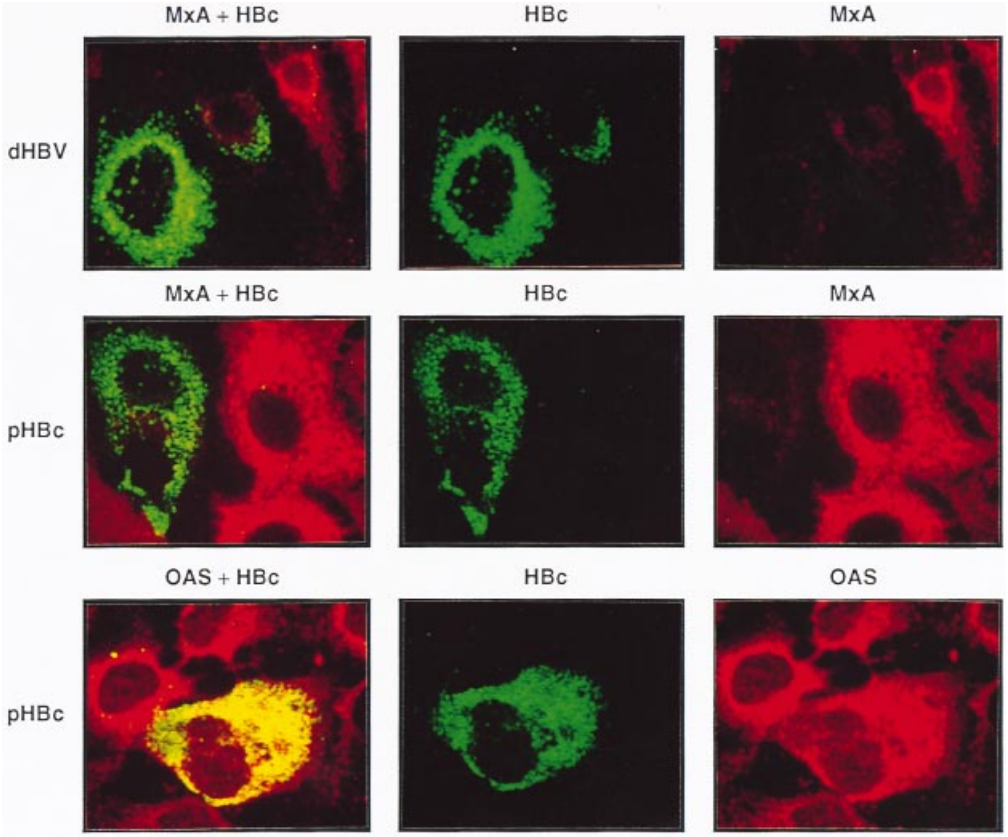

Fig. 4. Immunofluorescence double-staining and confocal microscopy analysis of cells transfected with dHBV DNA or pHBc. Huh7 cells were transiently transfected with dHBV DNA (upper panels) or pHBc (middle and lower panels) and treated with 500 IU/ml 2aIFN for 20 h. The MxA and the HBc proteins were simultaneously detected by double-staining IF, using a mouse anti-MxA antibody and/or a rabbit anti-HBc antibody (upper and middle panels). Similarly, the p100 OAS and the HBc protein were simultaneously detected, using a mouse anti-p100 OAS antibody and/or the same rabbit anti-HBc antibody (lower panels). The red fluorescence corresponds to MxA protein (upper and middle panels) or to p100 OAS (lower panels). The green fluorescence, detected in the same cells using a different coloured filter, corresponds to the HBc protein (all panels).

we analysed the MxA-coding RNAs in the different transfected cells with and without 2aIFN treatment. After treatment with 500 IU/ml 2aIFN, the cellular RNAs encoding the MxA protein were clearly induced. As shown in Fig. 5, the amount of these transcripts was lower (4- to 5-fold) in the cells expressing dHBV DNA, compared to cells transfected with HBV DNA or the neomycin-resistance gene. In contrast, the amount of transcripts encoding two other IFN-inducible proteins (namely OAS p40-46 and 6-16) was found to be comparable in transfected cells as well as in negative controls (Fig. 6). Thus, these results suggest a specific inhibition of MxA protein induction in dHBV DNA-expressing cells at a pre-translational level.

Luciferase and CAT assays

In order to determine the level of the trans-suppressive effect of HBc protein, we have constructed a plasmid expressing the luciferase reporter gene under the control of the minimal 550 bp MxA promoter. This plasmid (pMxA550-Luc) was transfected into Huh7 cells alone or co-transfected with dHBV DNA, pHBc or HBV DNA. After treatment with 2aIFN, the induced expression of the luciferase gene was increased by about 4·5-fold in cells transfected with pMxA550-Luc alone, and 6-fold when co-transfected with HBV DNA. In contrast, induction of luciferase gene expression was only 2-fold higher after co-transfection with dHBV DNA, and there was no induction following pHBc co-transfection (Fig. 7*a*). We also investigated the influence of the HBc protein on the ISRE of the human 6-16 gene. The HBc protein induced a weak (16%) decrease in p6-16 CAT expression upon IFN treatment (Fig. 7*b*). Thus, these results demonstrate a strong transcriptional inhibition of the MxA minimal promoter activity by the HBc protein.

Discussion

We have shown previously that dHBV DNA expression was associated with a chronic course of HBV infection *in vivo*

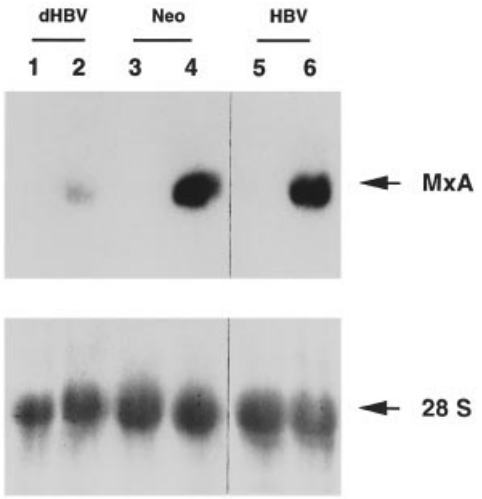

Fig. 5. Northern blot analysis of the MxA transcripts in stably transfected cells. Total RNA was extracted from dHBV DNA-transfected cells (dHBV; lanes 1 and 2), neomycin-resistance gene-transfected cells (Neo; lanes 3 and 4), and HBV DNA-transfected cells (HBV; lanes 5 and 6), without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) treatment with 500 IU/ml 2aIFN. After Northern blotting, the membrane was hybridized with a specific ^{32}P-labelled MxA DNA probe. Standardization was achieved using a 28S probe.

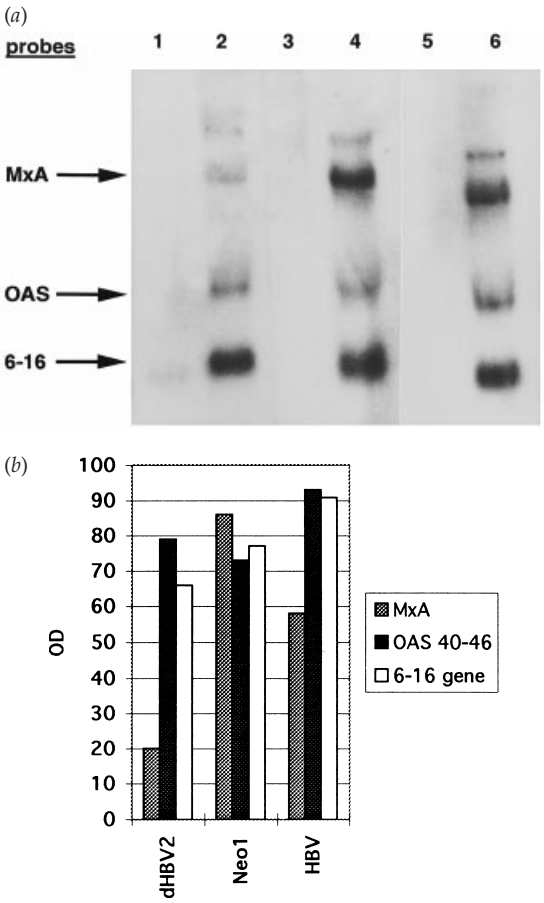

Fig. 6. Northern blot analysis of the MxA, OAS p40-46 and 6-16 gene transcripts in stably transfected cells. In an independent Northern blot experiment (*a*), total RNA was extracted from dHBV DNA-transfected cells (lanes 1 and 2), neomycin-resistance gene-transfected cells (lanes 3 and 4), and HBV DNA-transfected cells (lanes 5 and 6), without (lanes 1, 3 and 5) or with (lanes 2, 4 and 6) treatment with 500 IU/ml 2aIFN. The membrane was sequentially hybridized with specific ^{32}P-labelled MxA, OAS p40-46 and 6-16 gene probes. The histogram (*b*) presents semi-quantification results using OD scanning analysis (dHBV DNA-transfected cells, dHBV2; neomycin-resistance gene-transfected cells, Neo1; HBV DNA-transfected cells, HBV).

and leads to cytoplasmic accumulation of HBc protein. In this study, we have now demonstrated that the expression of dHBV DNA modulates the antiviral effects of IFN-α through a selective inhibition of MxA protein induction by 2aIFN. We have also shown that the HBc protein is implicated in this inhibition, partly through a trans-suppression of the MxA promoter.

We have previously shown that the amount of circulating dHBV DNA was correlated with ongoing HBV multiplication (Rosmorduc *et al.*, 1995) and a recent study showed that patients with high viraemia have a lower MxA but a greater OAS activity inducibility in PBMC (Fernandez *et al.*, 1997). These results suggest a role of dHBV DNA in the lower responsiveness to IFN in patients with high viraemia. This is in accordance with the well-recognized predictive factors for responses to IFN therapy *in vivo* (Perrillo *et al.*, 1990).

In cells stably transfected with dHBV DNA, inhibition of the antiviral effect of 2aIFN was detected against VSV, but not against EMCV, suggesting that the 2aIFN induced-antiviral activities against these viruses are different, and that expression of dHBV DNA only modulates an antiviral pathway specifically directed against VSV. Accordingly, we have observed diminished induction of the MxA protein and normal induction of p68 PK and p100 OAS in dHBV DNA-expressing cells treated with 2aIFN. These results are consistent with previous data showing that the MxA protein displays antiviral activity against VSV, but not against EMCV (Pavlovic *et al.*, 1990). In addition, it has already been shown that the antiviral effect of IFN against picornaviruses (EMCV) implicates OAS (Chebath *et al.*, 1987), further supporting a different pathway. Unexpectedly, we have found increased expression of the MxA protein, an increased antiviral effect of IFN against VSV and increased luciferase expression in HBV DNA-transfected cells as compared to negative controls. One possibility is that HBV DNA, unlike dHBV DNA, might slightly enhance the antiviral activity of exogenous IFN-α until significant intracellular accumulation of HBc protein occurs, whatever the mechanism may be (i.e. induction of endogenous IFN).

Following 2aIFN treatment, we have also found an additional band of 69 kDa in size using the anti-p100 OAS antibody in HBV DNA-transfected cells. This protein was not recognized by a specific anti-p69 OAS antibody (data not shown); however, this observation suggests that this p100 OAS-related protein could be highly induced by 2aIFN in HBV DNA-expressing cells, but not in those expressing dHBV

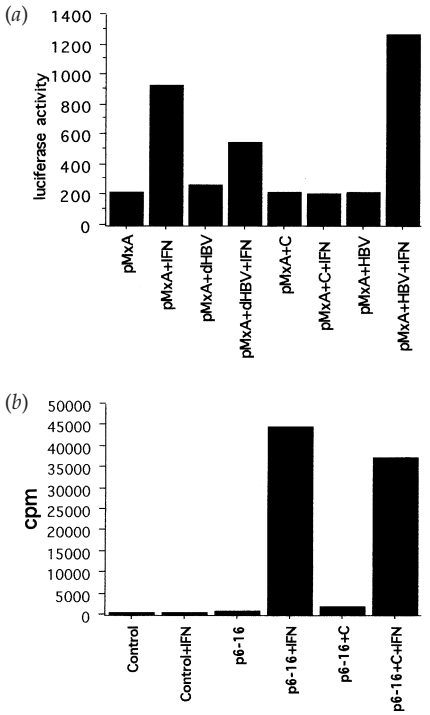

Fig. 7. Influence of the HBc protein on IFN-inducible promoter expression. (*a*) Functional analysis of the MxA promoter using the luciferase assay. Proteins were extracted from Huh7 cells transfected with either pMxA550-Luc alone (pMxA) or co-transfected with dHBV DNA (dHBV), pHBc (C) and HBV DNA (HBV). As indicated, luciferase activity is presented for untreated cells and cells treated with 500 IU/ml 2alFN (IFN). (*b*) Functional analysis of 6-16 ISRE using the CAT assay. Huh7 cells were transfected with p6-16 CAT alone (p6-16) or co-transfected with pHBc (C). The negative control is represented by untransfected cells (Control). CAT expression is presented for untreated cells and cells treated with 500 IU/ml 2alFN (IFN).

DNA. It is difficult at the present time, in the absence of a specific probe, to investigate this point further. Interestingly, this protein has also been detected in mononuclear cells from patients treated with IFN for cancer (Witt *et al.*, 1993).

Inhibition of MxA protein induction clearly involves the HBc protein. Indeed, we have observed, both in transiently and stably transfected cells, an inverse correlation between the amount of intracellular HBc protein and the expression of the MxA protein. In addition, HBV DNA-transfected cells, where no accumulation of the HBc protein was observed, do not show any inhibition of the MxA protein both in stable and transient systems. The specific role of the HBc protein in this inhibitory effect was demonstrated by immunofluorescence analysis after transfection experiments using a plasmid coding only for this HBc protein. However, we cannot rule out, as yet, additional regulatory effects of other dHBV DNA-encoded proteins (Rosmorduc *et al.*, 1995). It will be important in the future to investigate the mechanism involved in this effect.

We have found a decreased amount of RNA encoding the MxA protein in the cells stably transfected with the dHBV DNA, suggesting an inhibitory effect of the HBc protein at a pre-translational level. Interestingly, RNAs encoding two other IFN-induced proteins (OAS p40-46 and 6-16) were found at normal levels in these cells. In addition, we have found a strong inhibitory effect of the HBc protein on the 550 bp MxA promoter activity. This result is consistent with a previous report demonstrating a trans-suppressive effect of the HBc protein on IFN-β gene expression (Whitten *et al.*, 1991). The protamine-like C-terminal part of the HBc protein seems to be necessary for this inhibitory effect and may act by binding to a particular DNA sequence present in a 353 bp IFN-β regulatory DNA region (Whitten *et al.*, 1991). In addition, it has been shown that human immunodeficiency virus could directly induce the expression of the MxA protein, even in absence of IFN, after *in vitro* infection of monocytes (Baca *et al.*, 1994). The hypothesis of a direct trans-suppressive effect of the HBc protein requires nuclear translocation of this protein. However, so far, the subcellular localization of the HBc protein both *in vivo* and *in vitro* is still debated (Blum *et al.*, 1984; Guidotti *et al.*, 1994; Roingeard *et al.*, 1990; Yeh *et al.*, 1993, 1994) and our results show a predominant cytoplasmic localization of HBc protein (unpublished results). Thus, other mechanisms for the trans-suppressive action of the HBc protein must be considered, including in particular an indirect action mediated by cellular factor(s).

The antiviral activity of the MxA protein has been demonstrated against influenza virus, VSV, tick-borne Thogoto virus and measles virus, but not against EMCV, Semliki Forest virus, mengo virus and herpesvirus (Frese *et al.*, 1995; Pavlovic *et al.*, 1992, 1995). The function of MxA proteins, apart from antiviral activities, remains elusive (Staeheli *et al.*, 1993). The N-terminal part of the MxA protein contains three domains with GTP binding and hydrolysing activity, and shows significant homology to other members of a family of GTPases, that includes rat microtubule-associated dynamin, *Drosophila shibire* and the yeast vacuole sorting proteins (Horisberger, 1992; Pitossi *et al.*, 1993; Rothman *et al.*, 1990). This N-terminal feature thus suggests that MxA proteins may also have a function in intracellular protein transport. However, the precise mechanism of the MxA antiviral activity remains unclear. It might act at different steps of the virus replication cycle, according to the species of the virus (Pavlovic *et al.*, 1992; Zürcher *et al.*, 1992). In particular, it has been suggested that the murine Mx1 protein might bind to the PB2 subunit of the influenza virus RNA polymerase and lead to inhibition of virus replication (Huang *et al.*, 1992; Staeheli *et al.*, 1993) or that the MxA protein expression is associated with hyper-phosphorylation of VSV P protein leading to interference with primary transcription of the VSV (Schuster *et al.*, 1996). Further studies are now required to investigate the potential role of the MxA protein in the defence against HBV.

In conclusion, our study provides a first demonstration of a direct inhibition of the IFN-induced MxA protein expression by the HBc protein. This result, together with previous studies, suggests that the HBc protein might modulate the expression

of genes implicated in the antiviral and/or antiproliferative activity of IFN. This would fit with the *in vivo* and *in vitro* reciprocal interaction between IFN and HBV, as described previously (Ikeda *et al.*, 1986; Jakschies *et al.*, 1993; Nishiguchi *et al.*, 1989; Onji *et al.*, 1989; Poitrine *et al.*, 1985). Our results are also consistent with our previous *in vivo* observations that suggest a role for dHBV DNA in the mechanism of HBV persistence.

We thank Dr Ara Hovanessian (Institut Pasteur, Paris, France) for providing anti-p68 PK, anti-p69 and anti-p100 OAS antibodies and the OAS p40-46 DNA probe and Dr M. A. Petit (INSERM U 131, Clamart, France) for providing anti-HBc antibodies. This work was supported by grants from Institut National de la Santé et de la Recherche Médicale, Caisse Nationale d'Assurance Maladie, Association pour la Recherche sur le Cancer, and Ligue Nationale contre le Cancer, Villejuif and Paris, France. H.S. was recipient of a scholarship from Deutsche Forschungsgemeinshaft.

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Received 21 August 1998; Accepted 7 January 1999