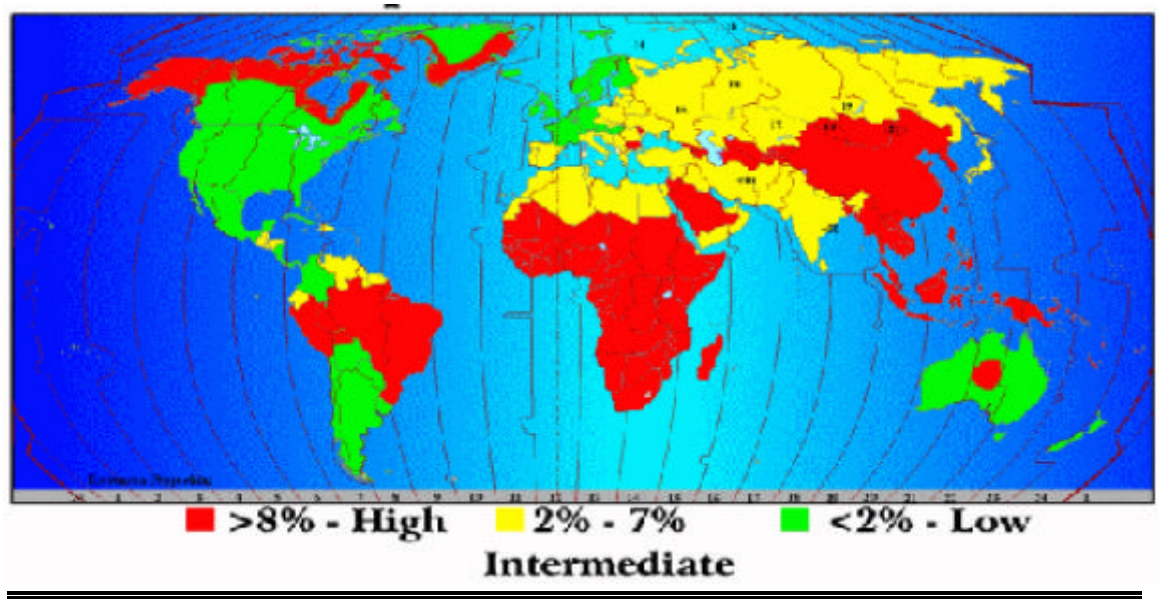
1- Introduction

1.1- Human Hepatitis B virus (HBV)

1.1.1- Pathogenesis of Hepatitis B

Hepatitis B is one of the most common diseases of mankind and a serious problem for global public health. It is preventable with safe and effective vaccines which had been available since 1982 (Michael ML. Et al., 1984; Carloni G. et al., 1984). The initial use of HBV vaccines was limited by their high cost. Two billion people have been infected with hepatitis B virus (HBV) worldwide (Lee WM, 1997). Among those, more than 350 million have chronic (lifelong) infection, 150 million are carriers and live in the Western Pacific region and 125 million carriers are in United States (WHO Fact Sheet, revised October 2000). The global prevalence of hepatitis B is shown below (map 1). These chronically infected persons are at high risk of developing liver cirrhosis and hepatocellular carcinoma, that accounted for about one million deaths each year.



Map 1: geographical distribution of HBV endemicity (WHO report, 2000)

Viral Hepatitis

Hepatitis is an inflammation of the liver that results primarily from infection with one of six viruses called hepatitis A, B, C, D, E and G (Fischler B. et al., 1997). All of these viruses can cause an acute disease with symptoms lasting several weeks including yellowing of the skin and eyes. It can take several months to a year to feel recover

again. Hepatitis B virus can cause chronic infection of the liver, a condition where the patient is unable to eliminate the virus and may develop liver cirrhosis or liver cancers many years later. HBV is the most serious type of viral hepatitis and the only type causing chronic hepatitis for which a vaccine is available.

Mode of infection

In the majority of developing countries (sub-Saharan Africa, most of Asia and the Pacific), most people get infected with HBV during childhood and 8-10% of the general population are chronically infected. In these regions, liver cancer caused by HBV is the leading cause of cancer deaths in men (the second most frequent cause in our industrialized countries following tobacco abuse). The consequences of HBV infection depend on the age at the time of infection. The risk of an infected person to become a carrier drops from 90% in the first six months of life to about 25% by the age of five years, and to 10% by the age of 15 years. On a hand, it is unusual (2-5%) for those infected later during adult life to become chronic carriers. On the other hand, the older the person when infected, the more likely he will develop an acute illness.

Hepatitis B virus is transmitted by contact with blood or body fluids of an infected person in the same way as human immunodeficiency virus (HIV). However, HBV is 50-100 times more infectious than HIV. The most common ways of getting infected with HBV are perinatal infection (from mother to baby around birth), unsafe injections or transfusions and sexual contact, but not by oral contact with contaminated food or water.

Treatment of chronic hepatitis B and liver cancer

Liver cancer is almost always fatal, and develops between 35 and 65 years of age. In our countries, surgery and chemotherapy can prolong the life of an infected individual for a few years, but the disease can rarely be cured. In absence of immunoprophylaxis, the cumulative rate of recurrence of hepatitis B infection has exceeded 90% (Samuel D. et al., 1993; Terrault NA., 1999). In some patients, chronic hepatitis B can be treated with drugs called interferon alfa-2b ($30x10^6$ I.U. per week for 4 months) and lamivudine, a nucleoside analog (once-daily dosing with 100mg) (Lin O. and Keeffe E.B., 2001). However, interferon or lamivudine therapy has many side effects, costs thousands of euros and will never be available to most patients in developing countries.

In chronic hepatitis B with end-stage liver disease lamivudine therapy may serve as a bridge to liver transplantation. However, despite an effective pre-liver transplantation anti-HBV vaccination, cases of HBV recurrence are reported in a patient transplanted with an anti-HBcAg-positive liver graft (Dumortier J. et al., 2002). A long-term prophylaxis to this patient was essential to prevent HBV recurrence.

Vaccine

Although the vaccine will not cure chronic hepatitis, it is 95% effective in preventing the development of chronic infections, and is considered to be the first vaccine preventing a major human cancer. Since 1982, over one billion doses of hepatitis B vaccine have been used worldwide. As of March 2000, 116 countries had included hepatitis B vaccine in their national vaccination programs. However, the cost of this vaccine has been one of the main obstacles that prevented the introduction of this vaccine to many of the countries in sub-Saharan Africa, Indian subcontinent and the Newly Independent States.

1.1.2- Genome organization of HBV

HBV is the prototype member of the hepadnaviridae family which is comprised of members found in woodchucks (Summers J., 1978), ground squirrels (Marion PL et al., 1980), Pekin duck (*Anas domesticus*) (Mason W.S. et al, 1980) and grey herons (*Ardea cinerea*) (Sprengel R et al, 1988), and primates including wooly monkeys (Landford et al., 1998), orangutans, gorillas and gibbons (Schäfer S. et al, 1998; Robertson B.H. and Margolis H.S., 2002). Recently new hepadnavirus are characterized in cranes (Prassolov A. et al., 2003).

Besides their species and tissue specificity, all these viruses have the same genomic organization and virion structure and replicate their DNA genomes by reverse transcription of an RNA intermediate. With about 3.2 kb, the hepadnaviral genome is one of the smallest viral genomes known (Galibert F. et al., 1979). It shows an extremely compact organization and encompasses at least three extensively overlapping open reading frames (ORF) (fig.1a). These ORFs encode proteins with essential functions for the viral life cycle: the DNA polymerase/reverse transcriptase (P) with RNase H activities, the core or capsid protein (C) that assembles into an icosahedral nucleocapsid and at least two surface proteins present in the viral envelope. Mammalian

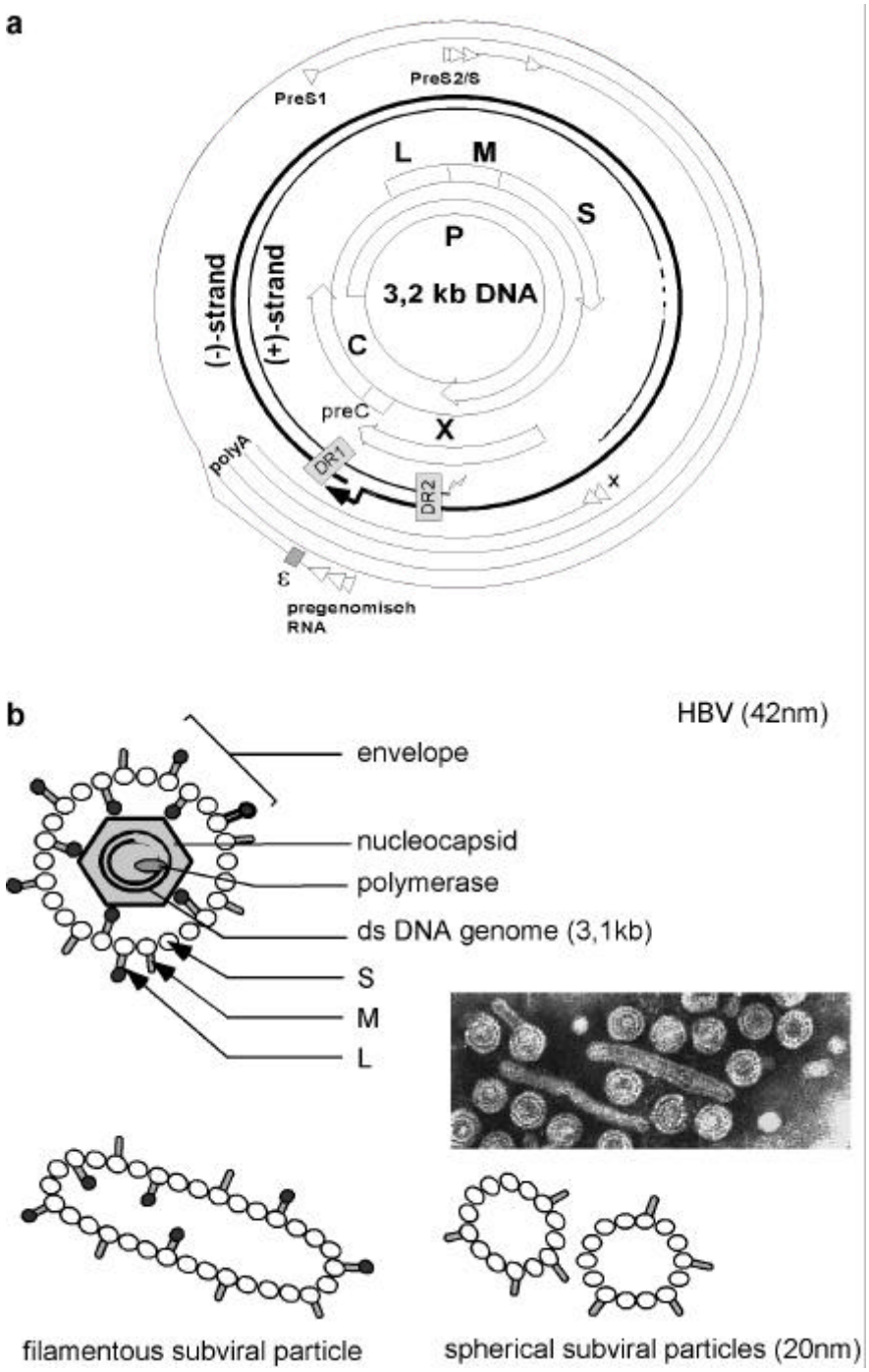


Fig.1: Structural and genetic organization of hepatitis B virus (HBV) (**a**) Genome organization of HBV (based on fig.1 of (Nassal M. and Schaller H., 1993)). The outer lines represent the different classes of transcripts, the bold inner circles the DNA genome as present in the virion. (**b**) The infectious HBV virions (42nm Dane particle) consists of an outer envelope, which contains the large (**L**), middle (**M**) and small (**S**) surface proteins, and an inner **nucleocapsid**, or core, which harbours the double-strand DNA genome with the viral DNA polymerase. No-infectious spherical or filamentous subviral particle are excreted. Electron micrograph of virions and subviral particles is shown.

viruses carry an additional ORF encoding the nonstructural X protein that has been shown to act as a pleiotropic transcriptional transactivator by influencing signaling pathways. While the X gene product is apparently not required for virus production, but it appears to be essential for the establishment of infection *in vivo* (Zoulim F. et al., 1994).

1.1.3- Structure of HBV virion

The HBV virion (Dane particle) is a double-shelled sphere, 42 nm in diameter, with an inner nucleocapsid enclosing the viral genome with covalently bound polymerase proteinand an outer lipoprotein envelope (Fig.1b). The envelope of mammalian hepadnaviruses

contains three closely related viral proteins, the large (L), middle (M) and small (S) envelope protein (Heermann K.H. et al., 1984), while members of the avian genera carry only the L and S polypeptides. The envelope proteins are likely to mediate the first contact between the virus

and the hepatocyte. However, at least for HBV, the early steps of the infection cycle, viral adhesion, entry and uncoating are still poorly understood due to the limited host range of the virus and lack of an *in vitro* infection system.

1.1.4- HBV life cycle

Apart from the restricted possibility to employ *in vitro* infection of primary human hepatocytes (Galle P.R. et al., 1994; Gripon P. et al., 1993) and newly of a human hepatoma cell lines (Gripon P. et al., 2002), much of what is know about the HBV life cycle has come from transfection studies of cloned HBV genomes into suitable human liver cell lines (HepG2 or HuH7) that support virus production, but are refractory to infection (Sureau C. et al., 1986).

Most infection studies *in vitro* and *in vivo* were performed with the duck HBV (DHBV) animal model. Cellular uptake of hepadnaviruses is generally assumed to include receptor binding which is predominantly mediated by the large viral envelope protein, endocytosis, subsequent capsid release by membrane fusion and nuclear import of the viral genome (fig.2).

Following delivery of the partially double-stranded viral DNA genome to the nucleus, it is filled in to yield a covalently closed circular DNA (cccDNA) molecule (Tuttleman

J.S. et al., 1986; Köck et al., 1993) that serves as a template for transcription by host RNA polymerase II (fig.3) (for a review see: (Ganem D. and Varmus HE., 1987)). In HBV, several sets of 0.7, 2.1, 2.4 kb subgenomic and 3.5 kb genomic transcripts are produced and transported to the cytoplasm, where they are all used as mRNAs. Nuclear export of viral transcripts is regulated by means of a post-transcriptionally regulatory element present in all viral mRNAs (only know for HBV and WHV). One of the transcripts, the 3.5 kb terminally genomic RNA is used for translation of the core protein and the viral polymerase, and serves as a template for reverse transcription. This RNA molecule is termed the RNA pregenome. Reverse transcription is initiated by a protein-priming mechanism through binding of the viral polymerase to a cis-acting recognition signal on the RNA, called encapsidation signal **e** (Junker-Niepmann M. et al., 1990; Bartenschlager R. and Schaller H., 1992; Wang G.H. and Seeger C., 1992). Formation of this complex is facilitated by a host cell chaperone, the heat shock protein Hsp90, that maintains the viral polymerase in a specific conformation required for its

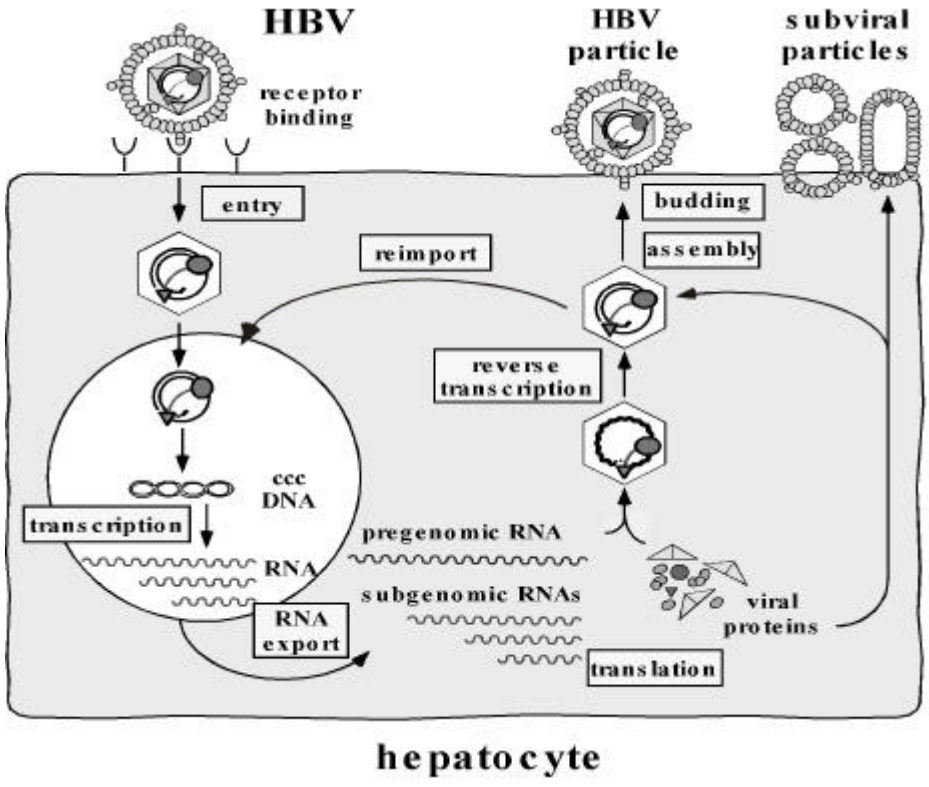


Fig. 2: The basic replication cycle of HBV in hepatocytes

functional activity (Hu J. and Seeger C, 1996). Covalent attachment of the polymerase to ε triggers nucleocapsid assembly, a highly ordered reaction, in which 90 or 120 core

protein dimers form an icosahedral shell around the replication complex (Crowther R.A. et al., 1994; Böttcher B. et al., 1997).

Within these premature core particles, the pregenome is then transcribed into minus-strand DNA by the action of the P protein for which the cis-acting direct repeat DR1 and DR2 are required (Seeger C. et al, 1991). Concomitantly, the RNA template is degraded except a small fragment which serves as a primer for plus-strand DNA synthesis. During plus-strand elongation, the genome is circularized but DNA polymerization ceases before the strand is completed. This is likely due to envelopment of nucleocapsids which may prevent further dNTP supply (Ganem D., 1996). During the early phase of infection, the mature nucleocapsids transport the replicating viral genome back to the nucleus to amplify the intracellular pool of viral DNA (Tuttleman J.S. et al., 1986).

Later during infection, the nucleocapsid associates with viral envelope proteins that are synthesized as polytopic membrane proteins of the endoplasmic reticulum (ER) membrane, where they rapidly oligomerize. The assembly of HBV is thought to occur at post-ER/pre-Golgi membranes where preformed cytosolic nucleocapsids are packaged by the transmembrane viral envelope proteins. The preS region of L and the cytosolic S loop play a pivotal function in this process (Bruss and Ganem, 1991; Ueda et al., 1991; Huovila et al., 1992 Löffler-Mary et al., 2000). Virions then leave the cell via the constitutive pathway of secretion without lysing the host cell. Accordingly, hepatitis B virus infections are non-cytopathic. Rather, HBV-associated liver damage and disease are thought to be mediated by cellular immune response to 10^{13} spherical or 10^{10} filamentous particles per ml serum of HBV-infected individuals and appear in huge excess over 10^{9} virion particles containing HBV DNA genome per ml serum (Heermann .H. and Gerlich W.H., 1991). The role of theses particles in the viral life cycle is poorly understood. Subviral HBsAg particles are noninfectious. Possibly, they present decoys to drape neutralizing antibodies thereby facilitating the progression of infections by virions and inducing such as immune tolerance.

1.1.5- Experimental models for the study and therapy of HBV infection

Studies on the interaction between HBV and the immune system have been hampered by the fact that HBV exhibits a very narrow host range and shows a strong tropism for liver parenchymal cells. The susceptibility to HBV infection is restricted to

differentiated cells. Only primary human hepatocytes are susceptible to HBV infection (Gripon P. et al., 1988). Several human hepatoma-derived cell lines support HBV replication after HBV DNA transfection (Sells M.A. et al., 1987; Sureau C et al., 1986), but none are susceptible to HBV infection. Stable cell lines with integrated HBV genomes, e.g., HepG2.2.15 cells (Sells A. et al., 1987) are used to characterize the action of drugs on HBV replication. A recent hepatoma-derived cell line HepaRG, that expresses a representative panel of liver-specific genes, seems to be susceptible to HBV infection (Gripon P. et al., 2002).

HBV infects only humans and higher primates like chimpanzees. However ethic laws and higher costs for chimpanzees have been one of the main obstacles for clinical/animal trials. Closely related viruses only infect animals, e.g. ducks, woodchucks or ground squirrels, whose immune system is poorly defined. Although some work helped in the characterization of woodchuck or duck cytokines (Lohrengel B. et al., 1998; Schultz U. et al., 1995).

HBV1.3 transgenic mice, whose hepatocytes replicate the virus at levels comparable to patients with chronic hepatitis have been developed without any evidence of cytopathology (Guidotti L.G. et al., 1995). The HBV transgenic mouse model creates the opportunity to examine many aspects of HBV immunobiology and pathogenesis that have not been analyzed before. The disadvantage is that HBV replicates from an integrated genome, which can't be eliminated. An tissue culture system is established based on immortalized, highly differentiated hepatocytes prepared from mice transgenic for both c-Met and HBV (Pasquetto V. et al., 2002). Alternatively adenovirus vectors have been used to transfer replication-competent hepatitis B virus genomes into liver cells and to initiate HBV replication in experimental animal (Ren S. and Nassal M., 2001; Sprinzl M.F. et al., 2001). So DHBV replication is initiated in C57/BL56 mice, which is injected with adenovirus AdDHBV (Sprinzl M.F. et al., 2001).

Using the HBV1.3 transgenic mice, Chisari F.V. and coworker have shown that the intrahepatic induction of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interferon alpha/beta (IFNα/β)downregulates HBV replication noncytopathically in the liver (Guidotti and Chisari, 1996; Guidotti and Chisari, 1999). This antiviral effect was achieved by injecting HBV transgenic mice with HBV-specific cytotoxic T lymphocytes (CTL) (Guidotti L.G. et al., 1996a) or infecting them with an unrelated hepatotropic virus, such as lymphocytic choriomeningitis virus (LCMV) or an

recombinant replication-deficient adenovirus (Guidotti L.G. et al., 1996b; Cavanaugh V.J. et al., 1998). The CTL-dependent effect occurs within 24 hours and appears to be mediated by both IFNγ and TNFα (Guidotti L.G. et al., 1996a). The LCMV- and adenovirus-dependent effect occurs in two distinct phases. The first phase occurs within 12 to 24 hours and is mediated by IFNα/β and/or TNFα induced by the infecting virus. The second phase occurs 5 to 7 days post-infection and is associated with the intrahepatic induction of IFNα/β and TNFα as well as IFNγ produced during the cellular immune response to each virus (Cavanaugh V.J. et al., 1998). Using this same transgenic mouse model, IL-12 inhibits HBV replication via an IFNγ-dependent pathway (Cavanaugh V. et al., 1997). These noncytopathic antiviral events described in the HBV1.3 transgenic mice model are confirmed in the liver of chimpanzees acutely infected with HBV (Guidotti L.G. et al., 1999).

1.2- Cytokines as mediator immune modulation and antiviral responses

Cytokines are low-molecular-weight regulatory proteins or glycoproteins secreted by white blood cells and various other cells in the body in response to a number of stimuli. Cytokines serve as local messengers of the immune system. To exert their biological effects, cytokines must first bind to specific receptors expressed on the membrane of responsive target cells. Because these receptors are expressed by many types of cells, the cytokines can affect a variety of cells. The cytokine-binding receptors are classified in fives families: immunoglobulin superfamily receptors, class I cytokine receptor (hematopoietin receptor), class II cytokine receptor (interferon receptor), the TNF receptor and chemokine receptor families. Interferons are member of cytokines produced by virus-infected cells with function to induce a generalized antiviral state in nearby non-infected cells. Interferons have other effects including the capacity to induce cell differentiation, to inhibit proliferation by some cell types, to inhibit angiogenesis, and to function as immunoregulators (for review see chap. 12 in (Goldby R et al., 1999)).

1.2.1- Large family of interferons

Interferons (IFN) are a large family of multifunctional secreted proteins involved in antiviral defense, cell growth regulation and immune activation. The interferons may be

classified into two types: type I and type II interferon. They share no structural homology.

1.2.1.1- Type I interferon

They are produced in direct response to virus infection and consist of the products of the INFα multigene family and the product of the INFβ gene.

IFNa forms are produced by monocytes/macrophages, lymphoblastoid cells, fibroblasts, and a number of different cell types following induction by viruses, nucleic acids such as double-stranded RNA or polyinosinic-polycytidylic acid poly(I-C) (De Clercq E., 1981; Pitha P.M., 1981), glucocorticoid hormones, and low-molecular weight substances (n-butyrate, 5-bromodeoxy uridine). At least 23 different variants of IFNα are known. The individual proteins have molecular masses between 19-26 kDa and consist of 156-166 and 172 amino acid long proteins. There are at least 23 different IFNα genes. They have a length of 1-2 kb and are clustered on human chromosome 9p22 (Snows T.B. et al., 1982). It is not known whether all these genes are expressed following stimulation of the cells. IFNα genes does not contain intron sequences found in most other eukaryotic genes. Based on structural differences, two types of IFNα genes, designated class 1 and 2, are distinguished. They encode proteins of 156-166 and 172 amino acids, respectively. In some cell systems expression of some subtypes (IFNα-1, IFNα-2 and IFNα-4) is stronger than those of others. IFNα is used for the treatment of chronic hepatitis B and C. In combination with other agents, IFNα is also used in cancer therapy .

IFNb is produced mainly by fibroblasts and some epithelial cell types. The synthesis of IFNβ can be induced by common inducers of interferons, including double-stranded RNA, viruses, other micro-organisms. It is also induced by some cytokines such as TNFα and IL-1 . IFNβ weights about 20 kDa and has a length of 166 amino acids. Glycosylation is not required for its biological activity *in vitro*. The human gene encoding IFNβ has a length of 777bp and maps to chromosome 9p22 in the vicinity of the IFNα gene cluster. The IFNβ gene does not contain introns. IFNβ binds to the same receptor as IFNα but in contrast to IFNα, IFNβ is strictly species-specific, so that IFNβ of other species is inactive in human cells. IFNβ is involved in the regulation of nonspecific humoral immune responses and immune responses against viral infections.

IFNβ stimulates the activity of NK-cells and hence antibody-dependent cytotoxicity. It also increases serum concentrations of Beta-2-Microglobulin. IFNβ selectively inhibits the expression of some mitochondrial genes. IFNβ shows antiproliferative activity against a number of cell lines established from solid tumors. In combination with IFNα, IFNβ has been used in the treatment of chronic hepatitis B and it appears to be most promising if the disease has not lasted longer than 5 years. IFNβ has emerged as the first drug capable of producing clinical improvement in multiple sclerosis (MS).

1.2.1.2- Type II interferon

Type II interferon is the product of IFNγ gene (Allen G. and Fantes KH., 1980),and rather than being induced directly by virus infection, is synthesized in response to the recognition of virally infected cells by activated T lymphocytes and natural killer (NK) cells. IFNγ is mainly produced by T-cells and natural killer cells activated by antigens, mitogens, or alloantigens. It is produced by lymphocytes expressing the surface antigens CD4 or CD8. The synthesis of IFNγ is induced, among other things, by IL2, bFCF and EGF. IFNγ is a dimeric protein with subunits of 146 amino acids. IFNγ is synthesized as a precursor protein of 166 amino acids including a secretory signal sequence of 23 amino acids and is glycosylated at two sites. Two molecular forms of the biologically active protein, the 20 and 25 kDa, have been described. Both of them are glycosylated at position 25. The 25 kDa form is also glycosylated at position 97. Recombinant IFNγ isolated from *E. coli* is biologically active and glycosylation therefore is not required for biological activity. At least six different variants of IFNγ have been described by variable lengths of the carboxyterminal ends. No biological activities of these variants were observed. In contrast to type I interferons, IFNγ is labile at pH 2. Murine and human IFNγ show approximately 40% sequence homology at the protein level. The human gene has a length of approximately 6 kb. It contains four exons and maps to chromosome 12q24.1. IFNγ has antiviral and antiparasitic activities and also inhibits the proliferation of a number of normal and transformed cells. IFNγ synergies with TNFα and TNFβ in inhibiting the proliferation of various cell types. The growth inhibitory activities of IFNγ are more pronounced than those of other interferons. However, the main biological activity of IFNγ appears to be immunomodulatory. IL-2 induces the synthesis of IFNγ and other cytokines by T-helper cells. IFNγ regulates the expression

of MHC class II genes and is the only interferon that induces the expression of these proteins. IFNγ specifically induces the transcription of a number of genes. These genes contain regulatory DNA sequences within their promoter regions (interferon-stimulated response element ISRE) that function as binding sites for a number of transcription factors. Some of these genes are also expressed in response to other inteferons. Like other interferons, IFNγ can be used as an antiviral and antiparasitic agent and has been shown to be effective in the treatment of chronic polyarthritis. This treatment, which probably involves the modulation of macrophage activities, significantly reduces joint aches and improves various clinical parameters and allows reduction of corticosteroid doses. IFNγ may also be of value in the treatment of opportunistic infections in AIDS patients. It has been shown to reduce inflammation, clinical symptoms, and eosinophilia in severe dermatitis.

1.2.2- Induction of interferon expression

INFβ expression is induced by intracellular double-stranded RNA (dsRNA) which is provided by the viral genome itself or as result of replication or transcription of viral genomes. This induction of IFNβ gene expression occurs at the level of transcriptional initiation. The key induction event is the redistribution of the transcription factor NF-κB from cytoplasm to nucleus (Lenardo et al., 1989), which is activated via the dsRNA-dependent protein kinase R (PKR) (Maran A. et al., 1994).

IFNα can be upregulated by virus infection, but unlike IFNβ, the IFNα promoters do not have an NF-κB site, but contain elements that are related to the positive regulatory domain I- (PRD I) and ATF-2-binding sites which can bind interferon regulatory factors (IRF). However, the induction mechanism is poorly characterized.

IFNγ expression is induced via transcriptional activation in immune cells. The IFNγ promoter is under control of two distinct regulatory elements (proximal and distal). The mechanism of activation is also poorly characterized, but involves the p38 and JNK2 mitogen-activated protein kinase (MAP kinase) pathways (Rincon M. et al., 1998). IFNγ produced by T-cells, is dependent on IL-12 production by antigen-presenting cells and is stimulated by IL-18 (Singh S.M. et al., 2000). Molecular mechanism may involve activation of STAT4 by IL-12 and NF-κB by IL-18.

1.2.3- Interferon-inducible intracellular mechanisms as antiviral response

IFNα and IFNβ activate a variety of intracellular antiviral mechanisms and more than one mechanism can be activated at the same time in an infected cell. Many steps in the viral life cycle can be target: transcription, translation, assembly and secretion. A large variety of interferon-inducible genes have been identified that are activated by the JAK-STAT signal transduction cascade (Kalvakolanu D.V. and Borden E.C., 1996). Among the interferon-inducible genes, the 2´5´oligoadenylate synthetases, the Mx proteins, the double-stranded-RNA-activated protein kinase (PKR), and the double-stranded-RNA-specific adenosine deaminase systems are the best characterized.

The 2´5´-oligoadenylate system mediates antiviral activities mainly by induction of endoribonuclease L (RNAse L), which forms dimers to degrade single-stranded RNA including cellular and viral mRNA (Silverman RH, 1994; Dong B. and Silverman R.H., 1999) thereby leading to inhibition of protein synthesis. These enzymes are induced by both interferon type I and type II (Chebath J. et al., 1987).

The MxA protein is an interferon-induced GTPase that selectively inhibits the trafficking or activity of viral polymerases. Influenza and bunyaviruses probably are inhibited probably via direct binding of MxA to viral ribonucleoprotein complexes (Kochs G. and Haller O., 1999) and blocking their import into the nucleus, as has been reported for Thoghoto virus infection.

The interferon-inducible PKR is a serine/threonine kinase that has multiple functions in the control of transcription and translation (Clemens M.J. and Elia A., 1997). Activated PKR phosphorylates the α subunit of the eukaryotic translation initiation factor eIF2 and prevents recycling of initiation factors. In cells exposed to interferon, the level of activated of PKR is elevated and causes an enhancement of signal transduction via NF-κB activation. Induction of Bcl2- and caspase-dependent mechanism of apoptosis was also reported (Lee S.B. et al., 1997). But PKR is not sufficient to mediate full antiviral response (Yang Y.-L. et al., 1995).

Other factors that play a role in the interferon-induced antiviral response are caspases inducing apoptosis and the dsRNA-dependent adenosine deaminase (ADAR), which recognizes dsRNA as a substrate and replaces adenosines with inosine (Bass B. et al., 1989; Patterson J.B. et al., 1995).

Interferons like IFNγ can inhibit cell growth and thereby inhibit the replication of some viruses. The sensitivity of cells to the antiproliferative effects of interferons is cell-type dependent.

1.3- Adenovirus as vector for gene transfer

The year 1953 witnessed the isolation of adenovirus, which was soon recognized as an invaluable tool for investigating mammalian molecular biology. Several of the distinguishing features of adenovirus had made it the preferred vehicle for gene transfer and transgene expression in mammalian cells.

1.3.1- Pathogenesis of adenoviral infection

Members of the adenovirus family (Adenoviridae) infect a great variety of post-mitotic cells, even those associated with highly differentiated tissues such as skeletal muscle, lung, brain and heart. Adenoviruses are associated with a number of disorders, most of which are mild. The pathology is primarily from inflammation and loss of infected epithelial cells. Viruses of subgroup C (serotype 2, 5) cause various respiratory tract infections in confined groups (elderly, military recruits and children).

1.3.2- Structure and genome organization of Adenovirus

Adenovirus is a non-enveloped, 80-110 nm diameter virus with an icosahedral capsid consisting of three major proteins, hexon (II), penton base (III) and a knobbed fibre (IV), along with a number of other minor proteins, VI, VIII, IX, IIIa and IVa2 (fig.3a). Human adenoviruses contain a linear, double stranded DNA genome, with a terminal protein (TP) attached covalently to the 5’ termini. The DNA, which has a length of approximately 36 Kb, is wrapped in a histone-like protein and has inverted terminal repeats (ITRs) of 50-200 bp, which act as origins of replication. The virus contains a virus-encoded protease (Pr), which is necessary for processing some of the structural proteins to produce mature infectious virus. The hexon, penton base, and knobbed fiber, are the most important capsid proteins for gene delivery. Hexon is the major protein forming the 20 triangular faces of the viral capsid. The 240 hexon capsomers in the capsid are trimers, each interacting with six other trimers. The 12 vertices are formed by the penton capsomere, a complex of five copies of the penton base, and three copies of fiber. Each penton capsomere interacts with five hexon capsomeres, one from each of

the five faces that converge at the vertex. The knobbed fiber protrudes from the fiber base.

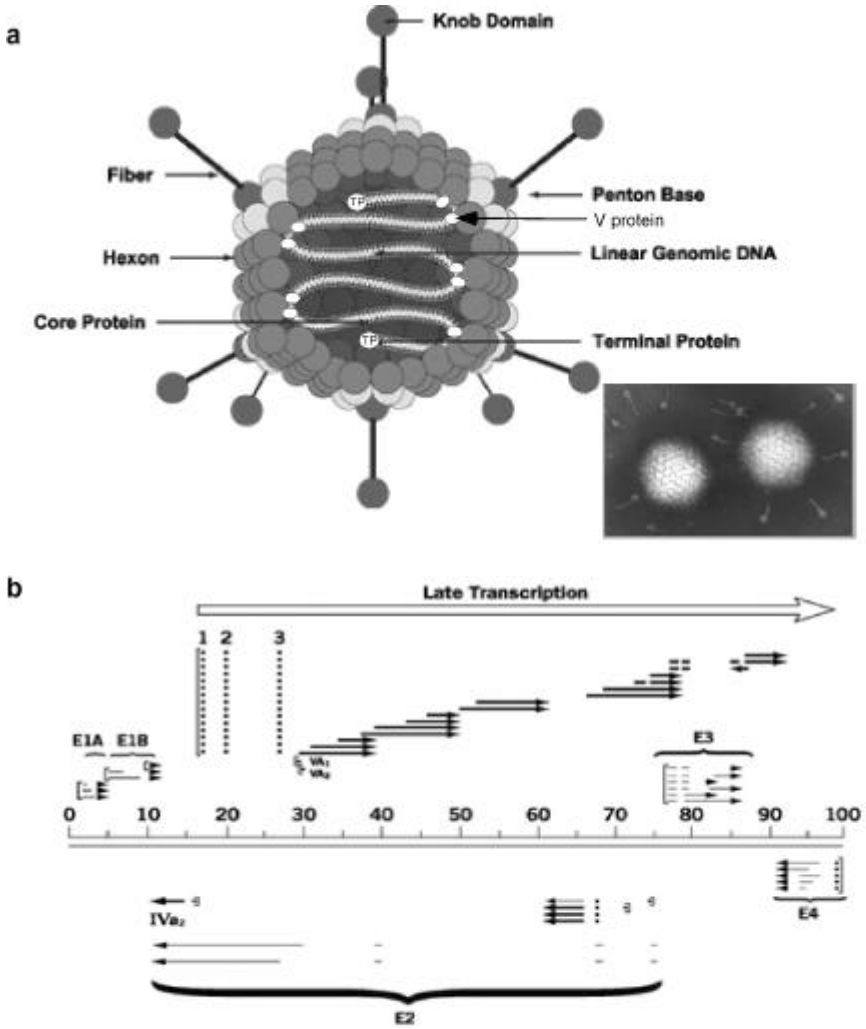


Fig.3: Structural model of adenovirus virion and transcription of adenovirus genome. (**a**) A stylized section of the adenovirus particle with an electron micrograph of adenovirus.
(**b**) Transcription of the early genes (E1A, E1B, E2, E3 and E4) and most late mRNAs originate at 16.3 map units and contain the tripartite leader whose components are labeled 1, 2 and 3. Heavy lines also designated mRNA species derived from early transcription units that are synthesized at elevated levels late after infection.

A complex series of splicing accompanies transcription of viral RNA´s, and genes are transcribed from both strands of the DNA genome. Adenovirus transcription is a two-phase event, early and late, occurring before and after viral DNA replication, respectively. The viral chromosome carries five early transcription units (E1A, E1B, E2, E3 and E4), two delayed early units (IX and IVa2) and one late unit (major late), which

is processed to generate five families of late mRNAs (L1 to L5), all of which are transcribed by RNA polymerase II (fig.3b). The E1 gene products can be further subdivided into E1A and E1B. E1 gene products are involved in the replication of the virus and are necessary to activate expression of the remaining viral genes. To gain replication deficiency of the virus used as gene delivery tool, and to prevent cell lysis, first generation recombinant adenoviruses are E1 deleted. Once packaged into a complementing cell line, i.e. a cell line that provides the E1 products *in trans* (e.g. in 293 cells), viral replication will be enabled.

The E2 region is subdivided into E2A and E2B. These proteins provide the machinery required for viral DNA replication and transcription of late genes.

Most of the E3 proteins are involved in modulating the immune response of infected cells, a function not essential for viral growth in vitro. Therefore, in addition to being E1 deleted, the first generation of adenoviruses are most often E3 deleted (E1/E3).

Gene products encoded by the E4 region (called ORFs 1-6/7) are involved in the metabolism of viral messenger RNA and provide additional functions that promote virus DNA replication and shut-off host protein synthesis. Furthermore, E4 products prevent viral DNA concatenation.

1.3.3- Adenovirus life cycle

The infectious cycle of adenovirus can be divided into an early and a late phase. The early phase covers the entry of the virus into the host cell and passaging its genome to the nucleus, followed by selective transcription and translation of the early genes. These early genes modulate the cellular machinery to facilitate replication of viral DNA which triggers transcription and translation of the late genes. The late phase is characterized by the assembly of the structural proteins of the virus in the nucleus and the maturation of infectious virus.

The adsorption of the virus to target cell receptors involves high-affinity binding via the knob portion of the fiber. The prime receptor for human adenovirus is identical to that for coxsackie B virus and has been named the coxsackie adenoviral receptor (CAR). After the attachment step, interaction between the penton base and the cell surface $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin coreceptors (Wickham T.J. et al., 1993; Bai M. et al., 1993) leads to internalization of the virus through endocytosis. Once inside the cell, and with help from the penton base, the virus escapes the endosome and translocates to the nuclear

pore complex, where the viral DNA is released into the nucleus and transcription begins. Transcription, replication and viral packaging take place in the nucleus of the infected cell. After encapsidation of viral DNA, a major change occurs in the nuclear infrastructure resulting in permeabilization of the nuclear membrane. This facilitates the egress of the virus into the cytoplasm and is followed by the disintegration of the plasma membrane and release of adenovirus from the cell.

1.3.4- Recombinant Adenovirus type 5 as vector for gene transfer

Adenoviruses can infect a wide variety of cell types and tissues in both dividing and non-dividing cells. Adenovirus do not integrate their genome into the cellular chromosomal DNA (Bett AJ. et al., 1993; Romano G. et al., 1999). This characteristic has led to their extensive use as a vector for gene transfer in about 80 clinical trials since 1993 in the United States (Liebert M., 2002). The virus can incorporate only 2 kb of foreign DNA without significant effect on virus stability or infectivity. To introduce larger DNA sequences into adenoviral vectors, some non-essential viral genes have been removed.

Replication-defective adenoviral vectors based on serotype 5 (Crystal et al., 1994; Harvey et al., 1999), which has an E1A/E1B (480-3534 bp) and E3 (28683-30469 bp) gene deletions, were the first generation vectors to be evaluated for *in vivo* gene transfer in a wide variety of preclinical models. Adenovirus serotype 5 is not associated with severe illness and do not cause tumors in animals, in contrast to other serotypes. In addition, adenoviral vectors based on serotype 5 is an excellent candidate for liver-directed gene therapy approaches, because it preferentially localized in the liver post-intravenous injection in rodents (Jaffe HA et al., 1992; Prevec L. et al., 1989). About 6% of the wild-type adenoviral genome (7-8 kb) of transgene DNA can be inserted in the E1-E3-deleted adenoviral vector. Adenoviral plasmids can be generated by homologous recombination in eukaryotic cells (293 packaging cell line), using recombination in bacteria (AdEasy system (He T-C et al., 1998)) or during productive lambda phage infection (McVey D. et al., 2002). Propagation and titration of first generation adenovirus required complementation for the missing E1 functions by a variety of cell lines, such as the human 293 cell line (Graham F.L. et al., 1977) or the 911 cell line (Fallaux F.J. et al., 1996).

However, replication-defective adenovirus vector induce a substantial inflammatory response in naive animals, that limits the expression of transduced genes as well as readministration of vectors (Smith T.A. et al., 1993; Gallin J.I., 1989). Host immune responses to the low level of adenoviral gene expression after vector administration are thought to be responsible for the majority of the adverse events in studies employing experimental animals (Yang Y. et al., 1995). For this reason, there has been considerable efforts in gene therapy field to develop advanced forms of adenoviral vectors in which many or all viral genes have been deleted for the goal of diminishing vector-induced host immune responses (Engelhardt J.F., 1994; Amalfitano A. and Chamberlain J.S., 1997). More sophisticated vectors (second- and third generation) have been constructed in which nearly all of the virus genes has been removed with "gutless" or helper-dependent adenoviral vectors (Amalfitano A. et al., 1998; Kochanek S., 1999; Schiedner G. et al., 2002).

1.4- Tetracycline-based inducible system: the Tet-off and Tet-on system

Regulatory elements from the Tn10-encoded tetracyclin (tet) resistance operon from E. coli (Hill W. and Wissmann, 1989; Hill W. and Berens C., 1994) have been implemented in gene expression systems that can be regulated by tetracycline. One of these tet inducible systems utilizes a tet-transactivator, tTA to regulate gene expression. This chimeric protein consists of a fusion between the C-terminal domain of VP16 from herpes simplex virus, known to be essential for transcription of immediate early viral genes (Trizenberg S. et al., 1988) and the tetracycline repressor from E.coli, tetR (Gossen M. and Bujard H., 1992; Gossen M. et al., 1995). tTA binds to the tet operator (tetO) when tetracycline is absent. In the presence of even low concentration of tetracycline, however, a conformational change in the tet repressor domain prevents the binding of tTA to tetO. When multiple tetO sequences are placed upstream of a reporter gene, expression of tTA results in activation of gene expression in the absence of tetracycline. This system has been used *in vitro* (Früh K. et al., 1994; Wimmel A. et al.,1994) and *in vivo* (Furth P.A. et al., 1994; Kistner A. et al.,1996). A problem with this system is that tetracycline must be present to repress gene expression, and tTA protein is toxic to mammalian cells (Gossen M. et al., 1993). A second problem is that in several instances a high level of basal expression independent of tTA binding in this

so called Tet-off system was observed (Furth P.A. et al., 1994; Howe J.R. et al., 1995; Kistner A. et al., 1996).

Another version of the tetracycline inducible system was established as Tet-on system using a reverse tet-transactivator, rtTA, which binds tetO in the presence of tetracycline. Four amino acid exchanges in TetR sequence provided a strong tetracycline or doxycyline dependence to binds tetO (Gossen M. et al., 1995). Target gene expression is induced in response to treatment with doxycycline at low dose: from 10 ng/ml to 1 µg/ml *in vitro* (Gossen M. et al., 1995) and from 200 µg/ml to 2 mg/ml in drinking water *in vivo* (Schönig K.,et al., 2002). A new reverse tet-transactivator analogue rtTA2^{S}-M2 was generated which is better inducible by doxycycline, more stable and shows lower residual affinity to tetO in the absence of doxycycline in transgenic animals (Urlinger S. et al., 2000).

1.5- Aims of study

The Aims of my study were to establish a set of first generationadenovirus vectors, which mediate cytokine gene transfer into livers of mice, and to use them to cntrol HBV replication.

First, cytokines were constitutively expressed under control of a CMV promoter. As antiviral cytokines mouse interferon gamma (mIFNγ) and interferon beta (mIFNβ), and as an anti-inflammatory cytokine the viral interleukin 10 analogue (vIL10) encoded from EBV (Zdanov A. et al., 1997) were selected. Cytokine secretion from transduced hepatocytes had to been shown and their activity on HBV replication *in vitro* and *in vivo*. The following questions were asked:

i) What is the efficiency of gene transfer into the livers of mice after a single i.v. injection and does it dependent of the dose of the adenoviral vector ?

ii) Which amount of the vector is sufficient to strongly repress hepatic HBV replication without induction of massive inflammation ?

iii) What does the kinetics of the antiviral effect on HBV replication look like in mice infected with the minimal effective dose of adenoviruses mediating mIFNγ and mIFNβ expression ?

iv) What does the kinetics of expression and secretion of cytokine from transduced hepatocytes in these mice look like ?

v) How can we distinguish the effect of the expression of cytokines from host-immune responses to adenoviral particles ?

Using the first set of adenoviruses, the last question could not be answered (v) due to continuous expression without tissue specificity. To allow such studies, regulated and liver specific gene expression was needed.

Therefore, the second part of this study focussed on incorporating a bidirectional tet-responsive promoter element into the adenoviral vector and on establishment of a liver specific and tetracycline-regulated cytokine expression in transgenic mice. To characterize this system, the induction of a reporter gene expression has to be determined in livers of mice transduced with these adenoviral vectors. Low basal activity and repeatedly regulation of gene expression *in vivo* would be important for the last part of my project.

In a third part of the study, the tetracycline regulated gene expression should be used to distinguish the effect of cytokine expression on HBV replication from an effect of the host immune response against the adenoviral vector. Using the tet-regulated gene expression system, the antiviral effect on HBV replication should be quantitatively characterized indirectly via the luciferase activity.