4. Other Relevant Data

4.1 Pathology

The pathology of infection by HBV involves an acute phase, which may be recognized clinically as acute viral hepatitis, and then a long chronic phase with development of chronic active hepatitis and often cirrhosis. HCC may evolve during some phase of chronic hepatitis, usually after cirrhosis has supervened but in some cases without cirrhosis. The majority of HCCs associated with HBV appear to arise in a clinically silent way, with few symptoms and no evidence of chronic hepatitis until the carcinoma is in a late stage.

4.1.1 *Acute hepatitis*

The histological features of acute viral hepatitis are highly variable, and hepatitis A, B, C, D and E viruses cannot be distinguished. They are also highly variable for the same agent in patients of different ages and immune status. In the neonate and other immunocompromised individuals, the histological changes are usually very mild with no significant hepatocellular cytopathic change and no significant hepatocellular hydropic swelling, acidophilic necrosis or cholestasis. The typical histological changes in an adult with symptomatic, icteric acute viral hepatitis include: (i) portal expansion with lymphoid hyperplasia (as occurs in many systemic viral infections); (ii) lobular inflammatory reaction with proliferation of sinusoidal lining cells; and (iii) marked hepatocellular changes, including hydropic change (especially in the perivenular areas), acidophilic necrosis and hepatocellular dropout. In the most severe cases of acute viral hepatitis, extensive hepatocellular necrosis occurs (called submassive or massive acute hepatic necrosis), and this is usually fatal. Such severe viral reactions are rare in the very young. In young people with severe viral hepatitis and necrosis, regeneration of hepatocytes is rapid, and recovery occurs with no chronic sequelae (Peters, 1975). There are few histopathological data on the transition of acute viral to chronic hepatitis in large series of patients.

4.1.2 *Chronic hepatitis*

Chronic hepatitis B is defined as HBV infection for more than six months; the corresponding histological features are extremely variable. Many different terms are applied to the histological patterns, which range from nearly normal to mild inflammatory changes to progressive fibrosis to severe necrotizing reactions. The terminology has been based not on large series of patients with carefully documented courses, virological studies and multiple biopsies but often on small numbers of patients with a few years of observation.

Two commonly used categories of chronic hepatitis are chronic persistent hepatitis and chronic active hepatitis. Chronic persistent hepatitis is used for portal lymphoid hyperplasia and HBV infection without lobular degenerative and inflammatory features (which are required for application of the term chronic active hepatitis). A third term for chronic hepatitis, chronic lobular hepatitis, was redefined by Scheuer and Thaler (1977) as predominantly intralobular inflammation and necrosis with no significant portal lymphoid hyperplasia or piecemeal necrosis (which is periportal hepatocellular necrosis and lymphocytic infiltration).

The separation of chronic hepatitis B into chronic persistent hepatitis and chronic active hepatitis may be misleading and has been challenged (Scheuer, 1991). As a single patient may demonstrate both patterns at the same time or over time, neither pattern is prognostically valid. Since cirrhosis is a common outcome of chronic active hepatitis and because it is irreversible and is not associated with evolution from chronic progressive hepatitis alone, chronic active hepatitis is valuable as a category. In 1989, an international group indicated that many factors, such as age, immunocompetence, infection status, drug use and cirrhosis, can be used to predict the severity of chronic hepatitis (Sherlock, 1989). Scheuer (1991) called for a reassessment of the classification and indicated that the terms chronic persistent hepatitis and chronic active hepatitis were introduced in the absence of adequate knowledge about the natural history of HBV. Cirrhosis is the major form of irreversible severe chronic liver disease that is related to chronic infection with HBV. The number of patients with acute viral hepatitis that progress to cirrhosis is not clear, and the rate of progression is highly variable. Many patients acquire HBV in childhood, and the initial episode (acute viral hepatitis) is clinically silent (Bortolotti *et al.*, 1990): of a series of 76 children with chronic hepatitis B, eight had a clinical event identified as acute viral hepatitis, but the rest were detected in screening programmes. Liver biopsy samples taken in this series showed a wide range of patterns, many having chronic persistent hepatitis and some having chronic active hepatitis. One of the patients had cirrhosis which subsequently became inactive (i.e. fewer inflammatory and hepatocellular degenerative changes). A few cases of cirrhosis resulting from HBV are detected in childhood, but the majority seem to be detected in late adult life. Clearly, many adults have chronic hepatitis B infection and do not progress to cirrhosis (Sugimura *et al.*, 1991).

4.1.3 *Cirrhosis*

Cirrhosis is defined as irreversible hepatic fibrosis with regenerative nodule formation (Popper, 1977). The etiology of cirrhosis may be very difficult to discern by histological means. The pattern of inflammatory activity may be a clue to the underlying agent, but

cirrhosis is often recognized at a late stage with little hepatocellular degeneration or inflammatory change. Hepatitis B viral cirrhosis is often identified on the basis of the presence of ground-glass cells, which are hepatocytes with a distinctive eosinophilic cytoplasmic change due to accumulated hepatitis B surface protein (Buendia, 1992), or by specific immunoperoxidase staining for HBV markers (Nayak & Sachdeva, 1975). The late stage of chronic hepatitis is associated with macronodular cirrhosis (formerly called postnecrotic cirrhosis). As cirrhosis progresses, the size of the nodules increases (Popper, 1977).

4.1.4 *Evolution of hepatocellular carcinoma from cirrhosis*

The strong correlation between HCC and cirrhosis is dependent on the etiology of the cirrhosis. Autopsy results suggest that HCC occurs with greatest frequency (38%) in association with cirrhosis due to chronic HBV infection, haemochromatosis and chronic HCV infection, with intermediate frequency (5–10%) in alcoholic cirrhosis and very low frequency in cirrhosis due to Wilson's disease, autoimmune chronic active hepatitis (< 5%) and primary biliary cirrhosis (Craig *et al.*, 1991).

The mechanism of progression of cirrhosis to HCC is much debated. Dysplastic hepatocellular changes have been described that may be associated with HCC in patients with HBV (Ho *et al.*, 1981). Liver-cell dysplasia is hepatocellular enlargement with nuclear pleomorphism and multinucleation; dysplastic cells occur in clumps or small nodules. Ho *et al.* (1981) reported that 60% of 558 cases of cirrhosis had liver-cell dysplasia and this change was strongly correlated with development of HCC; however, some nodules may be premalignant without liver-cell dysplasia. The transformation of regenerative nodules to atypical hyperplastic nodules and then to HCC is well described. The small regenerative nodule may have a 'nodule within the nodule' growth pattern, and some observers consider that such small nodules are precursors of carcinoma (Arakawa *et al.*, 1986).

Adenomatous hyperplasia (Takayama *et al.*, 1990), also called macroregenerative nodule, is a discrete nodule of hepatocytes that is apparent by gross examination because it is slightly larger and usually also has a different colour from the surrounding nodules of the cirrhotic liver. These nodules are usually 0.8–3.0 cm and not larger, as then the lesion is likely to be a small HCC. Light microscopy reveals that the nodule is composed of hepatocytes in regular trabeculae (not solid), lacks the overt features of carcinoma (such as acinar formation, mitosis and high nuclear:cytoplasmic ratios) and includes foci of small blood vessels and bile ducts. These 'trapped' bile ducts and vessels, usually at the periphery, distinguish adenomatous hyperplastic nodules from hepatocellular adenoma. Some enlarged nodules in cirrhotic livers are HCC, and other nodules demonstrate some features of both hepatocellular and adenomatous hyperplasia (and are thus called atypical adenomatous hyperplasia). These atypical nodules have small areas of acinar formation, an area of thicker cord development or focal hepatocytes with increased nuclear:cytoplasmic ratio. Additional levels of a tissue block and/or more sections of a single nodule reveal that some atypical nodules are small HCCs. The transition of benign hepatocytes to atypical hepatocytes to carcinoma has been observed in a few nodules of cirrhotic liver, whereas the usual histological examination of HCC reveals only masses of carcinoma with no transitional growth areas. In a series of 110 cirrhotic liver explants (of many etiologies, including HBV

cirrhosis but excluding known HCC) examined carefully for distinctive (grossly apparent) nodules, 40 nodules were identified in 19 livers (Ferrell *et al.*, 1992). Microscopic examination allowed classification of 12 as small HCCs and 28 as adenomatous hyperplasia (some with atypia) and liver-cell dysplasia.

Several examples of atypical adenomatous hyperplasia have been shown to have the same clonal integration pattern as the HCC within the same liver. This clonal growth provides a link between atypical adenomatous hyperplasia and the evolution of HCC (Tsuda *et al.*, 1988). Furthermore, the same HBV DNA integration pattern detected in multiple nodules of HCC in the same liver with chronic hepatitis B has been interpreted as proof of clonal growth of HCC and consequent metastasis within the liver. The detection of an additional, new integration pattern in the tumour cells during its course suggests additional HBV-related mutagenesis (Hsu, H.-C. *et al.*, 1991). In experimental models of HCC, e.g. woodchuck with chronic WHV infection (Ogston *et al.*, 1982) and rats with various chemicals, oval cells are identified adjacent to the tumour which are considered to be possible stem cells for HCC. Similar oval cells were detected in human HCC associated with HBV in one laboratory (Hsia *et al.*, 1992).

4.1.5 *Hepatocellular carcinoma*

The morphological pattern of human HCC is usually a homogeneous tumour composed of cells resembling hepatocytes with a trabecular or solid growth pattern. Several other growth patterns are recognized, including spindle-cell, clear-cell and fibrolamellar carcinoma, which are not associated with HBV (Colombo, 1992). A few reports have been made, however, of coexistence of fibrolamellar carcinoma with HBV. Because this variant almost always arises in noncirrhotic, normal livers, the few HBV-infected patients are probably carriers of HBV. Cholangiocarcinoma, the other common primary hepatic carcinoma, does not have detectable HBV markers in the tumour or in the surrounding non-tumorous tissue (Peters *et al.*, 1977).

4.2 Molecular biology

The molecular biology of HBV in relation to HCC has been reviewed (Rogler, 1991; Buendia, 1992; Feitelson, 1992; Slagle *et al.*, 1992). The virus was first associated with HCC in epidemiological studies. During the last 10 years, the role of HBV in liver-cell transformation has been investigated by different approaches, either directly in human HCC or in experimental models, including human HCC cells in culture, transgenic mice carrying part or all of the viral genome (see section 3.2) and naturally occurring animal models of hepadnavirus infection (see sections 3.3 and 3.4). The studies have focused on three main subjects:

(i) integration of HBV: the integrated state of HBV DNA in human HCCs, integration of other hepadnaviruses, structure of viral inserts, cellular target sites for viral integration and insertional mutagenesis;

(ii) expression of HBV genes in human HCCs and their role in the tumorigenic process (mainly surface proteins and X transactivator); and

(iii) genetic alterations in human HCCs, activation of cellular oncogenes and inactivation of tumour suppressor genes.

4.2.1 *Integration of HBV DNA*

Viral integration has been detected only in hepatocytes, despite the presence of viral DNA in extrahepatic tissues (Bréchot, 1987).

(*a*) *Integrated state of HBV DNA in chronic hepatitis and hepatocellular carcinomas from hepatitis B surface antigen-seropositive patients*

Initial studies using Southern blot analysis showed the presence of integrated HBV sequences in cellular DNA of established HCC cell lines and in human HCCs from HBsAg-seropositive patients (Bréchot *et al.*, 1980; Chakraborty *et al.*, 1980; Edman *et al.*, 1980).

It is at present uncertain whether or not integration occurs in the early stages of natural acute hepatitis. In contrast, multiple integrations have been observed in tissues from patients with chronic hepatitis (Bréchot *et al.*, 1981b; Shafritz *et al.*, 1981; Boender *et al.*, 1985; Tanaka, Y. *et al.*, 1988), indicating that viral integration takes place prior to tumour development. Analysis of 83 cirrhotic nodules from the livers of 11 HBV carriers with cirrhosis revealed discrete bands at a higher molecular weight region in 26 of 83 nodules (31.3%), indicating clonal outgrowth of altered hepatocytes with viral integration (Yasui *et al.*, 1992). Single-site HBV insertions are common in childhood HCC but are less common later in life (Chang *et al.*, 1991), suggesting that multiple-site integration occurring during the course of long-term HBV infections might accumulate within single cells, as indicated by sequence divergence among HBV inserts in the same tumour (Imai *et al.*, 1987).

Integrated HBV DNA has been identified in HCC specimens from chronic HBV carriers in numerous other studies (Bréchot *et al.*, 1981a; Shafritz *et al.*, 1981; Hino *et al.*, 1984, 1985; Horiike *et al.*, 1989; Sakamoto *et al.*, 1989), indicating that HBV DNA integration is present in more than 80% of HCCs developing in HBsAg-seropositive patients.

(*b*) *Presence of HBV DNA in hepatocellular carcinomas from hepatitis B surface antigen-seronegative patients*

The presence of serum anti-HBs and anti-HBc in HBsAg-seronegative HCC subjects has been reported (Kew *et al.*, 1986b; Bréchot, 1987; Blum *et al.*, 1990). In spite of the fact that these antibodies generally reflect past, resolved infection, HBV DNA sequences have nonetheless been detected in some tumours in this group (Bréchot *et al.*, 1982, 1985; Hino *et al.*, 1985; Bréchot, 1987; Tabor, 1989; Lai *et al.*, 1990). Improvements in the sensitivity of assays for HBsAg and HBV DNA have made it possible to identify cases of chronic HBV infection with low viral replication (Bréchot *et al.*, 1985; Liang *et al.*, 1989; Bréchot *et al.*, 1991; Kremsdorf *et al.*, 1991; Liang *et al.*, 1991b). The finding of HBV DNA in HCCs in HBsAg-seronegative patients (Pontisso *et al.*, 1987; Paterlini *et al.*, 1993) has been questioned because the estimated copy number of HBV DNA sequences per cell is only 0.001–0.1. The significance of these findings is unclear.

(*c*) *Integrated hepadnavirus DNA in animal models*

Integrated WHV sequences have been detected in chronically infected woodchuck liver and in a majority (> 90%) of woodchuck HCCs in chronic carriers (Ogston *et al.*, 1982; Rogler & Summers, 1984; Hsu *et al.*, 1990), although Korba *et al.* (1989) reported a lower frequency of integration. Viral integration appears to be less frequent in tumours associated

with GSHV and even less in those associated with DHBV (Yokosuka *et al.*, 1985; Marion *et al.*, 1986; Imazeki *et al.*, 1988; Transy *et al.*, 1992).

(d) Structure and expression of viral inserts

Studies of the organization of cloned HBV inserts in liver tissues and HCCs show that HBV sequences are fragmented and rearranged and that integration and recombination sites are dispersed over the viral genome. These data indicate that HBV integration does not occur through a unique mechanism, as is the case for other retroelements and retroviruses. Virtually all HBV inserts consist either of linear subgenomic fragments or of rearranged fragments in different orientations, in the absence of a complete HBV genome, showing that these integrated sequences cannot serve as a template for viral replication. Integrated HBV sequences may be rearranged both during the integration process and after formation of viral inserts (Mizusawa *et al.*, 1985; Nagaya *et al.*, 1987; Tokino *et al.*, 1987). Integrated forms, made up of a continuous genome or subgenomic fragment, which are frequent in tissues from children with HCC and chronic hepatitis (Yaginuma *et al.*, 1987), are believed to represent primary products of integration.

Highly preferred integration sites have been mapped in the HBV genome within the 'cohesive end' region that lies between two 11-base pair direct repeats (DR1 and DR2) which are highly conserved in hepadnaviruses (Koshy *et al.*, 1983; Dejean *et al.*, 1984; Nagaya *et al.*, 1987). A narrow region encompassing DR1 has been shown to be particularly prone to recombination (Yaginuma *et al.*, 1987; Hino *et al.*, 1989; Buendia, 1992; Quade *et al.*, 1992). This region coincides with a short terminal redundancy of the minus-strand DNA, which confers a triple-stranded structure to the circular viral genome (Shih *et al.*, 1987). Integration sites are tightly clustered at both the 5′ and 3′ ends of minus-strand DNA, suggesting that replication intermediates and specially relaxed circular DNA might be preferential preintegration substrates (Nagaya *et al.*, 1987; Shih *et al.*, 1987). Invasion of cellular DNA by single-stranded HBV DNA, using mainly free 3′ ends, might take place through a mechanism of illegitimate recombination, also suggested by frequent patch homology between HBV and cellular sequences at the recombination breakpoints (Matsubara & Tokino, 1990). Different minor changes in flanking cellular DNA have been associated with viral integration, including microdeletions and short duplications (Yaginuma *et al.*, 1985; Dejean *et al.*, 1986; Berger & Shaul, 1987; Nakamura *et al.*, 1988; Hino *et al.*, 1989). The hypothesis that topoisomerase I might promote illegitimate recombination of hepadnavirus DNA *in vivo* has been proposed (Wang & Rogler, 1991). The mechanisms underlying HBV DNA integration have still not been fully identified. Analysis of a limited number of WHV and DHBV insertions suggests that a similar mechanism of integration occurs in these hepadnaviruses (Ogston *et al.*, 1982; Rogler & Summers, 1984; Hsu *et al.*, 1988; Imazeki *et al.*, 1988; Fourel *et al.*, 1990). No similar information is available for GSHV.

As a consequence of the viral integration process, sequences of the S and X genes and of the enhancer I element are present almost systematically in HBV inserts, whereas those of the C gene are less frequently represented (Buendia, 1992). It has been shown that the pre-S2/S promoter is transcriptionally active in its integrated form in human HCC (Freytag von Loringhoven *et al.*, 1985; Caselmann *et al.*, 1990) and that HBsAg might be produced from viral inserts (Zhou *et al.*, 1987). Highly rearranged HBV inserts show virus–virus

junctions scattered throughout the viral genome (Nagaya *et al.*, 1987), and recombination breakpoints have been mapped in the S coding region of some of them (Buendia, 1992). Truncation of the S gene between residues 77 and 221 (Buendia, 1992) confers transcriptional activation activity on the mutated pre-S2/S products (Caselmann *et al.*, 1990; Kekulé *et al.*, 1990). Other studies have shown that a large percentage of virus–host junctions are located in the carboxy terminal of the viral X gene, predicting a fusion of the X open reading frame with flanking cellular sequences in a way that might preserve the functional capacity of the X transactivator. Transcripts have been demonstrated from integrated X sequences in tumours and chronically infected livers (Miyaki *et al.*, 1986; Wollersheim *et al.*, 1988; Takada & Koike, 1990; Hilger *et al.*, 1991).

(*e*) *Cellular targets for viral integration in human hepatocellular carcinomas*

Studies of different viral insertions in many human HCCs have revealed that integration can take place at multiple sites on various chromosomes; insertion sites have been mapped on many different human chromosomes, with higher than average rates on chromosomes 11 and 17 (Tokino & Matsubara, 1991; Slagle *et al.*, 1991; Quade *et al.*, 1992). These studies did not demonstrate the presence of known dominant oncogenes or tumour suppressor genes in the immediate vicinity of any integration site. *Alu*-type repeats and minisatellite-like, satellite III and variable-number terminal repeat sequences have frequently been identified near HBV insertion sites (Shaul *et al.*, 1986; Berger & Shaul, 1987; Nagaya *et al.*, 1987; Buendia, 1992). A small cellular DNA compartment (H3), characterized by a base composition similar to that of HBV DNA and a high concentration of *Alu* repeats, has been designated as a major target for stable HBV integration (Zerial *et al.*, 1986; Buendia, 1992).

In many tumours, large inverted duplications, deletions, amplifications and chromosomal translocations have been associated with HBV insertions, suggesting that HBV DNA integration may enhance chromosomal instability (Koch *et al.*, 1984; Mizusawa *et al.*, 1985; Rogler *et al.*, 1985; Yaginuma *et al.*, 1985; Hino *et al.*, 1986; Tokino *et al.*, 1987; Hatada *et al.*, 1988). It has also been shown that HBV DNA promotes homologous recombination at a distance from the insertion site (Hino *et al.*, 1991). Roles for most of these chromosomal abnormalities have not been assigned as yet, however, although amplification of *hst*-1 loci has been associated with HBV integration in the same chromosomal region (Hatada *et al.*, 1988).

(*f*) *Insertional mutagenesis by HBV DNA*

Evidence for a direct *cis*-acting promoter insertion mechanism in HCC has been provided. In two cases, viral integration disrupted the structure of the gene (Dejean *et al.*, 1986; de-Thé *et al.*, 1987; Dejean & de-Thé, 1990; Wang *et al.*, 1990) in early tumours that developed in non-cirrhotic livers from clonal proliferation of a cell containing a single, specific viral integration. In one case, the HBV insertion occurred in an exon of the retinoic acid receptor β gene (*RARβ*) and fused the amino terminal domain of the viral pre-S1 gene to the DNA and hormone binding domains of the gene (Dejean *et al.*, 1986; de-Thé *et al.*, 1987; Buendia, 1992). The predicted chimeric HBV/RARβ protein might have altered transcriptional capacity and thus participate in the tumorigenic process (Buendia, 1992). In the second case, HBV sequences were found to be integrated in an intron of the human cyclin A

gene, resulting in production of spliced HBV/cyclin A fusion mRNAs initiated at the pre-S2/S promoter (Wang *et al.*, 1990, 1992). In the deduced polypeptide, the amino terminal domain of cyclin A (a target for proteolytic degradation of cyclin A at the end of the M phase) was replaced by an amino acid sequence from the terminus of pre-S1. Cyclins are important in the control of cell division, and disruption of the cyclin A gene by viral insertion probably contributed to oncogenesis (Buendia, 1992).

In a third human HCC, integration of HBV DNA into a cellular gene related to the epidermal growth factor receptor (c-*erb*B) has been described (Zhang *et al.*, 1992).

(*g*) *Insertional activation of* myc *family genes in woodchuck hepatocellular carcinoma*

The search for transcriptional activation of already known proto-oncogenes and for viral insertion sites in woodchuck HCCs has revealed that WHV acts as an insertional mutagen which activates *myc* family genes (c-*myc* and N-*myc*) in more than half of the tumours examined (Möröy *et al.*, 1986; Hsu *et al.*, 1988; Fourel *et al.*, 1990). Analysis of the mutated c-*myc* alleles in two tumours showed integration of WHV sequences in the vicinity of the c-*myc* coding domain, either 5′ of the first exon or in the 3′ untranslated region (Hsu *et al.*, 1988). Deregulated expression of the oncogene driven by its normal promoters resulted from deletion or displacement of c-*myc* regulatory regions known to exert a negative effect on c-*myc* expression and their replacement by viral sequences encompassing the enhancer I element (Buendia, 1992).

Insertional activation of N-*myc* genes was observed more frequently. In particular, the woodchuck N-*myc*2 gene (a functional processed pseudogene or 'retroposon') represents by far the most frequent target for WHV DNA integration. In about 40% of tumours, viral insertions were detected either upstream of the gene or in a short sequence of the 3′ untranslated region (Fourel *et al.*, 1990; Wei *et al.*, 1992). The N-*myc*2 gene is also present in ground squirrels, although insertional mutagenesis has not been demonstrated in this animal model (Transy *et al.*, 1992). Furthermore, woodchucks infected with GSHV do not show insertional mutagenesis (Hansen *et al.*, 1993). Therefore, a direct role of WHV DNA integration in *myc* gene activation might account for the higher incidence and more rapid onset of HCC in the woodchuck model. Finally, there is no evidence that HBV integrates into *myc* family genes in human HCC (Buendia, 1992).

4.2.2 *Expression and potential oncogenic properties of HBV gene products*

The HBV genome encodes seven proteins from four open reading frames. Experimental evidence has been presented for an oncogenic role of two of the viral proteins, the large surface (HBs) protein and the transcriptional transactivator X.

(*a*) *Surface proteins*

In natural HBV infection, the production of infectious virions and HBsAg particles depends on tight regulation of the relative levels of the three envelope glycoproteins. Neither liver lesions nor HCCs have been observed in any of the published transgenic lineages that carry and replicate complete HBV genomes or produce the middle and major surface (HBs) proteins from HBV-derived regulatory sequences (Babinet *et al.*, 1985; Chisari *et al.*, 1985; Burk *et al.*, 1988; Farza *et al.*, 1988; Araki *et al.*, 1989). The appearance and rate of deve-

lopment of preneoplasic nodules and liver tumours following administration of carcinogens are, however, slightly increased in HBsAg-seropositive transgenic mice over that in sero-negative littermates, suggesting that HBsAg expression might enhance the effects of hepatocarcinogens (Dragani *et al.*, 1990).

In contrast, when the endogenous pre-S1 promoter is replaced by an exogenous promoter (the metallothionein or albumin promoter), the production of roughly equimolar ratios of large HBs protein with respect to middle and major HBs proteins leads to intracellular accumulation of nonsecretable filamentous envelope particles within the endoplasmic reticulum of transgenic mouse hepatocytes (Chisari *et al.*, 1986, 1987). This leads to histological and ultrastructural features similar to those of 'ground-glass' hepatocytes, which have been described in chronic hepatitis B in humans. Cell death follows, accompanied by mild persistent hepatitis, which is followed by the development of regenerative nodules and eventually HCC by 12 months of age (Chisari *et al.*, 1989). The preneoplastic nodules and tumours display a marked reduction in transgene expression, suggesting that hepatocytes that express low levels of the large HBs polypeptide would have a selective survival advantage. Chemical carcinogens are not required for tumour induction in this model, but exposure of adult transgenic mice to hepatocarcinogens produced more rapid and extensive development of preneoplastic lesions and HCCs under conditions that do not alter the liver morphology of nontransgenic controls (Sell *et al.*, 1991). These data show that inappropriate expression of the large HBs protein can be directly cytotoxic to hepatocytes and may initiate a cascade of events that ultimately progress to malignant transformation (Buendia, 1992).

Studies of integrated HBV sequences in human HCC suggest a possible role for abnormal expression of rearranged viral S genes in the development of HCC. Deletion of the carboxy terminal region of the S gene generates a novel transcriptional *trans*-activation activity (Caselmann *et al.*, 1990; Kekulé *et al.*, 1990; Lauer *et al.*, 1992). Both integrated HBV sequences from a human tumour and from a hepatoma-derived cell line and different constructs bearing similarly truncated pre-S2/S sequences can stimulate the SV40 promoter in transient transfection assays; transactivation occurs at the transcriptional level and is dependent on the SV40 enhancer. The c-*myc* P2 promoter is also activated *in trans*. These findings support the hypothesis that accidental 3′ truncation of integrated pre-S2/S genes could be a causative factor in HBV-associated oncogenesis (Buendia, 1992).

(*b*) *HBx: a transcriptional transactivator*

Evidence for expression of the HBV *X* gene was obtained by Moriarty *et al.* (1985) and by Kay *et al.* (1985), who reported that the sera of HBV-related HCC patients recognize synthetic peptides made on X sequences. Expression of the *X* reading frame in prokaryotic and eukaryotic cells, using various vectors, allowed identification of a 17-kDa polypeptide that reacted with serum samples from a number of HBV-infected individuals (Elfassi *et al.*, 1986; Meyers *et al.*, 1986; Schek *et al.*, 1991). Anti-HBx has been detected in a minor proportion of acutely infected patients about three to four weeks after the onset of clinical signs, and more frequently in chronic HBsAg carriers who have markers of active viral replication. Very few patients are seropositive for anti-HBx after seroconversion to anti-HBs or at the time an HCC is discovered (Levrero *et al.*, 1991). Conflicting results have been

obtained regarding the association between anti-HBx and other viral markers and with HCC. The problem may be related to the weak antigenicity of HBx protein or to its sequestration into cellular compartments that render it inaccessible to the host immune system. HBxAg has been detected in the livers of HBsAg carriers and has been correlated with current viral replication and chronic liver disease (Levrero *et al.*, 1990; Haruna *et al*, 1991; Wang *et al.*, 1991). The HBx protein is located mainly in the cytoplasm of cells infected *in vivo*, at or near the plasma membrane and at the nuclear periphery (Vitvitski *et al.*, 1988; Levrero *et al.*, 1990; Wang *et al.*, 1991). The HBx protein has been detected in the nuclear compartment only in transfected cell lines (Höhne *et al.*, 1990; Seifer *et al.*, 1990).

The recent finding that the X gene product can *trans*-activate transcription from a number of HBV and heterologous promoters is of considerable importance in defining its role in the pathogenesis of HCC (for review, see Rossner, 1992). More clues to the possible role of HBx protein in HBV-associated pathogenesis were provided by three lines of evidence: in studies *in vitro* and *in vivo* and by direct analysis of biopsy samples of human liver and of HCC (Buendia, 1992).

High levels of expression of the *X* gene may induce malignant transformation of certain cultured cells, such as the mouse fibroblast NIH3T3 cell line (Shirakata *et al.*, 1989) and immortalized hepatocytes that express the SV40 large tumour antigen (Höhne *et al.*, 1990; Seifer *et al.*, 1990). It has been shown that the c-*myc*, c-*jun* and c-*fos* proto-oncogene promoters can be *trans*-activated by the *X* gene product (Balsano *et al.*, 1991; Seifer *et al.*, 1991; Twu *et al.*, 1993). Activation of protein kinase C (Kekulé *et al.*, 1993) and formation of a p53–HBx protein complex have been reported (Feitelson *et al.*, 1993).

Studies of transgenic mice carrying the *X* reading frame controlled by its natural HBV enhancer and promoter sequences or by heterologous liver-specific promoters have given rise to conflicting results. In three lines of mice (C11, H9 and E1) derived from the outbred CD1 strain (see Table 7) carrying a 1.15-kilobase HBV fragment (spanning the enhancer, the complete *X* coding region and the polyadenylation signal), preneoplastic lesions were observed in the liver, which were followed by carcinomas at 8–10 months of age (Kim *et al*, 1991). In contrast, a transgene in which the *X* coding domain was placed under the control of the α-1-antitrypsin regulatory region failed to induce tumours in ICR × B6C3F1 transgenic mice, although *X* mRNAs were detected in liver tissues (Lee *et al.*, 1990).

Analysis of integrated viral sequences in tumour DNA has shed new light on one of the mechanisms leading to overexpression of the *X* gene in chronically infected livers and in HCCs. HBV sequences are frequently interrupted between or around the viral direct repeats DR1 and DR2 upon integration into host cell DNA (Buendia, 1992), and overproduction of hybrid viral and host transcripts may result from HBV DNA integration in a hepatoma cell line (Freytag von Loringhoven *et al.*, 1985; Ou & Rutter, 1985). The presence of viral and host transcripts containing a 3′ truncated version of the HBx coding region fused with flanking cellular sequences and retaining *trans*-activating capacity was first described in a human HCC (Wollersheim *et al.*, 1988). Moreover, enhanced *trans*-activating capacity of the integrated *X* gene product has been related to substitution of viral carboxy terminal residues by cellular amino acids (Koshy & Wells, 1991). *trans*-Activating ability of similarly truncated *X* gene products made from fusion of integrated HBV sequences with adjacent cell DNA has also been shown in many tissues from patients with chronic hepatitis (Takada & Koike, 1990).

This suggests that the integrated *X* gene might be essential for maintaining the tumour phenotype that develops at the early stages of carcinogenesis. Consistent with this model is the finding that viral and host junctions can be mapped in the carboxy terminal region of *X* in most human HCCs (Nagaya *et al.*, 1987; Buendia, 1992).

4.2.3 *Genetic alterations in hepatocellular carcinoma*

Genetic alterations that cannot be associated clearly with a direct effect of viral infection have been observed in human HCCs. Such somatic changes include allele losses on several chromosomal regions, mutation and activation of cellular genes that show oncogenic potential and deletion or mutation of tumour suppressor genes.

(*a*) *Chromosomal losses and tumour suppressor genes*

Several groups have reported loss of heterozygosity in a large number of HCCs (for a review, see Buendia, 1992). Loss of heterozygosity on the distal 1p region and on chromosomes 4q, 11p, 13q and 16q has frequently been detected in human HCCs by restriction fragment length polymorphism (Pasquinelli *et al.*, 1988; Wang & Rogler, 1988; Buetow *et al.*, 1989; Tsuda *et al.*, 1990; Simon *et al.*, 1991). These studies showed no relation to tumour histology or grade, presence of HBV, cirrhosis or ethnic origin of the patient. Thus, it has been suggested that these parts of the human genome might contain genes, the functional loss of which might be involved in hepatocellular carcinogenesis. Allele loss of the short arm of chromosome 17p, which includes the *p53* gene, has also been observed frequently in human HCC (Fujimori *et al.*, 1991; Slagle *et al.*, 1991). Viral insertions at chromosome 17p in some HCCs are not physically linked to the *p53* gene (Hino *et al.*, 1986; Tokino *et al.*, 1987; Zhou *et al.*, 1988). Accumulation of allelic loss on different chromosomes (e.g. 13q, 16q) has been associated with advanced stages of HCC (Nishida *et al.*, 1992). Whether HBV DNA integration, which has been shown to promote genetic instabilily, contributes to these events has not been elucidated.

Tumour suppressor genes are identified on the basis of their loss or inactivation in tumour cells, as the product of such genes are negative regulators of cell growth (Marshall, 1991). Mutational inactivation of *p53* is the commonest known genetic alteration in human cancer (Hollstein *et al.*, 1991). *p53* and retinoblastoma genes are the only genes known to be involved in HCC (Bressac *et al.*, 1990, 1991; Hsu, I.C. *et al.*, 1991; Murakami *et al.*, 1991). The frequency of *p53* mutations in HCC was reported to be high ($\geq 45\%$) in Qidong, China (Hsu, I.C. *et al.*, 1991; Scorsone *et al.*, 1992; Li *et al.*, 1993) and Mozambique (Bressac *et al.*, 1991; Ozturk *et al.*, 1991); intermediate (15–35%) in Japan (Murakami *et al.*, 1991; Oda *et al.*, 1992; Nishida *et al.*, 1993) and in Shanghai (Buetow *et al.*, 1992; Li *et al.*, 1993), Xian (Buetow *et al.*, 1992) and Taiwan, China (Hosono *et al.*, 1991; Sheu *et al.*, 1992); and low (0–15%) in Germany (Kress *et al.*, 1992), the United Kingdom (Challen *et al.*, 1992), Thailand (Hollstein *et al.*, 1993) and Alaska, USA (Buetow *et al.*, 1992). As shown in Table 12, the relationship between chronic infection with HBV (as determined by serum HBsAg) and the frequency of *p53* mutation in HCC was addressed directly in three studies, and *p53* mutations were shown to occur at similar frequencies in HbsAg-seropositive and HBsAg-seronegative patients.

Table 12. Frequency of *p53* mutations in patients seropositive and seronegative for hepatitis B surface antigen (HBsAg)

Location	All patients			HBsAg seropositive			HBsAg seronegative			Reference
	Total	With mutants		Total	With mutants		Total	With mutants		
		No.	%		No.	%		No.	%	
Japan[a]	140	46	33	30	10	33	98	32	33	Oda *et al.* (1992)
Taiwan, China	61	20	33	41	15	37	20	5	25	Sheu *et al.* (1992)
Thailand	15	2	13	7	2	29	6	0	0	Hollstein *et al.* (1993)
Total	216	68	31	78	27	35	124	37	30	

Numbers of seropositive and seronegative do not add up because the HBV status was not known for all patients.
[a]128 Japanese, six Korean, four Indonesian and two Taiwanese patients

A 'hot spot' mutation (Buendia, 1992) at the last guanine residue of codon 249 (AG*G* to AG*T*) was identified in more than 45% of HCCs from Mozambique and Qidong, China (Bressac *et al.*, 1991; Hsu, I.C. *et al.*, 1991). The codon 249 mutation (AGG→AGT) of *p53* is not a mutational hot spot in non-HCC tumours (Hollstein *et al.*, 1991). The presence of codon 249 mutations in HCC was reported in both HBsAg-seropositive and HBsAg-seronegative patients (Ozturk *et al.*, 1991; Buetow *et al.*, 1992; Oda *et al.*, 1992; Sheu *et al.*, 1992; Hollstein *et al.*, 1993; Yap *et al.*, 1993) as well as in the presence and absence of tumour HBV DNA (Scorsone *et al.*, 1992; Li *et al.*, 1993). An association between HBxAg and *p53* both *in vitro* and *in vivo* has been described (Feitelson *et al.*, 1993).

(*b*) *Activation of cellular oncogenes*

The search for activated oncogenes in DNA from HCCs using the NIH3T3 cell transformation assay has not been successful in most cases. A transforming DNA, *lca* (liver cancer), was characterized in a very small number of tumours (2/4) (Ochiya *et al.*, 1986). This new oncogene is expressed at a proliferative stage in fetal liver; its expression in liver cancer has not been associated with gross rearrangement of the gene (Shiozawa *et al.*, 1988). Another transforming gene, *hst*-1, was identified by this method in a tumour from an HBsAg-seronegative patient (Yuasa & Sudo, 1987); and co-amplification of integrated HBV sequences and *hst*-1 was reported in another case (Hatada *et al.*, 1988). Conflicting results have been obtained concerning the *ras* genes in the NIH3T3 cell transformation assay. Direct sequencing of the c-Ha-*ras*, c-Ki-*ras* and N-*ras* genes has shown a very low incidence of point mutations in liver tumours (Tsuda *et al.*, 1989; Tada *et al.*, 1990; Ogata *et al.*, 1991).

Increased expression of c-*myc* protooncogene has been described in a majority of human HCCs but also occurs in cirrhosis (Gu *et al.*, 1986; Himeno *et al.*, 1988). In rare cases, it was associated with genetic amplification of the c-*myc* locus (Trowbridge *et al.*, 1988).

4.3 Other observations relevant to possible mechanisms of action of HBV in carcinogenesis

4.3.1 *Cell division and tissue regeneration in response to HBV infection*

Increased incidences of HCC have been reported in association with liver diseases other than that caused by HBV infection, in which cirrhosis occurs with accompanying regenerative nodules (e.g. alcoholic cirrhosis, haemochromatosis, HCV-induced cirrhosis), suggesting that the active cell division associated with cirrhosis may contribute in an important way to the development of HCC (Colombo *et al.*, 1989; Simonetti *et al.*, 1992). Although cirrhosis is not essential to HCC (see above), the necrosis and inflammation associated with both chronic active hepatitis and cirrhosis may be important. This would explain the absence of tumours in chronically HBV-infected chimpanzees (see 3.1.1(*a*)) in which inflammatory changes are usually limited to the portal spaces and cell necrosis is normally absent or minimal (Shouval *et al.*, 1980). In support of this view is the high incidence of HCC observed in mutant LEC rats in which fulminant hepatitis occurs four months after birth, followed by chronic hepatitis and HCC in association with low levels of ceruloplasmin and heavy hepatic copper deposits (Ono *et al.*, 1991). The changes that occur in the lineages of transgenic mice in which HBsAg accumulates and induces cell necrosis and regeneration, described above, constitute yet another observation in support of this hypothesis (Chisari *et al.*, 1989).

4.3.2 *Immune response*

In patients with chronic HBV infection, it is now clear that immunological mechanisms are involved in the lysis of infected hepatocytes. At least in acute HBV infection, CD8 cytotoxic T lymphocytes that recognize hepatocytes which express HBV core peptides in association with major histocompatibility class I proteins have been demonstrated (Penna *et al.*, 1991). In chronic HBV infection, however, cytotoxic T cells are difficult to demonstrate. It is suggested that the secretion of processed nucleocapsid proteins, such as HBeAg, are involved in inducing tolerance of T cells that can react to HBe and HBc proteins (Thomas *et al.*, 1988). During the course of chronic infection, e antigen/antibody seroconversion may occur at a rate of approximately 5–10% of cases per year. This results in elimination of hepatocytes, indicating that HBV replication perhaps occurs by immune recognition of HBe by anti-HBe. In some patients, e-negative virus then emerges, and viraemia and inflammatory liver disease continue (Carman *et al.*, 1993). These patients progress to cirrhosis, and it has been argued that the associated cycles of liver-cell necrosis and regeneration regulated by growth factors contribute to the multi-step process that leads to the development of HCC in patients with chronic hepatitis with or without cirrhosis. The immunological selection pressure operating against the hepatocytes that support productive HBV infection (expressing nucleocapsid proteins) will favour selective regeneration of uninfected hepatocytes and of hepatocytes containing only integrated HBV sequences, not expressing c and e but perhaps expressing HBsAg (Fowler *et al.*, 1986). These cells are under a strong regenerative stimulus, and it is proposed that it is these cells that give rise to regenerative nodules and HCC (Shafritz *et al.*, 1981; Shafritz, 1982; Thomas *et al.*, 1982).

4.3.3 *Hepatocellular carcinoma-associated tumour markers*

The best-studied marker of HCC is α-fetoprotein, which is used to diagnose the tumour (Abelev, 1974). Whether there is a specific relationship between HBV and expression of this protein is uncertain (Chan *et al.*, 1980; Trichopoulos *et al.*, 1980b; Kew & Macerollo, 1988).

4.3.4 *Role of aflatoxins and possible modification of the effect of HBV*

Little information is available as to whether there is a biological interaction between HBV and aflatoxin, although it is established that aflatoxin is mutagenic, and both factors can induce liver injury resulting in increased cell proliferation. One hypothesis is that HBV infection and the associated hepatitis may alter carcinogen metabolism, and some evidence has been provided in humans and woodchucks to support this idea (De Flora *et al.*, 1985, 1989). Aflatoxin B_1 is immunosuppressive (Pier & McLoughlin, 1985; Richard, 1991), but its influence on the immune response to HBV infection has not been investigated.