Latent Hepatitis B Virus (HBV) Infection and HBV DNA Integration is Associated with Further Transformation of Hepatoma Cells *in vitro*

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Summary
Hepatitis B virus (HBV) is the major cause of chronic liver disease and hepatocellular carcinoma in the world, with more than 400 million people infected worldwide. To date, there is no reliable model for the study of the many aspects of HBV infection, despite the use of the chimpanzee. Although several alternative methods have been previously developed for the in vitro study of HBV infection, there is still an urgent need for new in vitro infection models, including for the ability of HBV to integrate into the host cell genome.
Here we describe a process to improve infection of the human hepatoma cell lines HepG2 and HuH-7 in vitro with HBV originating from human blood. As shown previously for infection of hepatocytes with hepatitis C virus (HCV), the removal of the cell-bound lipoproteins prior to the addition of the viral inoculum to the cells could also be critical for the uptake of HBV via lipoprotein (LDL)-related receptors. Induction by insulin and dexamethasone led to an increase of HBsAg expression at the cell surface in association with the integration of the viral DNA into the host genome and HBx RNA detection. This integration process was also shown to be associated with cytopathic changes and further phenotypic transformations of the cells.

Zusammenfassung: Latente Hepatitis-B-Virus Infektion und HBV DNA-Integration führen zu weiterer Transformation von Hepatomzelllinien *in vitro*
Hepatitis B Virus (HBV) ist weltweit die Hauptursache chronischer Leberleiden und von Leberkrebs mit mehr als 400 Millionen infizierten Menschen auf der Welt. Bisher gibt es noch kein verlässliches Modell für die Untersuchung diverser Aspekte der HBV Infektion, trotz des Einsatzes von Schimpansen als Versuchstiere. Obwohl mehrere Alternativmethoden für die in vitro Untersuchung von HBV entwickelt worden sind, gibt es noch immer eine dringende Nachfrage nach neuen in vitro Infektionsmodellen, inklusive für die Integration von HBV in das Wirtszellgenom. Hier beschreiben wir einen Prozess, durch den die Infektion der humanen Hepatomzelllinien HepG2 und HuH-7 in vitro mit HBV aus humanem Blut verbessert wird. Wie schon für die Infektion von Hepatozyten mit Hepatitis C Virus (HCV) gezeigt, kann die Entfernung von zellgebundenen Lipoproteinen vor der Zugabe des viralen Inokulums auf die Zellen kritisch sein für die Aufnahme von HBV durch Lipoprotein (LDL)-verwandte Rezeptoren. Induktion durch Insulin und Dexamethoson führte zu einer Steigerung der HBsAg Expression an der Zelloberfläche in Verbindung mit der Integration von viraler DNA in das Wirtsgenom und der Detektion von HBx RNA. Dieser Integrationsprozess führte ferner zu zytopathischen Veränderungen und weiteren phänotypischen Transformationen der Zellen.

Keywords: Hepatitis B Virus, HBV, in vitro, infection, integration, hepatocyte, lipoproteins, cancer, hepatocellular carcinoma

1 Introduction

Hepatitis B virus infection is one of the most prevalent viral diseases in the world, since around 400 million individuals are infected (Arbuthnot and Kew, 2001). Infection with HBV can lead to hepatocellular carcinoma (HCC) (Parkin et al., 1999; Wild and Hall, 1999). During this natural history of HBV infection in man, the integration of the viral DNA into the genome of hepatocytes is classically associated with the non-replicative late phase of the chronic infection which precedes the onset of liver cancer (Dejean et al., 1984; Shafritz and Kew, 1981; Shafritz et al., 1981).

To date, mostly chimpanzees, but also recombinant mice, marmosets, woodchucks and ducks have been used as animal models for the study of HBV infection (Schinazi et al., 1999). Animals have been employed particularly for the evaluation of antiviral compounds; a non-exhaustive list of scientific articles is provided in Table 1.

This hardly compensates for the lack of an efficient and reliable *in vitro* infection system to study the different phases

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Tab. 1: Numbers of animals employed in research for the screening of antiviral compounds against the hepatitis B virus (HBV) or against the cognate duck hepatitis B virus (DHBV) infection model. The list comprising scientific articles published during the last ten years is non-exhaustive.

Scientific articles	Numbers of animals
Mason et al., 1994	19 ducks
Fourel et al., 1994	10 ducklings
Severini et al., 1995	at least one duck
Rajagopalan et al., 1996	3 woodchucks
Howe et al., 1996	24 ducklings
Luscombe et al., 1996	18 ducklings
Zoulim et al., 1996	13 ducklings
Witcher et al., 1997	3 woodchucks
Cullen et al., 1997	25 woodchucks
Cavanaugh et al., 1998	56 transgenic mice
Nicoll et al., 1998	18 ducklings
Genovesi et al., 1998	23 woodchucks
Lin et al., 1998	18 ducks
Ilan et al., 1999	between 432 and 680 transgenic mice
Seigneres et al., 2000	29 ducklings
Le Guerhier et al., 2001	67 ducklings
Engler et al., 2001	review
Muchmore, 2001	review
Lewin et al., 2002	review

of HBV life cycle, especially integration of HBV DNA into the host cell genome. The infection of cells with a viral inoculum, or the transfection of cells (mostly hepatocytes) with nucleic acids, and the generation of a recombinant baculovirus expression system, have been employed in order to produce HBV infection in eukaryotic cells *in vitro* (Delaney and Isom, 1998; Galle et al., 1994; Ganem, 1996; Hirschman et al., 1980; Hohne et al., 1990; Schinazi et al., 1999; Weiss et al., 1996). So far little attention has been devoted to genomic integration and its consequences *in vitro*.

It has been suggested that putative receptors for HBV, annexin V binding protein and apolipoprotein H, bind to lipid components of the virus (Neurath and Strick, 1994). We have hypothesised that when the cell-bound lipoproteins, synthesised by the hepatocytes themselves (Dixon and Ginsberg, 1992; Glickman and Sabesin, 1988) or included in the serum employed for the cell culture, are removed from their receptor(s) prior to the addition of the viral inoculum to the cells, this procedure could be critical to favour the infection of hepatocytes with HBV. Following the early linearisation of the viral genome, integration of the viral DNA into the cellular genomic DNA occurred rapidly after 8 days of infection with the addition of two hormones, insulin and dexamethasone, to the cell culture medium. It is notable that in this hepatoma infection system, no production of progeny virions in the cell culture supernatant was observed, whatever the cell culture conditions employed. Using this method we have shown that HepG2 and HuH-7 cells infected with HBV were still positive for HBV DNA, as revealed by polymerase chain reaction, two years after the onset of the infection. Some cell culture formulations enriched with hormones and/or oligoelements, increased the expression of the HBsAg at the cell surface of the infected cells. Finally, the infection of the cells resulted in dramatic cytopathic changes such as vacuolisation, increase in cell size and growth in soft agar, suggesting a potential transforming effect of HBV.

2 Materials and methods

2.1 Cells

HepG2 cells (American Type Culture Collection; passage number over 150) were grown as described previously (Favre et al., 2001). The doubling time was around 8 to 10 days in the conditions employed. The totality of the cell culture medium was changed every 2 to 3 days. HuH-7 cells (Nakabayashi et al., 1982) were grown in Eagle's Modified Essential Medium supplemented with 10% fetal calf serum, 1% sodium pyruvate, 2 mM L-glutamine and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin) (complete medium).

2.2 Infection of cell cultures

The cells ($7x10^5$ to $1x10^6$) were grown in 6-well plates (Costar) until confluency. Infection of the cells was performed essentially as described recently (Favre et al., 2001). Briefly, the cells were washed with PBS and then treated on ice with 10 mg/ml high molecular weight dextran sulphate (AppliChem, Darmstadt, Germany) plus 1 mM of the calcium chelator ethylene glycol-bis (beta-aminoethyl ether) N,N, N', N'-tetra-acetic acid (EGTA; ethylene-bis (oxyethylenenitrilo) tetraacetic acid; egtazic acid) in ice-cold PBS for 10 minutes, in order to remove the cell-bound lipoproteins (Rayyes and Florén, 1998) and to maintain putative LDL receptor(s) free of lipoproteins at the cell surface. The cells were then washed extensively with ice-cold PBS to remove traces of the dextran sulphate-lipoprotein complex. The viral inoculum, consisting in normolipidemic human serum containing up to 2000 pg of HBV DNA per ml (up to $8x10^8$ particles per ml) either undiluted or diluted in a final volume of 500 µl PBS, was then added on ice to the cells for an incubation period of 45 min to 1 h in order to allow adsorption of the virus to the cells. The viral inoculum was then removed, and the cells were extensively washed with PBS. To allow penetration of the virus, the cells were then incubated at 37°C in complete cell culture medium. The cell culture medium was changed after an overnight incubation in order to remove unbound or loosely bound input virus. The infected cells were split into new tissue culture flasks every 2 to 3 weeks. Where indicated, the cells were also grown in the presence of additional 100 mIU human insulin per ml and/or $5x10^{-6}$ M dexamethasone.

2.3 Analysis of viral HBV DNA

At the indicated times, cultures were rinsed twice with phosphate-buffered saline. To isolate the covalently closed circular (ccc) DNA-enriched fractions, we used a previously described procedure (Summers et al., 1990). Briefly, cell layers were washed once with HEPES buffer and lysed with buffer containing 0.5% SDS. Protein-bound replicative forms were precipitated as a protein-detergent complex by addition of KCl to a final concentration of 0.5 M. After centrifugation, ccc DNA, along with genomic DNA, was recovered from the soluble fraction by phenol extraction and ethanol precipitation. Other relaxed circle (rc) and single stranded (ss) HBV DNA forms were dissolved by digestion of the pellet with proteinase K (0.8 mg/ml) for 4 hours at 55°C and purified by phenol extraction followed by ethanol precipitation. For the isolation of the total cellular DNA, hepatocytes were lysed in a solution containing 0.8 mg/ml proteinase K, 0.1% SDS, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 20 mM EDTA and incubated at 55°C for 4 hours. This lysate was extracted twice with phenol-chloroform, and nucleic acids were collected by ethanol precipitation.

Ten µg of total cellular DNA, or aliquots of ccc DNA extracts containing 10 µg of genomic DNA, which copurifies with the ccc DNA, were analysed by 1% agarose gel electrophoresis and transferred to an amphoteric nylon membrane (Biodyne A-Pall filtron). For rc DNA extracts, which contain only contaminant cellular genomic DNA and cannot therefore be quantified accurately by DNA content, aliquots equivalent to the corresponding ccc DNA extracts were analysed. Hybridisations were performed with a ^{32}P-labelled full-length genomic HBV probe (Turin et al., 1996). The membranes were analysed by autoradiography. Linear HBV DNA (5 ng) obtained by *Eco*R1 digestion of plasmid and equivalent amount of ccc DNA obtained from infected hepatocytes as described above were used as standard.

2.4 Analysis of viral HBx RNA by RT-PCR

Total RNA was isolated from cellular pellets (2x10^5 cells) using Trizol (Gibco-BRL) and treated with RNase-free, DNase I for 15 min at 37°C. Analysis of viral HBx RNA was performed exactly as described previously by reverse transcription followed by nested PCR and Southern blot analysis (Chemin et al., 2001).

2.5 Soft agar assay

Agar suspension culture is a selective clonogenic assay for the transformation of cells by a virus (Macpherson and Montagnier, 1964; West, 1997). The HepG2 cell line is not tumorigenic and does not grow in 0.4% soft agar. Thus, if this cell line is infected with a virus, the latter being able to transform the cells, these cells might grow in soft agar. In order to determine whether HBV alone is a transforming virus, the HepG2 and HuH-7 cell lines were infected as described above, incubated for around three weeks in complete cell culture medium, trypsinised, washed in complete medium, processed for the soft agar assay as described above in the presence of 0.4% soft agar (Noble Agar, Difco, Detroit, USA) in complete medium, and finally incubated for a three week incubation period.

2.6 Detection of HBsAg

For the detection of the HBV surface antigen (HBsAg), the AXsYM (Abbott Laboratories, Illinois, USA) and MONOLISA AgHBs PLUS (BioRad, Hercules, USA) routine tests were employed.

2.7 Immunofluorescence

Infected HepG2 cells (around 10^6) were trypsinised and washed in complete cell culture medium. The cells were then resuspended in 80 µl of PBS containing 0.2% fetal calf serum (FCS) and fluorescein isothiocyanate (FITC)-labelled, goat anti-hepatitis B surface antigen (Anawa Trading, Wangen, Zürich, Switzerland) at a 1:50 dilution. The cells were then incubated for 30 min on ice, followed by three washes with ice-cold PBS. The cells were then fixed in 500 µl of PBS containing 1 to 2% paraformaldehyde, or acetone. As negative control, uninfected cells were employed. The cells were observed with a microscope equipped with epifluorescence (Carl Zeiss, Axiophot 35, Oberkochen, Germany).

2.8 Flow cytometry

The cells that were processed for immunofluorescence, as described above, were also analysed by flow cytometry (FACSCalibur, Becton Dickinson).

2.9 Propidium iodide staining

After harvesting and washing, the cells (10^6) were fixed in ice-cold 70% ethanol for 30 min. After two washes with PBS containing 1% fetal calf serum, the cells were kept overnight at 4°C in PBS containing 20 µg propidium iodide and 1 mg DNase-free, RNase A per ml. Cells were then analysed by flow cytometry using 488 nm excitation by gating out doublets and clumps using pulse processing and collecting fluorescence above 620 nm.

3 Results

3.1 Latent HBV infection: effect of cell culture conditions on HBsAg expression and HBV DNA integration

According to our working hypothesis, the cell-bound lipoproteins were removed from their receptor(s) by using dextran sulphate prior to the addition of the viral inoculum to HepG2 and HuH-7 cell lines. The expression of the viral proteins in the HBV-infected cells was initially assessed by immunofluorescence and flow cytometric analysis using an anti-HBs antibody. The percentage of cells which stained positive for HBsAg at their cell surface 18 days post-infection was around 75% and 60% for the HepG2 and HuH-7 infected cells, respectively. Cells infected without the removal of the cell bound lipoproteins from the cell surface prior to the HBV infection were not stained (not shown). One major peak of fluorescence was observed with HBV-infected HepG2 cells (Fig. 1A), whereas two peaks of fluorescence were observed with HuH-7 cells, thus suggesting two HBsAg-expressing cell populations (Fig. 1B).

One month post-infection under basal cell culture conditions (without hormones) HBsAg expression was almost not detectable. Five months later, when insulin was freshly added to the basal cell culture medium and HuH-7 and HepG2-infected cells were incubated for a period

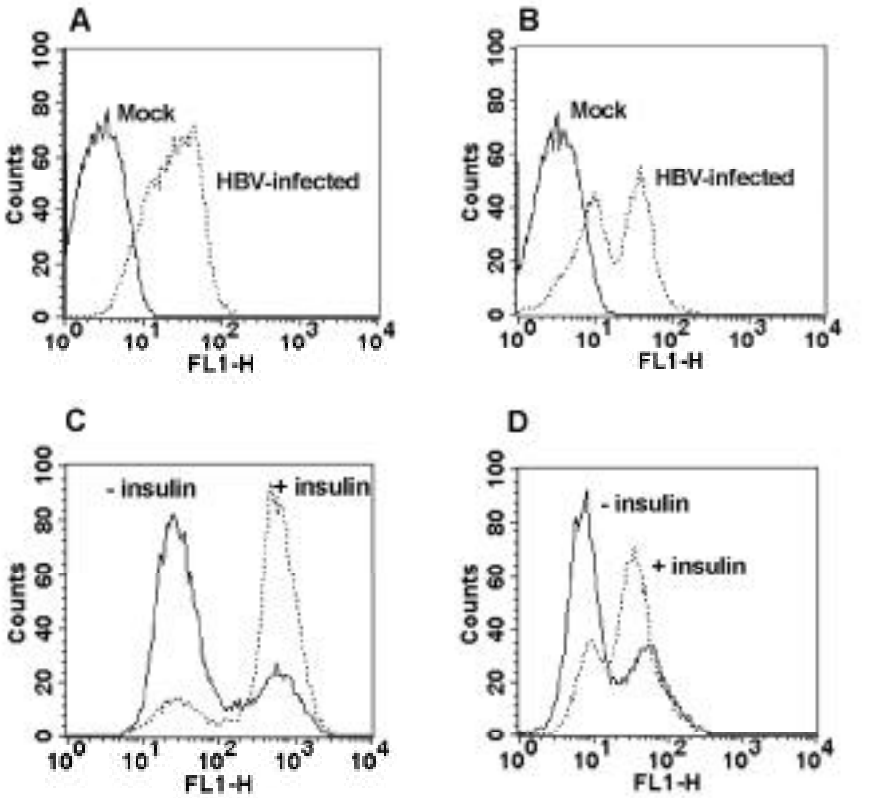

Fig. 1: Expression of HBsAg on the cell surface of HBV-infected HepG2 (A and C) and HuH-7 (B and D) cells.
After 18 days of infection (A and B) or six months after infection of the cells maintained in complete cell culture medium followed by two weeks incubation with additional human insulin at 100 mIU/ml (C and D), the cells were processed for analysis by flow cytometry. Relative immunofluorescence (FL1-H).

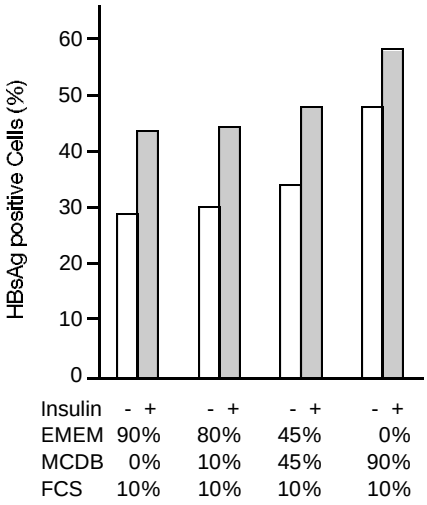

Fig. 2: Role of insulin and cell culture medium formulations on the expression of cell surface HBsAg in HBV-infected HepG2 cells.
HBV-infected HepG2 cells were incubated for two days in classic EMEM containing 10% FCS, and thereafter for ten days in various medium formulations in the absence or presence of additional insulin at physiological concentrations (100 mIU/ml), as indicated. Flow cytometric analysis on 20 000 cells was performed using a FITC-labelled antibody directed against HBsAg. The percentage of the positive, gated cells is given.

of 14 days, a dramatic increase in the cell surface HBsAg expression occurred in both infected cell lines (Fig. 1C and 1D).

Since various cell culture media and chemical formulations are important for the growth of hepatocytes *in vitro*, we investigated whether various medium formulations could also modulate the expression of HBsAg at the cell surface. Hepatocytes were thus treated with dextran sulphate and then infected with HBV as described above, incubated in initial basal cell culture medium (90% EMEM, 10% FCS) without insulin for two days, and thereafter incubated for 10 days in various medium formulations in the absence or presence of additional insulin at physiological concentrations (100 mIU/ml). Flow cytometric analysis confirmed that the addition of insulin into the basal cell culture medium induced an increase of HBsAg expression at the cell surface of HBV-infected HepG2 cells (42.6% positive in comparison to 28.1% without insulin induction). The addition of dexamethasone ($5x10^{-6}$ M) to the complete cell culture medium led to similar results (not shown). When a cell culture medium enriched with oligoelements (such as the MCDB-104 medium) was added at different proportions to the basal medium (EMEM), we observed the highest expression of HBsAg when EMEM was completely replaced by MCDB-104, even in the absence of additional insulin (Fig. 2).

Southern blot analysis of the relaxed circular (rc) DNA form in the HBV-infected HuH-7 cells under the basal cell culture conditions containing 10% of MCDB-104 medium in the presence or absence of additional hormones was performed. Four days post-infection, the viral DNA was detected mostly as a linear form (3.5 kb) but also as a relaxed single stranded form (Fig. 3A). These HBV DNA forms were only observed in the presence of insulin plus dexamethasone in the cell culture medium (lane 5). Interestingly, 8 days post-infection, the linear form of the viral DNA was no longer detected (Fig. 3B). However, the viral DNA was now found to be integrated into the cellular genomic DNA, but only in the presence of insulin plus dexamethasone (lane 5). Such linear and integrated DNAs were not observed when either insulin or dexamethasone was added alone (lanes 3 and 4). The closed covalent circular (ccc) DNA was never detected during the infection process (not shown). The HBV-infected HepG2 and HuH-7 cells were shown to contain discrete amounts of viral DNA only detected by PCR followed by Southern blot

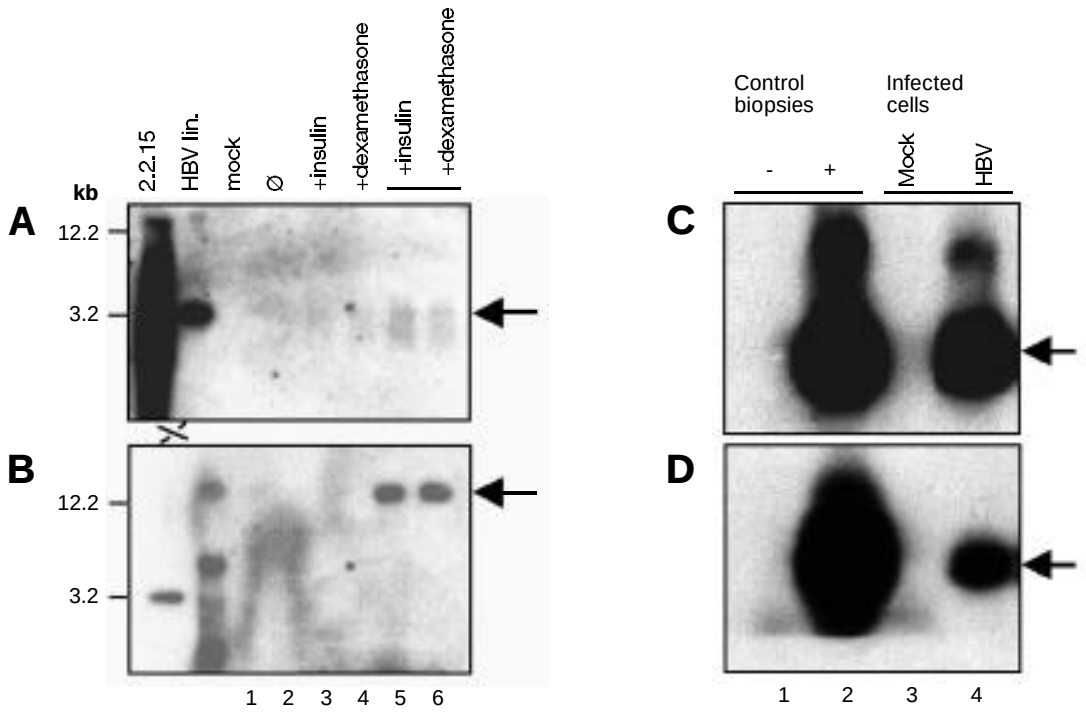

Fig. 3: Southern blot analysis of DNA extracted from HuH-7 cells infected with HBV.
DNA was electrophoresed on a 1% agarose gel, transferred onto a nylon membrane, and hybridised with a purified HBV DNA probe. A, B: HuH-7 cells four and eight days post-infection, respectively. Lane 1: mock-infected cells. Lane 2: HBV-infected cells, incubated in complete cell culture medium. Lanes 3, 4: HBV-infected cells, incubated with additional insulin or dexamethasone, respectively. Lanes 5 and 6: HBV-infected cells, incubated with both insulin and dexamethasone. Positive controls used were DNA from 2.2.15 cells, and linear HBV DNA (HBV lin.). Note the inversion of the 2.2.15 and HBV lin. controls between panels A and B. C, D: Southern blot detection of the nested-PCR amplification products with primers located in the S gene in HBV-infected HepG2 and HuH-7 cells, respectively, two years post-infection. Negative controls were mock-infected cells (mock; lane 3). Negative (-) and positive (+) liver control biopsies are represented in lanes 1 and 2, respectively.

analysis two years after initiation of the infection (Fig. 3C and D, respectively).

3.2 Phenotypic changes of HBV-infected hepatoma cells

Interestingly, cytopathic changes were observed in HBV-infected HepG2 cells: the cells were enlarged and contained dense nuclear inclusions and cytoplasmic vacuoles of various sizes. The cells also showed long cytoplasmic processes (Fig. 4A). These features were neither observed with mock-infected HepG2 cells (Fig. 4B) nor in the infected HuH-7 cells (not shown). The HBV-infected HepG2 cells did not grow as a true monolayer (as compared with the uninfected HepG2 cell line), showing loss of contact inhibition and formation of large clumps containing up to several hundreds of cells (Fig. 5A, a to c). This suggested that the virus *per se* could have a potentially transforming effect. To document this, the HepG2 cells, which were maintained in optimised cell culture conditions during three weeks after infection, were trypsinised and thereafter grown in 0.4% soft agar for the clonogenic assay. The aberrant growth of the HepG2 cells was confirmed with the appearance of large clumps containing hundreds of cells after three weeks of incubation (Fig. 5B), contrary to non-infected and mock-infected HepG2 cells similarly treated with dextran sulphate (data not shown).

To study the status of the HBV-infected HepG2 cells, the DNA content was analysed using propidium iodide. The staining of the mock-infected HepG2 cells four days after confluency (around day 14 after plating) revealed that about 6% of the cells were in the S-phase of the cell cycle. However, under the same plating and cell culture conditions, the staining of the long-term HBV-infected HepG2 cells revealed that between 16 to 22% of the cells were in S-phase (Fig. 6). We finally assessed, whether HBx RNA was present in the long-term HBV-infected HepG2 cells. Reverse transcription followed by nested-PCR and Southern blot analysis revealed the presence of viral HBx RNA sequence in the HBV-infected cells (Fig. 7).

4 Discussion

4.1 General considerations on the generation of an *in vitro* infection model for HBV infection

Reliable ways and means can be employed in order to investigate human biology and health issues, especially in

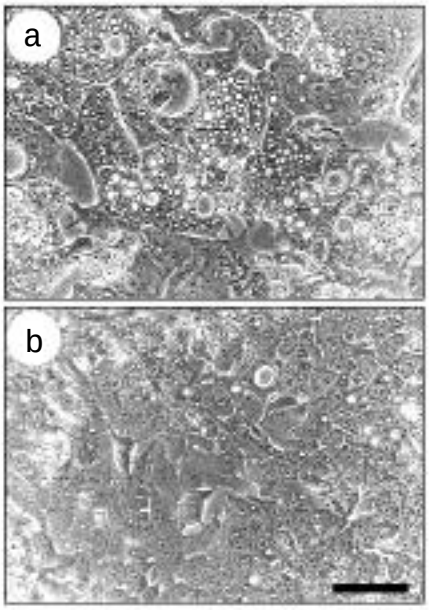

Fig. 4: HBV-infected cells in monolayers.
a: the HBV-infected HepG2 cells 12 days post-infection are enlarged and contain dense nuclear inclusions of various sizes and cytoplasmic vacuoles. Some cells present long cytoplasmic processes. b: mock infected HepG2 cells. Bar, 60 µm.

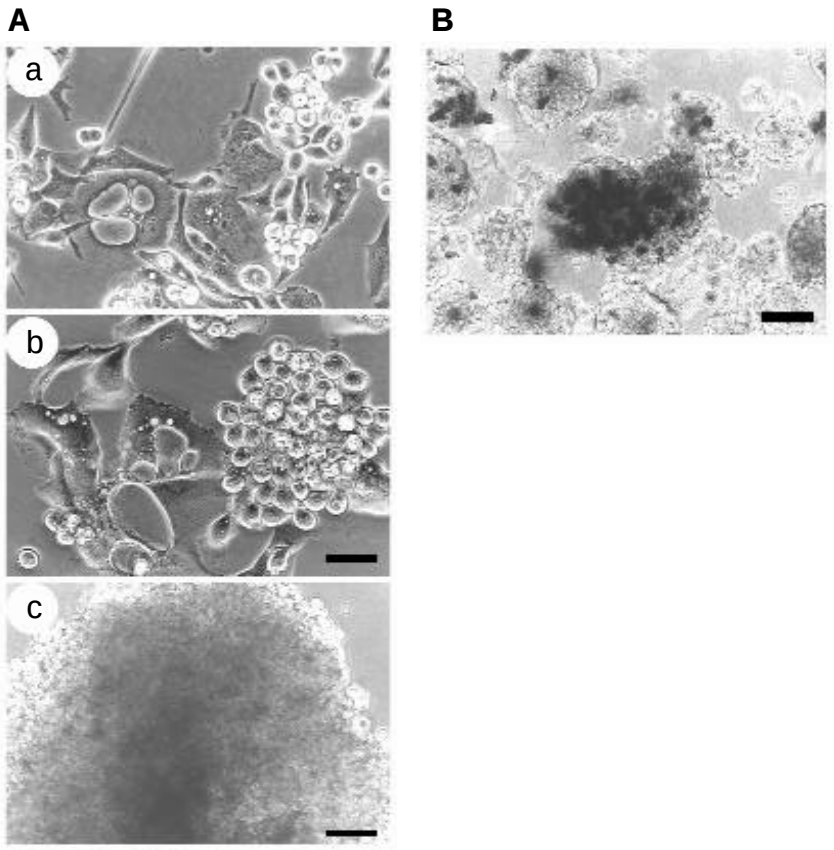

Fig. 5: Growth of HBV-infected HepG2 cells in hormone-supplemented cell culture medium (A) and as suspensions in soft agar (B).
A: Infected cells were grown for two weeks, trypsinised, washed, and grown for two more weeks in complete medium supplemented with a physiological concentration of insulin (100 mIU/ml). a, b: aberrant morphologies of adherent, HBV-infected cells with appearance of clusters of refringent, rounded cells; c: rounded cells proliferate and produce large clumps of cells. B: HepG2 cells were infected with HBV for an incubation period of three weeks, and were thereafter grown in soft agar for the clonogenic assay. Large clumps of cells grew in the soft agar after three weeks. Dark cells are probably necrotic. Non-infected cells and mock-infected cells do not grow in soft agar (not shown). Bars: 40 µm (A: a, b); 100 µm (A: c); 120 µm (B).

the field of viral infections. The so-called assessment method builds up from molecules and cells *in vitro* to the individual via tissues and organs. It is in strong contrast to the top-down approach of the animal model, which faces the full complexity of the animal from the outset. This complexity is as formidable in ducks, marmosets or mice as it is in primates. By performing *in vitro* infection of eukaryotic cells with HBV, it might be envisioned that the molecular responses of the cells to xenobiotics and antiviral compounds can be studied at the level of global genetic expression, for example by means of biochips and proteomics. Several individual genes, either cellular or viral, can be selected for their specific response involved in the metabolic pathways during the infection. This will undoubtedly lead to a better comprehension of the processes involved in the onset and the maintenance of the viral infection, and help in the search for new antiviral therapies. Until now, the chimpanzee and the transgenic mouse have been the most relevant models for HBV infection studies. A drastic replacement of these animal models is rather unlikely, however, a considerable reduction of testing could be envisioned, e.g. by performing basic research and drug screening *in vitro* and testing solely the most promising compounds *in vivo* in a limited number of animals.

4.2 Putative role of dextran sulphate on *in vitro* HBV infection of hepatoma cells

It was tempting to speculate that the crucial event leading to the *in vitro* infection of hepatic cells with HBV might lie at the level of the cellular receptor(s) and/or plasma membrane binding protein(s) of the virus. Several receptors and binding proteins have been proposed to be the mediators of HBV entry into target cells (reviewed in Duclos-Vallee et al., 2000; Treichel et al., 1997). Among those, the LDL receptor and the annexin V binding protein, which are present in the plasma membrane of liver cells (Cooper and Ellsworth, 1996; Geffen and Spiess, 1992; Hertogs et al., 1993), have been proposed to be the mediators of the binding and the internalisation of HBV into human hepatocytes (de Bruin et al., 1996; De Meyer

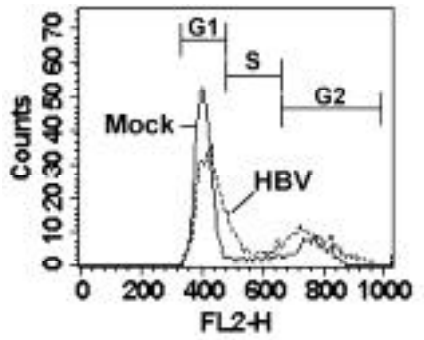

Fig. 6: Propidium iodide staining of HepG2 cells.
Mock-infected cells (mock) and HBV-infected cells (HBV) were analysed by flow cytometry 14 days after plating. Cells were in G1, S and G2 phase of the cell cycle. FL2-H: relative fluorescence.

and Yap, 2000; Neurath and Strick, 1994; Treichel et al., 1997; Treichel et al., 1994). Transfection of a rat hepatoma cell line with a construct expressing human liver annexin V binding protein conferred susceptibility to hepatitis B virus infection *in vitro* (Gong et al., 1999). Moreover, small hepatitis B virus antigen epitopes were shown to be involved in binding to human annexin V (De Meyer et al., 1999). The LDL receptor and the annexin V binding protein bind Ca^{2+} and lipoproteins (Cooper and Ellsworth, 1996; Geisow et al., 1987; Neurath and Strick, 1994; Steer, 1996). Moreover, the hepatitis B surface antigen binds to apolipoprotein H (Mehdi et al., 1994; Stefas et al., 2001). Altogether, there was thus circumstantial evidence that HBV might enter cells via the LDL receptor and/or the annexin V binding protein (Fig. 8). We thus hypothesised that the cell-bound lipoproteins might somehow hamper the efficient binding of the viral particles to the low density lipoprotein receptor *in vitro*, because the viral inoculum contains free lipoproteins, and because the lipoproteins are synthesised by the hepatocytes themselves (Dixon and Ginsberg, 1992; Glickman and Sabesin, 1988).

In the past, the removal of high density lipoprotein-bound cholesterol for the preparation of fractionated human blood components was performed routinely (Burnstein and Samaille, 1960; Finley et al., 1978; Fredrickson et al., 1968; Warnick et al., 1982) by mixing human blood with either heparin or dextran sulphate and by sedimenting the insoluble lipoproteins. A related procedure was also applied to remove cell-bound low density lipoproteins from the hepatocyte cell line HepG2 *in vitro* by dextran sulphate (Rayyes and Florén, 1998). The removal of lipoproteins from HepG2 cells was previously shown to be a crucial step for the success of HepG2 cell infection with hepatitis C virus (HCV) *in vitro* (Favre et al., 2001).

During the last decades, primary hepatocytes have been used for *in vitro* infection with HBV (Galle et al., 1994; Gripon et al., 1993; Iacovacci et al., 1993; Lamas et al., 1991; Lanford et al., 1994). The initial events such as adsorption of the virus to the cells may have been facilitated by the fact that the cells originating from liver biopsies might have been handled as heparinised samples or were in contact with calci-

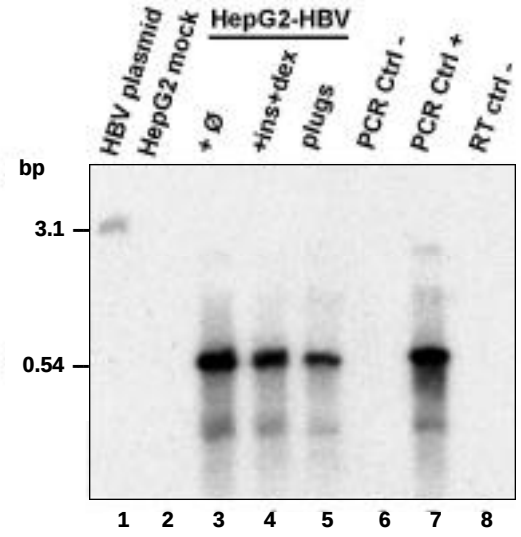

Fig. 7: Analysis of HBx RNA sequences by reverse transcription (RT)–PCR followed by Southern blotting.
RNA prepared from HBV-infected HepG2 cells was used as template for cDNA synthesis using a mixture of oligo dT and random hexanucleotides as primers after DNase I treatment. This cDNA was then amplified by PCR using specific primers of the X region of the HBV genome. PCR products were analysed by Southern blotting using a ^{32}P-labelled probe specific for HBV DNA.

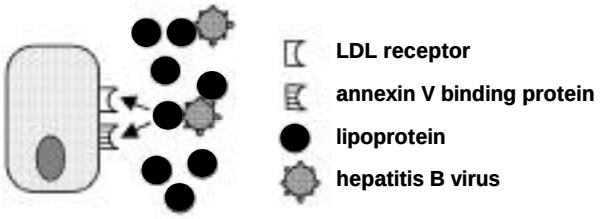

Fig. 8: Involvement of low density lipoproteins during the infection of hepatocytes with HBV *in vitro*.
The LDL receptor and the annexin V binding protein have been proposed to mediate HBV adsorption and penetration into the hepatocytes. Both molecules bind to low density lipoproteins (LDLs). Since HBV binds to the apolipoprotein H present in the LDLs, HBV might thus enter into the cells via the LDL receptor and/or the annexin V binding protein. The use of dextran sulphate prior to infection removes the free lipoproteins from the cell surface and facilitates subsequent binding and penetration of the LDL-HBV complexes into the hepatocytes.

um-chelating agents such as EGTA or ethylene diamine tetraacetic acid (EDTA) prior to the *in vitro* infection procedure, following collagenase treatment. It has been shown that heparin and calcium-chelating agents efficiently remove cell-bound lipoproteins from the hepatocyte cell line HepG2 (Havekes et al., 1983; Mulder et al., 1991; Wade et al., 1988). Heparin and EGTA might thus allow the removal of the cell bound lipoproteins prior to the addition of the viral inoculum composed of a viral lipoprotein complex. Furthermore, polyethylene glycol (PEG) has been shown to be critical for the *in vitro* infection of primary hepatocytes with HBV (Gripon et al., 1993). The effect of PEG during the infection of the cells with HBV might be explained by the fact that PEG readily precipitates high density lipoproteins (HDL) and other plasma proteins (Vikari, 1976; Warnick et al., 1979), however with several drawbacks (Warnick et al., 1982).

As the transfected HBV genome is transcribed efficiently in the HepG2 and HuH-7 cell lines after transfection (Galle et al., 1994; Ganem, 1996; Hirschmann et al., 1980; Hohne et al., 1990; Schinazi et al, 1999; Sells et al., 1987; Sureau et al., 1986; Weiss et al., 1996), attachment and entry were believed to be the restrictive steps during the infection process. Recently, it was shown that the treatment of hepatocytes with dimethyl sulfoxide (DMSO) might improve the expression and the presentation of differentiation-specific viral receptors, a process generally referred to as receptor activation (Paran et al., 2001). It is known that DMSO might act at multiple levels, such as in the generation of sustained, highly differentiated stages in the cultivated cells and in the arrest of hepatocytes in the G1 phase of the cell cycle. Indeed, these authors have shown that DMSO significantly improved the cell attachment of HBV via the preS1 sequence present on the HgSAg. DMSO activated at least two putative receptors at the cell surface, thus suggesting that HBV was binding to multiple receptors. Finally, we have not yet analysed, whether albumin played a trigger role in virus attachment to the host cell.

4.3 Improved effect of hormones on HBV infection and putative transforming effect of HBV

Insulin has been shown to modulate the expression of HBx through the activation of cellular transcription factors (Choi et al., 1998). A decrease of the expression of HBsAg-expressing cells was observed when insulin was employed in the cell culture medium during short-term incubation of cells transfected with HBV (Chou et al., 1989; Clementi et al., 1984). In contrast, our results strongly suggest that the long-term presence (two weeks) of insulin in the cell culture medium is followed by a dramatic increase of HBsAg expression (Fig. 1). Thus, insulin might play an important role in the HBV infection (Choi et al., 1998; Tur-Caspa and Laub, 1990). The exact mechanism by which cell-surface HBsAg expression, and possibly other viral polypeptides, are induced in long-term insulin-containing cell cultures remains to be established. However, it is known that promoters and enhancer elements are involved (Antonucci and Rutter, 1989; Hu and Siddiqui, 1991; Huan and Siddiqui, 1993; Karpen et al., 1988; Lee et al., 1998; Raney et al., 1992; Siddiqui et al., 1986; Su and Yee, 1992; Tur-Caspa and Laub, 1990; Vannice and Levinson, 1988).

We have shown that HepG2 cells following infection with HBV were able to grow in soft agar, thus strongly suggesting that the virus has a transforming potential. The exact molecular mechanisms by which this effect is obtained are unclear. It might involve the expression of the HBx protein (Arbuthnot and Kew, 2001; Diao et al., 2001; Feitelson and Duan, 1997; Hohne et al., 1990; Twu and Schloemer, 1987), even though this feature remains controversial (Gottlob et al., 1998).

4.4 Integration of HBV DNA after infection

As previously observed (Mabit et al., 1996), HBV infection of hepatoma cells can lead to the integration of the HBV genome into the cellular DNA. We confirm that the infection system described above indeed prompted the integration of the HBV genome into established hepatocyte cell lines after *in vitro* infection. Remarkably, this appears to be a fast process since it was already observed 8 days after the onset of infection, following facilitation of virus entry via the lipoprotein receptor and/or the annexin V binding protein, by pre-treatment of the cells with dextran sulphate. Interestingly, the process by which the viral HBV DNA integrates resembles that observed in duck hepatitis B virus (DHBV) infection (Yang and Summers, 1998). In this model involving primary duck hepatocytes, a phenomenon called "illegitimate replication" revealed the generation of a linear DNA intermediate prior to viral DNA integration. The same phenomenon was observed with our HBV-infected hepatoma cells, thus providing strong evidence that HBV can rapidly integrate into the genomic DNA of hepatocytes and become silent. Thereafter, the expression of HBsAg at the cell surface could be dramatically increased when a physiological hormone such as insulin was added to the cell cultures. Another interesting finding was the putative stimulatory effect of hormones and/or oligoelements (present in the MCDB-104 medium) on HBsAg expression, exerting a similar enhancing effect as insulin. Our results also provide evidence that the integration of the HBV genome into the genomic DNA of target cells might be a stable event. Indeed, the HBV infected HuH-7 and HepG2 cells analysed more than two years after the onset of the infection (with passages every two to three weeks into new cell culture flasks), still harbour integrated viral DNA. Evidence for such integrated DNA sequences in a majority of chronically infected livers and most cases of HBV-related HCC was already emphasised many years ago (Brechot et al., 1981; Kam et al., 1982). It was shown then that HBV integrates near regulatory cell cycle genes (de The et al., 1989; Wang et al., 1990) and, more recently, very close to the nuclear matrix genes (Shera et al., 2001). We have not seen the presence of replicative ccc DNA intermediates in our HBV-infected hepatoma cell lines; this feature was already observed with tumor-derived cell lines or hepatocellular carcinoma cells obtained from HBV-infected indi-

viduals (Rogler and Chisari, 1992; Shafritz, 1990). Thus, it is possible that peculiar HBV promoters and/or enhancers, yet switched off, have to be induced in order to express the HBV ccc DNA.

Work is in progress to determine the preferential sites of integration, if any, by working with cells of clonal origin. Many additional experiments are required to further study the sequential steps of the HBV *in vitro* infection process. Indeed, the potential inhibition of the adsorption and/or the penetration of the virus into target cells by using highly purified LDLs as competitor molecules, the fine analysis of the modulation of the HBsAg expression during time-course experiments, and the study of the mechanisms involved in the viral DNA integration, for example, are obvious goals for the future.

As yet, despite some progress, a major therapeutic breakthrough remains to be achieved (Locarnini, 1998; Lok, 2000; Lok, 2002). Hence new targets for antiviral therapy will have to be defined and obviously, detailed knowledge of the molecular mechanisms of hepatitis B virus (HBV) replication is required. To date, several types of regimens are available to reduce and/or inhibit the replication of the virus (Zoulim, 1999; Zoulim and Trepo, 1999). However, a strong need does exist for the screening and the identification of new antioncogenic agents during chronic, silent HBV infection when HBV DNA further integrates into the host genome, favouring cancerogenesis. The model system reported here may serve to look for new pharmacological targets to interfere with HBV adsorption and penetration and also DNA integration. This system thus has advantages over the transfection of naked HBV nucleic acids into the target cells, because with the use of the transfection procedure, these two first steps cannot be studied. It might also allow for the *in vitro* screening of candidate inhibitors of therapeutic potential, without the use of animals.

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