Hepatitis B virus downregulates the human interferon-inducible MxA promoter through direct interaction of precore/core proteins

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The human MxA protein is an interferon (IFN)-inducible GTPase with proven antiviral activity against diverse viruses. IFN responsiveness is impaired in chronic hepatitis B virus (HBV) infection. Accordingly, initial experiments showed that, in contrast to parental HepG2 cells, when HepG2-derived 2.2.15 liver cells carrying the HBV genome were treated with IFN, they could not synthesize the MxA protein. Furthermore, MxA expression was reduced in HepG2 cells transiently transfected with the HBV genome. To assess whether HBV-encoded precore/core (preC/C) proteins interact with the IFN-signalling pathway, HepG2, Chang and HeLa cells were transfected with preC/C expression plasmids; the levels of signal transducers remained unaffected. Next, full-length and deletion mutants fused to the CAT reporter gene were tested to investigate whether MxA inhibition occurs at the promoter level. In co-transfection experiments, IFN-induced CAT activity was inhibited by preC/C expression in a dose-dependent manner. Analysis of deletion mutants showed that the region affected by the preC/C proteins comprises IFN-stimulated response elements 2 and 3, upstream of the putative start codon of the MxA promoter. In addition, HBV preC/C proteins interacted directly with the MxA promoter, as shown by electrophoretic mobility shift assays. These results demonstrate a mechanism that HBV probably uses to downregulate an element of the IFN-induced host antiviral responses, which accounts for the impairment observed in HBV-infected patients.

INTRODUCTION

Interferon-α (IFN-α) is a pleiotropic cytokine with immunomodulatory and antiviral activities. With regard to its antiviral activity, it induces a special condition, termed the antiviral state, in which the cells are especially resistant to infection by bacterial or viral pathogens (Staeheli, 1990). This antiviral state is mediated through IFN-regulated cellular proteins (Revel & Chebath, 1986; Sen & Ransohoff, 1993) such as Mx, 2′,5′-oligoadénylate synthetase and protein kinase PKR, etc. These proteins are synthesized after IFN exposure while the protein levels of others are increased (Muller et al., 1994). All IFN-induced genes share a DNA element present in their 5′ promoter regions (Haque & Williams, 1998; Williams, 1991) called the IFN-stimulated response element (ISRE). This sequence binds a nuclear factor, designated IFN-stimulated gene factor (ISGF), which promotes the initiation of transcription and acts as an enhancer (Ihle, 1996).

The IFN signal transduction pathway is well known (Stark et al., 1998). The IFN-α receptor is a heterodimer composed of two subunits, IFNAR-1 and IFNAR-2. Appropriate ligand binding results in the phosphorylation and activation of two tyrosine kinases associated with the subunits of the receptors, Jak1 and Tyk2. Then, these proteins tyrosine-phosphorylate the signal transducer and activator of transcription (Stat) 1 and Stat2. Phosphorylated Stat1 and Stat2 form a complex known as ISGF3 with p48, a DNA-binding protein. The complex translocates to the cell nucleus where it binds to the ISRE sequences in the promoter region of IFN-stimulated genes, thus activating the transcriptional machinery. This pathway appears to be a target site for some viruses to escape host immune survey (Heim et al., 1999; Miller et al., 1999).

When patients suffering from chronic hepatitis B are treated with IFN-α, up to 40% of them show clearance of hepatitis B virus (HBV) serum markers and normalization of liver functions (Torresi & Locarnini, 2000; Lin & Keeffe, 2001; Zuckerman & Zuckerman, 2000). In this regard, it is not known what mechanisms are involved in HBV resistance to IFN treatment in the remaining patients. We have shown previously (Fernández et al., 1997) that the ability of peripheral blood mononuclear cells (PBMCs) from HBV chronically infected patients to express the MxA protein upon IFN stimulation in vitro is impaired. Recently,
Rosmorduc et al. (1999) have shown that HBV defective particles are implicated in a deficient response to IFN-α in Huh7 cells and that this behaviour appears to be mediated by the accumulation of the HBV capsid protein. Indeed, these authors have proposed the transcriptional inhibition of MxA promoter activity by the core protein. Therefore, in this study, we have analysed what mechanism could be used by HBV to escape from IFN-α-induced antiviral activities. We have examined the IFN signal transduction pathway in cell lines transfected with the precore/core (preC/C) proteins. In addition, we have tested for a direct interaction of these proteins, at a pre-transcriptional level, with the cloned MxA promoter, which is considered a good model for IFN-induced genes.

METHODOLOGY

Cell lines. The human hepatoblastoma cell line HepG2, the Chang liver cell line, which is derived from normal liver tissue, and the HeLa cell line, derived from a cervix adenocarcinoma, were used. In addition, the 2.2.15 cell line, which is derived from a clone of HepG2 cells stably transfected with a dimer of the complete HBV genome (Sells et al., 1987), was used also. 2.2.15 cells express constitutively hepatitis B surface (HBsAg), e (HBeAg) and core (HBcAg) antigens and support full HBV replication (Sells et al., 1987). All cells were propagated and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Imperial) supplemented with 10% foetal bovine serum (FBS) (Imperial) and support full HBV replication (Sells et al., 1987). The 2.2.15 cell line, which is derived from a clone of HepG2 cells stably transfected with a dimer of the complete HBV genome (Sells et al., 1987), was used also. 2.2.15 cells express constitutively hepatitis B surface (HBsAg), e (HBeAg) and core (HBcAg) antigens and support full HBV replication (Sells et al., 1987). All cells were propagated and maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Imperial) supplemented with 10% foetal bovine serum (FBS) (Imperial) and support full HBV replication (Sells et al., 1987).

METHODS

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HBV DNA. The HBV genome used in some experiments was obtained from the pGHBV2 plasmid, which contains two tandem copies of the complete HBV genome cloned in the EcoRI site of the pGEM3Z plasmid (Promega). To recover a recircularized HBV DNA genome, pGHBV2 was digested with EcoRI and the 3-2 kb DNA fragment, corresponding to one copy of the complete HBV genome, was purified and joined using T4 DNA ligase (Promega), according to the manufacturer’s instructions. Expression of HBV proteins in transfected cells was checked by testing culture supernatants for the presence of HBsAg and HBeAg by means of enzyme immunoassays available commercially (Roche). To control for transfection efficiency, cells were co-transfected with a green fluorescent protein (GFP) expression plasmid and the amount of GFP was measured by flow cytometry; results were corrected accordingly (average of 10% transfection efficiency).

HBC and HBe expression plasmids. Coding regions of the HBV core (HBc) and precore (HBe) proteins were generated by PCR amplification of pGHBV2. The primers used are shown in Table 1. PCR products were digested with Nhel and cloned directly in the pCi mammalian expression vector (Promega). Production of HBeAg by the corresponding expression plasmid was verified by transfecting all cell lines and detection of HBeAg in culture supernatants by an enzyme immunoassay (Roche); intracellular preC/C proteins were assayed by sensitive Western blotting (Campillo et al., 1992) using specific HBV preC/C mouse monoclonal antibodies (mAbs) (Chemicon International). To verify that cells transfected with the HBCag expression vector synthesized the protein, HBCag was tested in culture supernatants by the method described by Quiroga et al. (1985). Full-length recombinant (Escherichia coli-derived) HBC and HBe antigen preparations were purchased from Virostat (Portland) and run as standards; purity was checked by Western blotting, indicating 95% purity for HBc and a pattern compatible with incomplete maturation of HBe expression in E. coli.

Table 1. Sequences of the primers used in the amplification of the human MxA promoter and deletion mutants, precore (HBe) and core (HBc) protein expression plasmids and probes for EMSA

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Sequence (5’→3’)</th>
<th>Orientation</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pMx1CAT</td>
<td>CCCTGAGAGGCCAGATGCGC</td>
<td>Sense</td>
<td>62–82</td>
</tr>
<tr>
<td>pMx2CAT</td>
<td>CCCTGAGAGGCCAGATGCGC</td>
<td>Sense</td>
<td>66–82</td>
</tr>
<tr>
<td>pMx3CAT</td>
<td>CCCTGAGAGGCCAGATGCGC</td>
<td>Sense</td>
<td>534–555</td>
</tr>
<tr>
<td>pMx4CAT</td>
<td>CCCTGAGAGGCCAGATGCGC</td>
<td>Sense</td>
<td>691–712</td>
</tr>
<tr>
<td>pCi-HBc</td>
<td>ATGGACATTTGACCCGTAT</td>
<td>Sense</td>
<td>590–871</td>
</tr>
<tr>
<td>pCi-HBe</td>
<td>ATGCACTTTTTCACCT</td>
<td>Antisense</td>
<td>590–871</td>
</tr>
<tr>
<td>MxSRE3</td>
<td>AGCCCGAGGGGGTAAGAGGC</td>
<td>Sense</td>
<td>534–555</td>
</tr>
<tr>
<td>MxSRE2</td>
<td>GGAGAGGACCAATGGGCTT</td>
<td>Sense</td>
<td>534–555</td>
</tr>
</tbody>
</table>

*Nucleotide position according to the human MxA promoter sequence reported by Chang et al. (1991).
†Nucleotide position according to the HBV DNA genome sequence reported by Ono et al. (1983).
**MxA promoter plasmids.** A fragment of the human MxA promoter from nucleotide 62 to 1031 (Chang et al., 1991) was obtained by PCR amplification of total DNA isolated from PBMCs from a healthy donor. For that purpose, 1 μg total DNA was amplified in a buffer containing 2-4 mmol MgCl₂ 1⁻¹, 0-2 mmol dNTP mixture 1⁻¹, primer mix (0-4 μmol 1⁻³ each) and 0-2 μl Taq DNA polymerase (Promega) for 30 cycles at 94 °C for 1 min, 60 °C for 30 s and 68 °C for 2 min, and a prolonged incubation at 68 °C for 15 min. The 969 bp PCR product was cloned directly into the pCR 2.1 vector (TOPO-TA cloning; Invitrogen). Sequence and orientation of the insert were verified by sequencing (Sequenase; Amersham). The MxA promoter was excised from the pCR 2.1 vector by KpnI/XhoI digestion and subcloned in the KpnI/Xhol sites upstream of the CAT gene of the pCAT-3 basic vector (Promega) to obtain the pMx4CAT plasmid.

Deletion mutants of the MxA promoter were generated by PCR using pMx4CAT. The PCR conditions used to obtain pMx3CAT, which contains all ISRE sequences and a few bases after the putative start codon, were as follows: 25 cycles of 94 °C for 1 min, 55 °C for 1 min and 68 °C for 2 min, and a prolonged incubation at 68 °C for 15 min. The pMx2CAT and pMx1CAT plasmids, which contain ISRE1/2 and ISRE1, respectively, were obtained as follows: 25 cycles of 94 °C for 15 s, 60 °C for 30 s and 68 °C for 1 min, and a final extension step at 68 °C for 15 min. All PCR products were cloned into the pCR 2.1 vector (Invitrogen) and, after sequencing, they were subcloned into the KpnI/Xhol sites of the pCAT-3 basic plasmid. A schematic representation of all of these constructions is shown in Fig. 1. Primers used in the amplification of the MxA promoter and the deletion mutants are shown in Table 1.

**Cell transfections and CAT assays.** All cell lines were transfected with the Superfect Transfection reagent (Qiagen) according to the manufacturer’s instructions. In all experiments, 2 × 10⁶ cells of each cell line were seeded onto 6-well plates (Costar) in 4 ml of supplemented DMEM the day before transfection. After being incubated overnight, cells were transfected with the corresponding HBV DNA or plasmid, or were mock-transfected, cultured for 24 h and harvested or stimulated when indicated with IFN-α for an additional 24 h. Finally, the cells transfected for Western blot analysis were treated as described below. Cells transfected with pCAT plasmids were lysed using the CAT Enzyme Assay system (Promega), following the manufacturer’s instructions. Equal amounts of protein were used and adjusted to ensure that the enzyme activity remained within the linear range (≤ 50 % conversion). Quantification was performed by densitometric analysis of spots in thin layer chromatography plates (Whatmann) in a laser densitometer (Molecular Dynamics). All transfection experiments were repeated independently at least four times to verify and ensure the reproducibility of results. Transfection efficiency was monitored with the pGL3 control vector and the Dual-Luciferase Reporter Assay system (Promega). The results of CAT assays are given in arbitrary units (AU) of the adsorption obtained for each one of the independent experiments.

**Protein extraction and Western blotting.** To assay for the presence of the human MxA protein, PKR and actin (as a ‘housekeeping control’) or the signal transducers Jak1, Stat1 and Stat2, cells were rinsed in PBS and lysed in RIPA buffer containing 1 % Nonidet P-40 (ICN Biomedicals), 0.5 % sodium deoxycholate, 0.1 % SDS, 10 mg PMSF ml⁻¹, 10 μmol leupeptin 1⁻¹, 1 mmol benzamidine 1⁻¹ and 100 μM Na₃VO₄. Normalized volumes of whole cell extracts according to the protein concentration were boiled for 5 min with SDS-PAGE sample buffer and electrophoresed in gradient (4–20 %) Tris/glycine gels (Novex). At the end of the run, the gel was blotted onto a Western PVDF membrane (Schleicher & Schuell). After an overnight blocking step at 4 °C with 10 % non-fat dry milk, the membrane was incubated at room temperature for 1 h with the appropriate primary antibody at a 1/1000 dilution. Human MxA protein was detected with a mouse-specific mAb, Stat1, Stat2, PKR and actin were detected with rabbit or goat polyclonal antibodies from Santa Cruz. Jak1 was detected with a mouse mAb from Transduction Laboratories. After extensive washing, the membrane was incubated with a secondary rabbit anti-mouse, rabbit anti-goat or goat anti-rabbit peroxidase-conjugated antibody (Dakopatts) at a 1/10000 dilution for 30 min at room temperature. Finally, the blot was revealed by autoradiography using the enhanced chemiluminescent substrate Supersignal West Pico Chemiluminescent substrate (Pierce). For immunoprecipitation experiments, 300 μl whole-cell extracts from 20 × 10⁶ cells were pre-cleared with 30 μl protein A/G PLUS-agarose (Santa Cruz) for 1 h at 4 °C. The lysate was pelleted by centrifugation and the supernatant incubated overnight at 4 °C with 5 μg Stat1 or Stat2 antibodies. After precipitation with 30 μl A/G PLUS-agarose, samples were washed twice with RIPA buffer and three times with 50 mM Tris/HCl pH 7.5. Pellets were then boiled with SDS-PAGE sample buffer and analysed by Western blot using the PY20 anti-phosphorytosine antibody (Transduction Laboratories). The bands in the autoradiograms were scanned for identification and semi-quantified by densitometry using Scion Imaging software (Scion).

**Electrophoretic mobility shift assay (EMSA).** For each EMSA, 1 μg HBc or HBe (Virostat) were incubated with 3 μl 87 % glycerol, 1 μl poly(dI·dC) and 2 μl of binding buffer containing 20 mM Tris pH 7.5, 75 mM KCl, 1 mM DTT and 5 μg BSA ml⁻¹ on ice for 15 min. In the case of the ‘supershift’ assay, samples were incubated for 30 min with appropriate HBV-specific (or species-matched irrelevant) antibodies. Then, 50 000 c.p.m. ³²P-labelled DNA probes were added and incubated at room temperature for 30 min. Samples were separated on polyacrylamide gels made with MDE gel solution (FMCl) in 0.5 × TBE as running buffer. The gel was then dried and stained with ethidium bromide.
exposed to PDS film (Kodak). Two fragments were obtained by PCR from pMx4CAT as probes for EMSA. The MxISRE3 probe contains the first fully functional ISRE3 near the putative start codon of the human MxA promoter and MxISRE2 contains the second functional ISRE2 of the MxA promoter. Two additional probes, termed ΔMxISRE2 and ΔMxISRE3, which do not contain ISRE2 and ISRE3 sequences, respectively, were obtained by PCR also. Primers used for PCR amplification are those listed in Table 1.

RESULTS

Analysis of MxA expression in HepG2 and 2.2.15 cells

To investigate the possible mechanisms by which HBV interacts with the IFN system, we have analysed the capacity of HepG2-derived 2.2.15 liver cells carrying the HBV genome and their parental HepG2 cells to produce the MxA protein. Thus, we cultured both cell lines in the presence of 1000 IU IFN-α ml⁻¹. As shown in Fig. 2(A), there were no detectable baseline levels of MxA protein prior to IFN treatment. Upon induction with IFN-α, an increase in the production of MxA was observed in HepG2 cells and it reached a peak expression at 24 h (Fig. 2A). In contrast, 2.2.15 cells were unable to express the MxA protein at detectable levels.

To assess whether this effect could be attributed to the presence of HBV DNA in 2.2.15 cells, we transiently transfected HepG2 cells with increasing amounts of recircularized HBV DNA from pGBHBV2 and cultured them in the presence of 1000 IU IFN-α ml⁻¹. The increase in transfected HBV DNA resulted in a clear reduction in the synthesis of endogenous MxA protein (Fig. 3A), in spite of an unexpected induction observed in the unstimulated cells. Such induction was not observed when HepG2 cells were transfected with pCi-HBc or pCi-HBe (C or preC/C protein expression, respectively), which, nonetheless, resulted in a reduction in MxA synthesis compared to the control plasmid pCi (Fig. 3B). The reduced levels of MxA observed with higher plasmid concentrations could have been due to a non-specific (toxic) effect, but the levels of expression of human actin (housekeeping control) remained unaffected. Induction of the MxA protein in cells without IFN treatment could be explained by the recent data reported by Lee & Yun (1998), which showed that the HBx protein activates the Jak1-Stat signalling pathway. HBx protein is a well-documented transcriptional trans-activator (Caselmann, 1996) with an intracellular compartmentalization that may depend on the expression levels (Henklé et al., 2001). Nevertheless, it is remarkable that the overall effect is an evident inhibition that correlates with the amount of HBV DNA transfected. The inhibition of MxA expression in 2.2.15 cells might be attributable to clonal variation, but the specific reduction in MxA synthesis in HepG2 cells transfected with pCi-HBc or pCi-HBe argues against this possibility.

To ensure the proper expression of viral antigens, we measured the intracellular levels of HBcAg and HBeAg in the supernatants of transfected HepG2 cells. An increase in the amounts of HBcAg and HBeAg released was observed and correlated with the increase in HBV DNA transfected in each experiment (data not shown). We have also investigated whether the expression of PKR, another IFN-inducible protein, was altered in 2.2.15 cells as well as in HepG2 cells transfected with pCi-HBc or pCi-HBe. Inducibility of PKR was comparable in 2.2.15 cells and untransfected HepG2 cells (Fig. 2B). In addition, and consistent with previous reports (Rosmorduc et al., 1999), no changes in the levels of PKR were observed in HepG2 cells transfected with the C or preC/C expression plasmids (Fig. 3B).

Signalling through the IFN pathway

Recent studies have shown that nucleocapsid HBV protein could be involved in a deficient IFN response (Rosmorduc et al., 1999). Thus, we have tested for a possible interference of the HBc and HBe proteins with the IFN signal transduction pathway. Total cell extracts from HepG2, Chang and HeLa cells transfected with pCi-HBc or pCi-HBe were analysed for Jak1, Stat1 and Stat2 levels. Jak1 is a tyrosine kinase associated with the IFN-α/β receptor and is activated upon IFN binding. As shown in Fig. 4, no apparently quantitative differences in the levels of Jak1 expression were found in the Chang cell line. Stat1 and Stat2 are the cytoplasmic signal transducers that are tyrosine-phosphorylated by Jak1 and
they form an essential part of the ISGF3 complex. Total levels of both proteins did not seem to be affected by the expression of HBc or HBe protein (Fig. 4, lanes 3–6). Similar results were obtained in the HepG2 and HeLa cell lines (data not shown). In addition, the levels of tyrosine phosphorylation of Stat1 and Stat2 remained unchanged, irrespective of whether or not HBc or HBe was present; similar results were observed in all cell lines tested (data not shown).

**Inhibition of the MxA promoter by HBV preC/C proteins**

Since HBc or HBe did not affect the IFN-signalling pathway in our system, we analysed whether MxA inhibition occurs at the promoter level. The human MxA promoter was amplified by PCR from PBMCs and cloned upstream of the CAT gene of the pCAT-3 basic plasmid, giving rise to pMx4CAT. To assay its functionality, HepG2, Chang and HeLa cells were transfected with pMx4CAT and cultured for 24 h in the presence of 1000 IU IFN-α ml⁻¹. As shown in Fig. 5(A), a clear induction of CAT activity was observed after incubation with IFN-α but there was no CAT activity in unstimulated HepG2 cells. Next, in another set of experiments, pMx4CAT was co-transfected with increasing amounts of either pCi-HBc or pCi-HBe. After the initial 24 h, cells were cultured for another 24 h in the presence of 1000 IU IFN-α ml⁻¹. CAT activity was inhibited in HepG2 cells by either HBc or HBe in a dose-dependent manner (Fig. 5A). Both proteins led to a similar rate of inhibition in all cell lines tested. As a control, cell lines were co-transfected with the pCAT control vector and pCi-HBc or pCi-HBe. No changes in CAT activity were observed (Fig. 5B).

To investigate in what region the HBc and HBe proteins

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**Fig. 3.** Inhibition of the human MxA protein but not PKR in HepG2 cells. 2 × 10⁶ cells were transfected with the indicated amount of a functional HBV DNA genome (A) or with 5 μg of the plasmid indicated (B). After 24 h of incubation, cells were stimulated with 1000 IU IFN-α ml⁻¹ as indicated for an additional period of 24 h. Finally, the cells were harvested and lysed to analyse the expression of the human MxA protein (A) or MxA, PKR and actin (B) by Western blot with specific antibodies, as described. The graphs of each panel show average quantitative results of triplicate experiments (in arbitrary units, AU, ± SE) of the autoradiography.
interact with the MxA promoter, several deletion mutants of the promoter were cloned in the proper sense in the pCAT-3 basic plasmid (Fig. 1). pMx1CAT contains only ISRE1 and, in agreement with previous reports, appears to be non-functional; it showed no CAT activity upon IFN induction (Fig. 6, lanes 1–6). pMx2CAT, which carries ISRE1 and ISRE2, showed a weak IFN induction of CAT activity (Fig. 6, lane 8). When cells were co-transfected with pCi-HBc or pCi-HBe, pMx2CAT showed a clear inhibition of CAT activity (Fig. 6, lanes 9–12). The last construction, pMx3CAT, which contains all ISRE regions and a few nucleotides downstream of the putative start codon of the MxA gene, showed a very weak and almost undetectable basal CAT activity. In contrast, a strong induction of CAT activity was observed after culture with IFN (Fig. 6, lane 14). When pMx3CAT was co-transfected with either pCi-HBc or pCi-HBe, a similar inhibition as with pMx4CAT was observed (Fig. 6, lanes 15–18 and 21–24). Thus, it is suggested that the region comprised around ISRE2/3 and the putative start codon is involved possibly in the interaction between the MxA promoter and the HBc and HBe proteins.

EMSA of the MxA promoter with recombinant HBc and HBe proteins

An EMSA was performed to investigate any possible direct interaction between HBV core proteins and the MxA promoter. Thus, DNA-binding assays employing purified recombinant HBc and HBe proteins demonstrated the appearance of an MxA-binding protein complex when incubated with MxISRE3 (Fig. 7A, lanes 2 and 5) or MxISRE2 (Fig. 7A, lanes 13 and 15) probes containing only the ISRE2 or ISRE3 sequences, respectively, but not with ΔMxISRE3 (Fig. 7A, lanes 8 and 9) or ΔMxISRE2 (Fig. 7A, lanes 11 and 12) probes lacking the ISRE sequences. Competition assays showed the sequence-specificity of DNA–protein complexes because these were competed in the presence of molar excess of the cold probes (Fig. 7A, lanes 3, 6 and 14). Moreover, supershift assays employing HBc-specific antibodies demonstrated that the HBV core-dependent DNA–protein complex is supershifted by HBc antibody (Fig. 7B, lane 2) but not by species-matched irrelevant antibody (Fig. 7B, lane 1); the complex was competed in the presence of a molar excess of the cold probe (Fig. 7B, lane 3). Thus, taken together, these results indicate a direct interaction of HBV core proteins with the MxA promoter, which may account for the impairment in response to IFN.
DISCUSSION

We have reported previously that PBMCs from HBV chronically infected patients showed a diminished capacity to respond to IFN-α in comparison to mononuclear cells from healthy subjects (Fernandez et al., 1997). Moreover, this impairment was more evident in those patients with a high level of viraemia, which implies a high level of viral protein production, thus indicating a possible interaction of some of the viral proteins with the transcription machinery of the IFN system. Thus, at the molecular level, we have analysed and identified which viral proteins are involved in this effect. First, the inducibility of the MxA protein, a recognized indicator of IFN action (von Wussow et al., 1990), was inhibited totally in the 2.2.15 cell line, which carries an integrated HBV genome, and in HepG2 cells transfected with a functional copy of the recircularized HBV DNA. It is likely that an HBV gene product may interact with some component of the IFN-signalling pathway or at a pre-transcriptional level. Defective HBV genomes reduce the antiviral activity of IFN-α mediated by the HBV capsid protein and this involves a selective inhibition of the MxA protein but not other IFN-induced genes, such as PKR and 2',5'-oligoadenylate synthetase, another IFN-inducible gene, which suggests a specific effect over the MxA protein rather than a general interaction at a common signalling pathway.

Since neither HBc nor HBe appeared to affect the IFN-signalling pathway, we made an initial approach to investigate whether these proteins could be acting at a later step. In co-transfection experiments with the human MxA promoter and constructs expressing constitutively core (HBc) or precore (HBe) antigens, we have shown a clear inhibition of the reporter activity after IFN induction. This result was consistent in all cell lines tested, showing that the inhibition was not dependent on host cell factors and, thus, that it is not restricted to liver cells, which is in agreement with our previous observations in human mononuclear cells (Fernandez et al., 1997) and with the inhibition reported by others (Rosmorduc et al., 1999) in similar experiments made with defective HBV genomes. The major characteristic of this defective genome is the accumulation of the core protein in the cytoplasm of transfected cells and the authors concluded that this core protein might be involved in the reduced capacity to respond to IFN. In addition, we have also demonstrated the capacity of preC/C proteins to inhibit the IFN-dependent cell response.

Several previous works have shown how the human MxA promoter is organized (Aebi et al., 1989; Horisberger et al.,...
was confirmed by DNA-binding assays showing the formation of DNA–protein complexes with MxISRE2/3, but not with ΔMxISRE2/3 probes lacking ISRE, thus excluding a non-specific shift caused by the basic nature of both proteins (Heim et al., 1999). Therefore, these results confirm a direct interaction between HBC and HBe and two regions of the human MxA promoter. In co-transfection experiments with the deletion mutants, HBV proteins were able to inhibit the IFN-induced CAT activity of pMx3CAT as well as with pMx2CAT. The latter does not share the sequence downstream of the ISRE2 element but it was also inhibited. This observation, together with the EMSA results, suggest that the targets of the interaction of the preC/C proteins are probably the ISRE sequences of the human MxA promoter. The direct interaction of HBe with the human MxA promoter is unclear at present because the HBe construct has the capacity to express preC/C-related proteins. However, because of the nature of recombinant HBe, it cannot be ruled out that such an interaction occurs with other components of the IFN system. In this sense, using a similar approach with the IFN-β promoter, Whitten et al. (1991) concluded that HBe and HBe inhibit the activity of the IFN-β regulatory elements, although they failed to show any mechanism implicated in this effect.

HBC plays an important role in HBV maturation and its capacity to bind DNA sequences is not striking because there are several nucleic acid-binding motifs at the C-terminal region of the protein (Hatton et al., 1992). However, the secreted precore protein is not essential for replication or infection (Chang et al., 1987; Schlicht et al., 1987), although recent studies have implicated a non-secreted precore precursor in the regulation of HBV replication (Scaglioni et al., 1997). Nevertheless, for HBe to mediate transcriptional repression, the precore protein needs to be localized in the nucleus. The majority of the protein buds into the endoplasmic reticulum and is processed before secretion as HBeAg (Bruss & Gerlich, 1988). However, some of the precore protein may show a nuclear localization (Aiba et al., 1989). A mutation in the precore sequence has been reported that prevents formation of HBe (Carman et al., 1997). Collectively, these findings might explain differences in MxA inducibility among serum HBeAg-negative patients with the precore mutant (Fernandez et al., 1997).

IFN-α is efficacious in patients chronically infected with HBV but there are still some patients who do not respond to treatment or who relapse after having achieved an initial response (Torresi & Locarnini, 2000; Lin & Keffe, 2001; Zuckerman & Zuckerman, 2000). Little is known about the mechanism that mediates the IFN-induced elimination of HBV from a patient. However, if HBe and HBe bind to the ISRE sequences present in the IFN-induced genes, this effect could account for the interference observed in the IFN response in these patients and highlights the potential role of using combination therapies with other antiviral compounds that can evade this virus-evolved escape mechanism. Moreover, these interactions could explain why patients

**Fig. 7.** EMSA of the ISRE2 and ISRE3 sequences of the human MxA promoter with recombinant HBC and HBe. (A) 50 000 c.p.m. 32P-labelled DNA probes MxISRE3 (lanes 1–6) and MxISRE2 (lanes 13–16) or ΔMxISRE3 (lanes 7–9) and ΔMxISRE2 (lanes 10–12) probes lacking ISRE sequences (all were obtained by PCR from pMx4CAT) were incubated with 1 µg recombinant HBC (lanes 2, 3, 8, 11, 13 and 14) or 1 µg recombinant HBe (lanes 5, 6, 9, 12, 15 and 16), as indicated; lanes 1, 4, 7 and 10, controls (probes without protein). In lanes 3, 6, 14 and 16, samples were incubated in the presence of a molar excess of the corresponding cold probe. (B) The MxISRE3 probe was incubated with 1 µg recombinant HBC and the DNA–protein complex was analysed by supershift assay with HBC-specific antibody (lane 2) or species-matched irrelevant antibody (lane 1); in lane 3, the sample was incubated in the presence of a molar excess of the cold probe. The autoradiograph shows one representative of two separate experiments performed with similar results. Arrows indicate DNA–protein complexes; the arrowhead shows the supershifted DNA–protein complex.

1990; Ronni et al., 1998) and, by sequence analysis, it was found that the promoter contains three ISREs, although only ISRE2 and ISRE3 showed a functional capacity to respond to IFN-α (Horisberger et al., 1990; Ronni et al., 1998). Accordingly, in our study, the first deletion mutant, pMx1CAT, which contains only the ISRE1 sequence, did not exhibit any CAT activity. However, pMx2CAT, which also contains the ISRE2 sequence, showed a weak capacity to be induced by IFN. In addition, CAT activity was similar to the initial full-length cloned promoter represented by pMx4CAT, only when the ISRE3 sequence was added in pMx3CAT. These deletion mutants allowed us to confirm earlier reports in our system with several cell lines. Interestingly, when cells were co-transfected with the HBC or the HBe expression plasmids and treated with IFN-α, pMx2CAT and pMx3CAT showed a clear inhibition of CAT activity, as occurs with the full-length promoter. Together, these results suggested that a direct interaction of the core protein (HBC) and/or precore (HBe) might occur at the promoter region comprising ISRE2 and ISRE3 sequences. This hypothesis
with a low level of viraemia (e.g. low synthesis of viral proteins in the infected cells), which is a recognized predictive factor of response to drug therapy (Brook et al., 1989), respond better to IFN-α than patients with a high level of viraemia. The relevance of this downregulatory mechanism is substantiated further by the finding of Gordien et al. (2001), who have reported recently that induction of MxA expression in the human hepatoma cell line HuH7 followed by transient transfection with the full-length HBV genome leads to inhibition of HBV replication. However, it is possible that inhibition of HBV may also occur via an MxA-independent pathway, as shown recently for hepatitis C virus (Frese et al., 2001). Interactions of HBV preC/C with other components of the IFN system could not be excluded and should be studied in the future.

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