Mutant p53 but not Hepatitis B Virus X Protein Is Present in Hepatitis B Virus-related Human Hepatocellular Carcinoma

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ABSTRACT

Hepatitis B virus (HBV) X protein is thought to play an important role in the development of hepatocellular carcinoma. Recent studies on a transgenic mouse tumor model suggest that HBV X protein may contribute to transformation by binding to and inactivating the cellular growth suppressor protein p53. We have studied 31 hepatocellular carcinoma tissues from Chinese patients for the possible occurrence of such interactions. Although most of the samples contained markers of HBV infection, including free and/or integrated HBV DNA, there was no detectable expression of HBV X protein by Western blot, immunoprecipitation, or histochemical staining. There was also no evidence of HBV X protein associated with p53 immunoprecipitated from the tumors. These observations suggest that, in naturally occurring human hepatocellular carcinoma, such interactions are uncommon and, therefore, unlikely to be of relevance in the latter stages of tumor development. On the other hand, 29 of 31 (93%) samples contained mutated forms of p53, as determined by various antibodies that detect wild-type or mutant p53 or both, and by the association of heat shock protein 70 with immunoprecipitated p53. These results show that conformationally altered p53 protein is present in tumors at a much higher frequency than is suggested by the presence of known mutations in the gene. This mutant p53 is functionally inactive, as suggested by the lack of expression of the p53-induced Mif, 21,000 Cip/UpWaf1 protein in the tumors. Because this inactivation of p53 was not correlated with the expression of HBV X protein, any interaction of HBV X protein with p53 may be relevant only during acute infection. Such an interaction could serve to relax cell growth control at a time when virus replication requires hepatocyte destruction to be balanced by regeneration.

INTRODUCTION

Despite the long-recognized strong association of chronic HBV infection with the development of HCC (1), the specific oncopgenic mechanisms mediated by HBV persistence are unknown. Insertional mutagenesis of cellular genes by integration of HBV DNA may sometimes be a factor in oncogenesis (2–5). Several studies indicate a possible role of the HBV-encoded X protein in carcinogenesis. The Mr 17,000 HBV X protein has been shown to be essential for viral replication (6) and for the establishment of infection (7, 8). The HBV X protein can trans-activate the transcription of viral and cellular genes through various cis elements present in the promoters of genes transcribed by RNA polymerases II and III (9). These include genes involved in cell proliferation [e.g., IL-8 (10), ICAM (11), c-jun (12), and c-fos (13)] that may be activated through intracellular signaling pathways mediated by protein kinase C (14) and the proto-oncogene ras (15, 16). It has also been shown that HBV X interacts directly with factors involved in transcription [e.g., the cyclic AMP-responsive element-binding protein CREB/ATF2 (17), the TATA box-binding protein TBP (18), and a subunit of RNA polymerase (19)]. Recent reports describe the ability of HBV X to associate with the cellular tumor suppressor protein p53 in vitro and to decrease its specific DNA-binding activity (20–22) and possibly interfere with DNA repair functions (23). Various mutants of p53 bound HBV X with variable efficiency; one mutant with diminished binding was also poorly bound by the E6 protein of the human papillomavirus (24). On the other hand, another mutant that is most commonly found in areas with high aflatoxin and HBV incidence retained binding to HBV X (21). These results are significant in light of the fact that transformation antigens of DNA tumor viruses, such as the adenovirus E1A antigen and the SV40 large T antigen, incapacitate p53 by binding and sequestering it or, alternatively, cause its degradation, as mediated by the E6 protein of the human papillomavirus (25). Thus, an obvious question is whether HBV X is analogous to any of these viral proteins in eliciting cell transformation by compromising p53 function. In recent studies on adenomas and carcinomas in a strain of transgenic mice carrying the HBV X gene, the expression of p53 seemed to correlate exactly with that of HBV X in transformed cells (26). Biochemical studies showed binding and cytoplasmic sequestration of p53 by HBV X in transformed cells but not in normal cells (26). Down-regulation of HBV X expression in these tumors by IFN treatment of the mice restored nuclear localization of p53 (26). These observations suggested that HBV X binding of p53 may be the predominant mechanism for p53 inactivation and a primary initial requirement in multistep carcinogenesis. However, in naturally occurring HCC, as in other tumors, inactivation of p53 is believed to occur primarily as a consequence of mutations in the coding sequence of the gene. Most of these are point mutations in the conserved DNA-binding domain of the protein [i.e., exons 5–8 (27, 28)]. A variable frequency of p53 mutations in HCC has been reported, varying from 25 to 60% in different studies. These mutations are considerably more frequent in HBV-infected patients exposed to aflatoxin who also have a high frequency of a unique G-T transversion in codon 249 of exon 7 of the p53 gene (29–34). These mutations give rise to altered proteins that are unable to bind to its cognate DNA sites and activate specific transcription (32). The p53 protein is a flexible molecule that can naturally undergo function-dependent conformational changes, suggesting that such changes occur in normal physiological contexts (36). Mutations lock the resultant protein into an aberrant conformation, rendering it incapable of normal physiological functions (36). Transition of wild-type p53 into a mutant conformation can also be induced by heat shock proteins (37) and certain metal ions [e.g., sodium vanadate (38)]. Similar conformational changes have also been observed in vivo during keratinocyte differentiation (39). Wild-type and mutant p53 differ immunologically (40), and only p53 in a mutant conformation can associate with HSP70 (37, 41). In this study, we have analyzed a collection of HCC samples from China for expression of HBV X and...
for the possible interaction of HBV X and p53 in vivo. We also determined the wild-type or mutant status of p53, as defined by various antibodies to p53 and by their binding to HSP70, and their functional ability by the expression of the Cip1/Waf1 protein in the tumors.

**MATERIALS AND METHODS**

**Tumor Tissues.** HCC tissues obtained either after surgical removal or at autopsy were kindly provided by Professor Wu Mengchao (Institute of Hepatobiliary Surgery, Changhai Hospital, Shanghai, China), and they were stored at −70°C. Most of them had markers of HBV infection, as determined by ELISA and RIA using commercial detection kits, at Changhai Hospital. The presence of viral DNA was determined by Southern blot hybridization, as described (Table 1).

**Antibodies.** The following antibodies were used in this study: α-p53-MAbDO-1, PAb240, PAb1801, and CM-1 (kind gifts from Professor David Lane, Dundee); α-HSP70-AB-1 (purchased from Oncogene Science); polyclonal (PAb-X) and monoclonal (α-HBV-X) antibodies 16F1 and 18C1 (raised as described below); and monoclonal antibody α-Cip1/Waf1 (Purchased from Transduction Laboratories). MAb68, a monoclonal antibody that detects p43, a protein abundantly expressed in transformed cells (42), was used as a control to confirm that the proteins in the tumor lysates were intact.

**Preparation of X Protein.** The X open reading frame of HBV was cloned in plasmid vector pET8c (kindly provided by Dr. William F. Studier) to yield the construct pET8c-X. An overnight culture of Escherichia coli BL21 (DE3) containing 4 M urea for 30 min at 4°C, and the pellet washed in buffer A [50 mM Tris-HCl (pH 8.0)-lO mM EDTA-50 μg/ml aprotinin-l μM phenylmethylsulfonyl fluoride], resuspended in the same buffer, and lysed by sonication for 20 s. The lysate was centrifuged at 13,000 rpm for 30 min at 4°C, and the pellet washed in buffer A containing 30% sucrose. This was followed by a second wash in buffer A containing 4 M urea for 30 min at room temperature. After centrifugation, the pellet was dissolved for 30 min in buffer A containing 8 M urea. The solution was cleared by centrifugation at 13,000 rpm, and the supernatant containing soluble X protein was dialyzed several times against buffer A containing decreasing concentrations of urea. X protein was separated from remnants of bacterial protein by preparative SDS-PAGE, and the abundant M, 17,000 protein, identified by staining with Coomassie blue, was electroeluted from the gel and used for the immunization of rabbits and mice. A GST-X fusion protein was used for the screening of hybridoma supernatants. Plasmid pGST-2TX was constructed by cloning the HBV X gene in pGST-2T vector (Pharmacia, Piscataway, NJ) and was expressed in DH5α cells. GST-X fusion protein was purified on glutathione-agarose columns. A plasmid, pCMV-X, was constructed by cloning the HBV X gene in the pRc/CMV vector (Invitrogen, La Jolla, CA) for expression in eukaryotic cells.

**Preparation of Monoclonal Antibodies.** Myeloma cells (Sp2 cell line) were cultured in RPMI 1640 containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 1 mM sodium glutamate at 37°C and 10% CO2. A responder mouse immunized with recombinant X (pet-X) was sacrificed, and a splenocyte suspension was made and fused to Sp2 myeloma cells using polyethylene glycol as described (43). Primary screening of clones was performed 10 days after fusion. Positive clones were subcultured in 24- and 6-well plates, and supernatants were tested for specificity on Western blots of lysates of transfected CCL13 cells transiently expressing X. Two hybridomas secreting monoclonal antibodies suitable for ELISA, Western blot, immunoprecipitation, and cell staining were cloned. They were designated as 16F1 and 18C1.

**Screening of Hybridoma Supernatants.** Plastic microtiter plates were coated with 1 μg/ml GST-X in PBS (50 μl/well) at 4°C overnight, blocked with 3% BSA for 2 h at room temperature, and incubated with hybridoma supernatants for 2 h, followed by incubation with horseradish peroxidase-labeled antimouse antibody for 2 h. ELISA was performed using TMB chromogenic substrate (43), and the absorbance at 450 nm was determined in a Dynatech plate reader.

**Southern Blot Analysis.** DNA was extracted from tumor tissue using a DNA extraction kit (Pharmacia). Ten μg of DNA were digested with either HindIII, which does not cut within the HBV genome, or with EcoRI, which cuts once. The restriction fragments were separated by electrophoresis on 0.8% agarose gels and transferred onto nitrocellulose membranes. Placed HBV DNA labeled with [α-32P]dCTP using random hexamer primers was used as a probe for hybridization.

**Western Blot Analysis.** Tumor tissues and cultured cells were lysed and homogenized in EBC buffer [50 mM Tris-HCl (pH 8.0)-320 mM NaCl-1.5 mM EDTA-0.5% NP40] containing 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. Thirty μg of protein per lane were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes, and blocked with 5% nonfat milk. Blots were incubated overnight with the appropriate primary antibody at 4°C and then incubated with 1:1000 diluted horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing the blot as performed previously, the immunoblots were developed with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL).

**Immunoprecipitation.** Tissue lysates containing 200 μg of protein were precleared with agarose beads (Bio-Rad, Richmond, CA) for 1 h and centrifuged to remove the beads. The supernatants were incubated with either 50 μl of hybridoma culture medium containing the relevant monoclonal antibody or 5 μl of polyclonal rabbit antibody overnight at 4°C. Twenty μl of protein G beads (Calbiochem) were then added to each tube and incubated for 1 h. The beads were then washed with EBC buffer, boiled in 20 μl of loading buffer, and loaded onto SDS-PAGE. Immunoprecipitates were detected by Western blot analysis as described. For immunoprecipitation of HBV X, 250 μg of total protein were first denatured by boiling in 1% SDS and 10 mM Tris (pH 7.4) for 5 min. Denatured proteins were suspended in 10 volumes of 0.5% Triton X-100, 7.5 mM Tris (pH 7.4), 0.5 mM EDTA, and 0.2% NP40, and were immunoprecipitated with Mab 16F1. Immunoprecipitates were analyzed on Western blots as described using Mab 16F1 or PAb-X.

**Immunohistochemistry.** Tissues were fixed in methacarn and embedded in paraffin wax. Thin sections mounted on glass slides were dewaxed, rehydrated, reacted with anti-X antibody, and stained. Briefly, sections were treated for 20 min in 1% H2O2/PBS, extensively rinsed in distilled water, and preincubated in normal swine or rabbit serum (1:5 in PBS). Three rinses in PBS
were performed after each incubation. Serial sections of each sample were incubated separately with undiluted hybridoma supernatants 16F1 and 18C1 and rabbit antiserum PAb-X (diluted at 1:30) for 1 h. After rinsing, biotinilated rabbit antimouse or swine antirabbit immunoglobulins (DAKO Corp.) diluted 1:200 in PBS were applied for 45 min. Sections were rinsed again and incubated with horseradish peroxidase conjugated streptavidin biotin complex (DAKO Corp.) for 30 min. The sections were rinsed, and peroxidase activity was developed for 2–7 min with 0.05% DAB and 0.03% H2O2 in PBS, counterstained with Harris' hematoxylin, dehydrated, cleared, and mounted in Pertex. Control experiments were performed with MAb68 monoclonal antibody to p43, a cellular protein, on serial sections. CCL13 cells grown on glass coverslips were transfected with pCMV-X and used as positive controls. Untransfected cells were used as negative controls. Cultured cells were reacted with antibodies and stained as described after fixation in acetone:methanol (1:1).

RESULTS

HBV DNA and Other Markers of HBV in HCC Samples. HCC samples from 31 Chinese patients from Shanghai and its environs were used in this study. They had been tested previously in the clinic for various markers of HBV infection, and we determined the presence of HBV DNA in the tumors by Southern blot hybridization, as shown in Table 1. Using total HBV DNA as a probe, 4 samples showed the presence of free viral DNA, 19 had integrated HBV DNA, and 1 had both. The additional samples showed faint bands probably due to only small fragments of integrated viral DNA (Table 1). Twenty-two (71%) of the tumors were HBV positive by at least one marker.

Lack of Detection of X Protein in Tumor Samples. Previous studies have reported on the presence of X protein in chronically infected patients (45) and HCC (45). In those histochemical studies, positive staining was seen in only a percentage of samples examined and only in isolated groups of cells within the tumor. In this study, we analyzed each of the samples by Western blot using monoclonal and polyclonal antibodies to recombinant HBV X protein. These antibodies can detect recombinant X protein expressed both in bacterial and human cells. However, we did not detect immunoreactivity in any of the tumor samples (Fig. 1A). We also attempted to concentrate possibly trivial amounts of X protein by immunoprecipitation from 250 μg of total protein from each tumor lysate using the monoclonal antibody 16F1 under denaturing conditions, but we were still unable to detect the X protein (Fig. 1B). To exclude the possibility that the absence of X protein might be attributed to protein degradation, we analyzed these tumor samples for p43, a recently described protein of the elongation factor family, which is highly expressed in HCCs (42). Every sample contained intact p43 (Fig. 1A), confirming that the lack of detection of X protein is not because of general protein degradation. Moreover, in our experience, X protein is not a particularly labile protein. To determine the sensitivity of our assay, we performed Western blot analysis using several serial dilutions of lysates of CCL13 cells transfected with an expression plasmid for X protein. In this experiment, X protein was easily detectable when only 0.1 μg of total lysate protein was used, compared to 30 μg of tumor cell lysates (Fig. 1C). Assuming a transfection efficiency of 10%, based on a β-galactosidase assay in cells transfected with a β-galactosidase reporter plasmid, we estimate that levels of X protein in tumors 2–3 orders lower than that in the transfected CCL13 cells would be detectable. This result indicates that either there is no expression of X protein in these tumors, or that it is below this very low limit of detection. Finally, in consideration of the possibility that X protein may be expressed in only isolated groups of cells in the tumor, we studied 12 representative tumor samples by immunohistochemical staining of thin sections using two monoclonal antibodies, 16F1 and 18C1 under denaturing conditions, but we were still unable to detect the X protein (Fig. 1B).
Fig. 2. Histochemical staining of HCC tissues for the detection of X protein. A and C, untransfected CCL13 cells. ×40 and ×20, respectively. B and D, CCL13 cells transfected with pCMV-X plasmid. ×40 and ×20, respectively (see arrows showing diffuse and punctate cytoplasmic staining). E and F, representative samples of 12 HCC tissues analyzed. ×20. The anti-X antibodies used were MAb6Fl, MAb18C1 (shown here), and PAb-X. Sections were stained by the avidin biotin complex method using DAB as substrate and counterstained with hematoxylin.

18C1, and the polyclonal rabbit antiserum PAb-X in each case. CCL13 cells transfected with X protein plasmid or untransfected cells were used as positive and negative controls, respectively. A similar and specific pattern of staining was observed with each of the antibodies; the distribution of X protein was cytoplasmic, and there was no obvious nuclear staining. In addition to diffuse cytosolic staining, there is also very clear punctate staining, occasionally perinuclear in distribution, with a small number of large granules (see arrows in Fig. 2, B and D). This pattern of staining is similar to that described previously in other transfected hepatocyte cells [i.e., HepG2 and Huh-6 and nonhepatocytes; i.e., COS cells (46, 47)]. In comparison, no staining was observed either in vector-transfected CCL13 cells (Fig. 2, A and C) or in any of the tumor samples (Fig. 2, E and F, showing representative samples). For each slide, the entire section was examined carefully for the possible presence of isolated stained cells or groups of cells. On the basis of these different methods of analysis, we conclude that X protein is not present in the HCC tissue studied here, in contrast to other reported studies (45, 48).

Detection of p53 in HCC Samples. To evaluate the importance of possible associations of X protein with p53 in hepatocellular carcinogenesis, we first analyzed each of the tumor samples for the presence of p53. In Western blots using DO-1 antibody, every sample showed the presence of p53 (data not shown). To obtain a more sensitive and quantitative measure of the levels of p53, we used PAb1801 for immunoprecipitation. Like DO-1, PAb1801 reacts with both wild-type and mutant p53, making it ideal for this purpose. Again, p53 was readily detectable in abundance in every sample (Fig. 3). Despite the lack of detection of X protein in any of the tumors, we attempted to confirm previous observations of X protein binding to p53; the rationale was that if X protein is present in very low amounts and if it is complexed with p53, then immunoprecipitation would concentrate it and facilitate its detection. Accordingly, immunoprecipitates were prepared from the tumor samples with PAb1801 and analyzed on Western blots with PAb-X. Again, there was no signal corresponding to X protein in any of the 31 samples (data not shown), which is consistent with our inability to detect X protein directly by Western blot, supporting the conclusion that there is no X protein in any of the tumor samples. In recent studies on transgenic mouse tumors, the inactivation of p53 was suggested to occur by stoichiometric binding by X protein (26). This obviously cannot be true in the human tumors, even if we were to assume undetectably low amounts of X protein. Thus, this result challenges the idea that X protein p53 binding is either common or important in the tumor.

Determination of Mutant Status of p53 in HCCs. Inactivation of p53 seems to be a crucial step in carcinogenesis. However, our results
do not support the idea that inactivation of p53 in HCC occurs by binding to X protein. On the other hand, specific mutations in the p53 gene are responsible for the loss of p53 function in 50–60% of HBV-positive HCCs (26–31). In its mutant conformation p53, is unable to induce specific transcription and, therefore, cannot function as a tumor suppressor. One of the criteria used to define the mutant conformation of p53 is its reactivity with the monoclonal antibody PAb24O, which recognizes most mutant forms but not wild-type protein (40). Eleven of the tumor samples used in this study were immunoprecipitated with PAb24O. p53 was detected in 10 of 11 immunoprecipitates tested on Western blots by a rabbit polyclonal anti-p53 antibody CM-I (Fig. 4A). The binding of HSP70 to p53 was used as another parameter for studying the conformation of p53 in the HCC samples (37, 41). PAb1801 was used to immunoprecipitate total p53 protein, which was then tested for bound HSP70 using the anti-HSP70 antibody AB-1 in Western blot analysis. Twenty-nine of 31 samples clearly showed the presence of HSP70 in the immunoprecipitate, and the remaining samples (samples N, O, and D) showed marginal amounts (Fig. 4B). The binding seems to be specific for mutant p53 because p53 immunoprecipitated from COS cells that has only wild-type p53 and is used as a control does not bind HSP70. In the converse experiments, HSP70 was immunoprecipitated from the tumor lysates with AB-1 antibody, and p53 was detected in the immunoprecipitates by Western blot using CM-I antibody. Again, p53 was detectable in 27 of 28 samples analyzed (Fig. 4C); samples N, O, and D were convincingly positive in this reaction. A weak signal was detected in CCL13 cells used as a negative control. This may be because of the exceptional abundance of HSP70 immunoprecipitated from these cells and the possible presence of a very small proportion of p53 in these cells with mutant conformation. These experiments indicate that >90% of HCCs have conformationally altered (mutant) p53 at the protein level. To determine whether the mutant p53 in the tumors was functionally inactivated, we tested 20 of the lysates for the expression of the Mr 21,000 product of the Cip1/Waf1 gene (49, 50).
This protein is an inhibitor of the cell cycle that is induced in normal cells by wild-type but not mutant p53. In a Western blot analysis, no expression of p21 could be seen in any of the tumors. CCL13 cells used as a positive control were positive for p21 (Fig. 4D). These results strongly suggest that p53 in most HCC is functionally inactive.

**DISCUSSION**

Loss of function of the tumor suppressor protein p53 is the most consistently observed biochemical parameter in a wide range of human and animal tumors including HCC. In most HCCs, HBV is considered to be a major etiological factor. Several lines of investigations suggest that the HBV X protein is associated with tumor development in chronically infected patients (10–23). The X gene is frequently present in tumors, and, although truncated and linked to cellular domains through integration, it is often potentially functional as an activator, as shown by cloning and expression assays in cultured cells (51–53). However, these observations provide only circumstantial evidence and are based mostly on the function of X as a transcriptional transactivator. The closest direct evidence for such involvement is the high incidence of HCCs in a strain of transgenic mice expressing the X gene under its own promoter (54). It is suggested that the association of X with p53 is an important mechanism for hepatocarcinogenesis in these mice through p53 inactivation by quantitative sequestration in the cytoplasm by X (26). It was of interest that in other similar studies using the α1-antitrypsin promoter to express X in transgenic mice, no tumors were obtained (35). It has been argued that the absence of tumors in that case may be due to the lack of sustained expression of X in the mice after birth (26). If this model was applicable to HBV-induced human HCC, it would predict that fairly high amounts of X should be present in most tumors, in which it should be bound to p53.

In this study, we examined a large number of HCCs by a number of methods: (a) for expression of p53; and then (b) for any interaction between them. Western blot analysis, immunoprecipitation, and immunohistochemical staining were performed using two newly developed anti-X monoclonal antibodies and one PAB. It was surprising that in no instance could we detect X in the tumors, despite the high sensitivity of detection, as estimated using dilutions of X-transfected cell lysates. There have been few studies thus far on the detection of X protein in HCCs (45, 48). The results of those studies have been equivocal and the interpretations controversial because of conflicting evidence as to the frequency of detection of X protein in tumors, its cellular distribution, and its intracellular localization. On the basis of histochemical studies, X protein was reported to be present mostly in small clusters of cells and localized within the nuclei of dysplastic cells, where it was suggested to bind and inactivate p53 (45). This is in contradiction to the observation of cytoplasmic colocalization of X protein and p53 in X protein-induced tumors in transgenic mice (26). One way to reconcile these results is to consider different mechanisms of transformation in the two systems. We propose that the presence of X protein in the tumor is not a general or important feature of human HCC, but that whenever detected, it may either be a "footprint" of past replication or, rarely, of ongoing virus replication. Alternatively, it may be due to X cellular fusion proteins expressed from integrated HBV sequences. Thus, any detection of the X protein in tumors is mainly a reflection of virus replication rather than of the tumor state. It is likely that the X protein may have a role in initiation or another early step in hepatocyte transformation, rather than in the maintenance of the tumor.

Considering the importance of X protein in virus replication and infection, the efficient expression of X protein during acute infection would be consistent with a regulatory function. By this reasoning, in the absence of any significant virus replication, the expression of X protein should be negligible. Therefore, it is perhaps not surprising that X protein is not easily detected in tumors. Regarding the possibility that X protein binds p53 in tumors (20, 21), although abundant amounts of p53 were immunoprecipitated with PAB1801, no X protein was coprecipitated with it, indicating again the lack of X protein in the tumors. Although it would be impossible to rule out absolutely any expression below the sensitivity of detection, such amounts would seem insufficient for quantitative sequestration of p53. Thus, our results suggest that if X protein p53 binding does indeed occur, it would be unlikely to be important beyond the early stages of transformation. Our data support the contention that the mechanism of p53 inactivation in human HCC is different than that proposed for transgenic mouse tumors, in which there is a high expression of X protein during embryonic development through adult life and in the tumors (26). Although miniscule amounts of X protein can never be properly ruled out by available methods, quantitative inactivation of p53 by sustained expression of X protein at a high level for years or decades, as in the transgenic mouse model, seems unlikely to occur in human hepatocarcinogenesis.

Because of the lack of X protein binding to p53 in any of the tumors investigated in this study, we tested whether p53 is inactivated possibly by other mechanisms. Using the monoclonal antibody DO-1, which recognizes both wild-type and mutant forms, relatively high levels of p53 were detected in every sample, suggesting the presence of mutant forms because, unlike the wild-type protein, mutant p53 is very stable (25). Immunoprecipitation of the tumor lysates with monoclonal antibody PAB1801 confirmed the presence of p53 in every tumor. Immunoprecipitation with the monoclonal antibody PAB240, which recognizes most mutant but not wild-type forms, yielded p53 in 10 of 11 (90%) randomly selected cases, indicating again extremely high proportion of HCCs having p53 with mutant conformation. HSP70, which selectively binds mutant p53 (37, 41), was present in p53 immunoprecipitated with PAB1801 from 29 of 31 tumors. Evidence for the loss of function of p53 in the tumors was implied by the lack of expression of the Cipl/Waf1 Mr 21,000 protein that is specifically induced by wild-type p53. Thus, according to several criteria used, mutant p53 appears to be a predominant feature in most HCCs of Chinese origin studied here. In contrast, nucleic acid sequencing studies on the p53 gene in HCC among the Chinese and other populations revealed mutations in exons 5–8 of the p53 gene in only 50–60% of cases (26–31). Thus, in a significant proportion of tumors, the origin of the mutant p53 protein is not clear. It is possible that these mutant p53 proteins are caused by mutations in other regions of the gene, a question that obviously needs to be addressed in future studies. Alternatively, it is possible that mutant forms of p53 may be induced at the protein level by unknown factors, possibly environmental toxins. The ability of X protein to bind both wild-type (20–22) and various mutant forms of p53, including a mutant often correlated with high aflatoxin and HBV incidence (29–34), has been reported (21). This observation is significant because it questions the relevance of X protein binding to p53 function.

Finally, our results do not imply that X protein has no effect on wild-type p53 function. Indeed, preliminary evidence suggests that X protein may interfere with specific transcriptional activation by p53. Our detection of X protein in the cytoplasm of transfected cells is consistent with the possibility that X protein may bind and sequester...
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p53 in the cytoplasm. Codon 249 of the p53 gene is a frequent target of mutation by aflatoxin B1. This mutation is apparently favored in the presence of HBV (29-34), suggesting that HBV proteins such as X protein may enhance the mutagenic effects or specificity of aflatoxin and other environmental mutagens. X protein may act in concert with genotoxic substances at an early step in hepatocyte transformation in chronically infected cells. At the same time, X protein may also interfere with p53 protein function and inhibit repair mechanisms, consequently allowing the accumulation of mutations. It is possible that X protein may be important for HCC development via different mechanisms in geographical locations with fewer environmental carcinogens. On the other hand, during acute hepatitis, interference to infection. Our results show that, in human HCC, it is neither HBV X protein nor sequestered p53 but mutant p53 that is the predominant presence of HBV (29—34), suggesting that HBV proteins such as X protein may act in concert with genotoxic substances at an early step in hepatocyte transformation in chronically infected cells. At the same time, X protein may also interfere with p53 protein function and inhibit repair mechanisms, consequently allowing the accumulation of mutations. It is possible that X protein may be important for HCC development via different mechanisms in geographical locations with fewer environmental carcinogens. On the other hand, during acute hepatitis, interference to infection. Our results show that, in human HCC, it is neither HBV X protein nor sequestered p53 but mutant p53 that is the predominant hallmark.

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