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Hepatitis B Virus X-Protein Binds Damaged DNA and Sensitizes Liver Cells to Ultraviolet Irradiation

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The mechanism which is responsible for the association of chronic hepatitis B virus (HBV) infection with hepatocellular carcinoma (HCC) is poorly understood. The protein encoded by the HBV X-gene (HBx) has been identified as potentially oncogenic. HBx is a promiscuous indirect *trans*-activator of a wide range of cellular and viral *cis*-elements and may disrupt the maintenance of genomic integrity by inhibiting p53 function and binding a putative DNA repair protein (XAP-1). In this report, we show that there is preferential binding of recombinant HBx to damaged DNA through an association with nuclear proteins. We have used the transcriptional acivation by HBx of the β-actin promoter of a β-galactosidase reporter cassette to label cultured Chang liver cells expressing HBx. We demonstrate that cells expressing HBx are sensitised to the lethal effects of low dose ultraviolet irradiation. These data indicate that HBx interferes with liver cell DNA repair by binding damaged DNA and may predispose to the accumulation of potentially lethal or carcinogenic mutations.

Chronic infection with the hepatitis B virus (HBV) is common in sub-Saharan Africa and east Asia where it is often complicated by the development of hepatocellular carcinoma (HCC) (1). HBV is a partially double stranded hepatotropic DNA virus which has a compact genome comprising four open reading frames which include the core (C), surface (S), polymerase (P) and X-encoding sequences (2). The function of the small 17 kDa X protein (HBx) is not well understood although it is required for the establishment of viral infection and may be important in causing HCC (3,4). *HBx* transgenic mice develop HCC with increased frequency (5) and are more susceptible to the hepatocarcinogenic effects of exposure to diethyl nitrosamine (DEN) (6). Moreover, HBx encoding sequences are frequently found in malignant tissue from patients with HBV associated HCC (7).

Although a number of mechanisms has been proposed which implicates HBx in the transformation of hepatocytes, the exact causal relationship is not established. HBx stimulates the transcription of a spectrum of genes. These include viral and cellular growth regulatory genes. This *trans*-activating effect is mediated indirectly by stimulating the RAS-RAF-mitogen-activated protein (MAP) kinase pathway (8-10) and by acting on cellular transcription factors (reviewed in 11). A role for HBx in DNA repair has been suggested by the interaction of HBx with XAP-1, which is the human homologue of the monkey UV-damaged DNA binding protein (UV-DDB) (12,13). The wide range of transcriptional activating properties and possible involvement of HBx in cellular DNA repair indicate that HBx may exert its effects on a general transcription factor(s) required for transcription coupled DNA repair. Of the seven general transcription factors needed for initiation, TFIIH is the most complex and has several functions (14). Two of the integral subunits of TFIIH are the DNA repair proteins, XPB (15) and XPD (16). In response to DNA damage in a normal cell, the tumor suppressor protein p53 interacts with TFIIH-associated factors to modulate nucleotide excision repair (17) and arrests cell growth or induces programmed cell death (apoptosis) (reviewed in 18). HBx inhibits binding of XPB to p53 (19) and also intereferes with the function of p53 (20,21). Normal p53 is essential for the maintenance of genomic integrity, and binding of DNA repair proteins or inhibition of p53 function by HBx may allow the accumulation of mutations. Although the binding of HBx to proteins required for DNA repair has been demonstrated, a functional impairment of DNA repair which may result in lethal or hepatocarcinogenic mutations has not been established.

A direct way of investigating the effect of HBx on cellular DNA repair is to assess the binding of HBx to

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damaged DNA and the survival of cells expressing HBx following exposure to a mutagen. In this report, we present data which demonstrate that recombinant HBx binds ultraviolet irradiated DNA preferentially through an association with nuclear proteins. Furthermore, we show that cultured hepatocytes which express HBx are more susceptible to the lethal effects of low dose ultraviolet irradiation. These results are consistent with the hypothesis that HBx inhibits the normal cellular processes which are responsible for the maintenance of genomic integrity.

MATERIALS AND METHODS

Plasmids

DNA encoding HBx from HBV strain *ayw* was amplified using PCR. The sequences of the primers were 5′GGGAATTCCATATGG-CTGCTAGGCTG 3′ (HBV *ayw* coordinates 1375 to 1390, Accession J02203) and 5′TGATAGATCTTGAACAGTAGGAC 3′ (HBV *ayw* coordinates 1870 to 1855). The 5′ terminal redundancies incorporated unique *Eco*RI (5′), *Nco*I (5′) and *Bgl*II (3′) restriction digestion sites at the extremities of the amplified DNA. After restriction with *Eco*RI and *Bgl*II, the sequence was cloned into the *Eco*RI and *Bam*HI sites of the pBluescript II SK$^+$ (Stratagene) multiple cloning site to produce pBS-X. The sequence of the X open reading frame was verified by dideoxy sequencing, then excised with *Not*I and *Eco*RI and inserted at the equivalent restriction sites of the bacterial protein expression vector pGEX 4T2 (Pharmacia). In the resulting plasmid, pGEX-X, the entire *hbx* sequence was inserted downstream and in frame as a fusion with the glutathione S-transferase (GST) encoding sequence of pGEX 4T2. The eukaryotic expression vector, pCIneo-X was constructed by inserting the *hbx* sequence from pBS-X (*Xho*I-*Xba*I restriction fragment) into the eukaryotic expression vector pCI-neo (Promega). The plasmid pβgal has been described (22), and contains an expression cassette with the marker gene β-galactosidase under control of the β-actin promoter.

Bacterial Expression of the GST-X Fusion Protein

The *E. coli* strain BL21 pLys was transformed with either pGEX-X or pGEX 4T2 in media containing ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml). Liquid cultures in logarithmic phase of growth were induced to express recombinant proteins by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) to 0.1 mM and then cultured for a further 1 hour at 25°C for the preparation of GST-HBx or at 37°C for the preparation of GST. Bacteria were recovered by centrifugation, resuspended in lysis buffer (phosphate buffered saline with 0.1% Tween 20 and 2mM phenyl methyl sulphonyl fluoride (PMSF)) and lysed by freezing and thawing. The DNA from the bacterial lysates was fragmented by vigorous physical shearing, the recombinant protein was bound to agarose-glutathione beads (agarose-GSH, Sigma) and the beads washed according to standard procedures (23). The purity of the eluted recombinant proteins was confirmed by polyacrylamide gel electrophoresis. GST or GST-HBx attached to the agarose GSH was used for the DNA binding assays.

Recombinant HBx-DNA Binding Assay

i. Preparation of radiolabelled damaged DNA and nuclear protein extracts. *E.coli* (strain XL1 Blue) transformed with pβgal were cultured in Lauria Bertani medium which was supplemented with [6-^{3}H] thymidine (Amersham) to 1 nCi/ml of culture broth. Plasmid preparation was by alkaline lysis followed by silica matrix adsorption. Purified DNA was subjected to ultraviolet irradiation (5 000 μJ/cm^2) using a Stratagene UV cross linker. Nuclear proteins were prepared from guinea pig liver according to the method of Stuempfle *et al* (24) and extracts stored at −70°C.

ii. Binding assay. Direct and nuclear protein mediated binding of recombinant GST-HBx and GST protein to unirradiated and irradiated pβgal were assayed. For the evaluation of direct interactions, 1 μg of GST or GST-HBx coupled to GSH agarose in assay buffer (5 mM Tris-Cl pH 7.4, 125 mM KCl, 2.5 mM $MgCl_2$ and 1 mM DTT) was incubated with 100 ng radiolabelled irradiated or unirradiated pβgal plasmid (4500 cpm) for 30 minutes at 37°C. To assay for nuclear protein mediated binding, plasmid templates were incubated with 5 μg nuclear protein in assay buffer for 15 minutes at 37°C. This was followed by the addition of GST-HBx or GST bound to GSH agarose and a further incubation at 37°C for 30 minutes. Thereafter, the beads were washed three times by resuspension in an excess of assay buffer then recovered by centrifugation. After the final wash, the beads were resuspended and bound tritiated DNA determined by liquid scintillation counting. The Student t-test was used for statistical comparison of the amount of bound DNA. Differences observed under each of the binding conditions were considered significant when the P-value was less than 0.05 and the confidence interval greater than 95%.

Cell Culture and Transfection

Chang liver cells (ATCC No. CCL-13) were cultured in Dulbecco's minimal Eagle's medium (DMEM) supplemented with penicillin (50 IU/ml), gentamycin (50 μg/ml) and 10% fetal calf serum. On the day prior to transfection, approximately 10^6 cells were seeded at one tenth the confluent density. Transfection was with a combination of pCI neo (2.5 μg) and pβgal (0.5 μg) or pCI neo-X (2.5 μg) and pβgal (0.5μg) according to the calcium phosphate method (25). In some cases, cells were exposed to ultraviolet irradiation (1 000 μJ/cm^2) four hours after transfection. All cells were washed with DMEM and fresh culture medium added. The cells were fixed and stained 48 hours later with an X-gal solution (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, Sigma) (26). Cells which had a dominant blue coloration on microscopic examination were assessed as positive. Each transfection experiment was performed in triplicate and the number of positive cells calculated from examining an entire dish of transfected cells. Statistical analysis was performed using the Student t-test.

RESULTS

To investigate an association between HBx and damaged DNA, we assessed the binding of recombinant HBx to normal and UV irradiated DNA. The assay was performed in the presence and absence of a nuclear protein extract from normal liver tissue. The solid phase for our assay comprised GST-HBx or GST protein attached to GSH agarose beads and the bound recombinant proteins were of correct molecular weights and apparently homogenous according to PAGE analysis (Figure 1). We selected the pβgal plasmid as the substrate for the binding assay as this DNA contains a promoter which is activated transcriptionally by HBx (see below). The *trans* activation is probably mediated indirectly by the documented interaction between HBx and a range of transcription factors (11). We reasoned that this property could be used to confirm the binding of HBx to nuclear protein and DNA under our assay conditions.

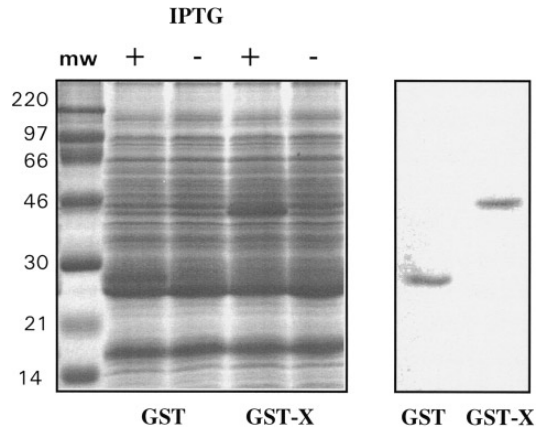

FIG. 1. Purification of recombinant GST and GST-X proteins. In panel A, the total bacterial lysates from cells expressing GST or GST-X are depicted before (−) and after (+) induction with IPTG. Panel B shows the proteins after purification using GSH-agarose affinity chromatography. The molecular weight of GST was 26 kDa and that of GST-X 43 kDa.

In Figure 2A, quantitative data for the binding of radiolabelled DNA to immobilised recombinant GST and GST-HBx are represented graphically. Similar minimal background binding between GST and irradiated or unirradiated DNA was observed in the presence and absence of nuclear protein. Interestingly, the binding between GST-HBx and irradiated or unirradiated DNA is similar and higher than the background ($P<0.01$) which suggests a direct interaction between GST-HBx and pβgal. However, the affinity of undamaged pβgal for recombinant GST-HBx in the presence of nuclear protein is significantly higher than the direct binding between HBx and pβgal ($P<0.05$). These findings are in keeping with an indirect association between HBx and the β-actin promoter which is mediated by a nuclear factor(s). In the presence of the nuclear protein extract, the affinity of HBx for damaged DNA is significantly higher than the binding to undamaged pβgal in the presence of nuclear protein ($P<0.0025$). These findings indicate that HBx associates preferentially to damaged DNA through an interaction which is mediated by nuclear proteins.

The transcriptional activating effects of HBx on the marker gene expression cassette of pβgal was used to label Chang liver cells expressing HBx. The cells shown in Figure 3 were cotransfected with a combination of either pCIneo and pβgal, or pCIneoX and pβgal. When cotransfection was with pCIneo-X, the ratio of the number of cells which was histochemically positive for the expression of β-galactosidase was eight-fold higher than when cotransfection was with pCIneo (Figure 2B). Thus under our assay conditions, not all cells transfected with pβgal and pCIneo were histochemically positive, and the number was increased significantly by the transcriptional activation by HBx. Moreover, the ratio indicates that at least 85% of the positive cells transfected with pCIneo-X express HBx and confirms that HBx is functional. Expression of HBx in the Chang cells transfected with pCIneo-X was confirmed by western blot analysis (data not shown). In our hands, the indirect histochemical method of detecting HBx through the activation of β-galactosidase expression was more sensitive and specific than immunocytochemical procedures.

The effect of low dose ultraviolet irradiation on Chang liver cells expressing HBx is demonstrated in Figures 2B and 3. The number of HBx expressing cells (reflected by the ratio) is decreased significantly after ultraviolet irradiation ($P<0.05$) indicating a suscepti-

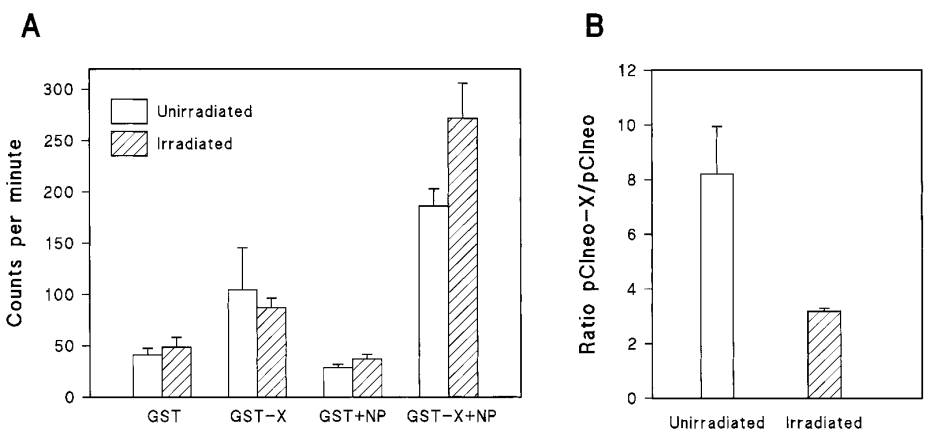

FIG. 2. (**A**) Binding of the pβgal plasmid to GST or GST-X immobilised on agarose-GSH. Each assay was conducted on irradiated and unirradiated pβgal in the presence or absence of the nuclear protein extract. The mean bound radioactivity (with standard error bars shown) was calculated from 6 assays. (**B**) The ratio of the number of histochemically positive cells transfected with pβgal and pCIneo-X to the number determined from transfection with pβgal and pCIneo. As indicated, the cells were either irradiated or unirradiated. The data represent the mean ratios with standard errors from three experiments.

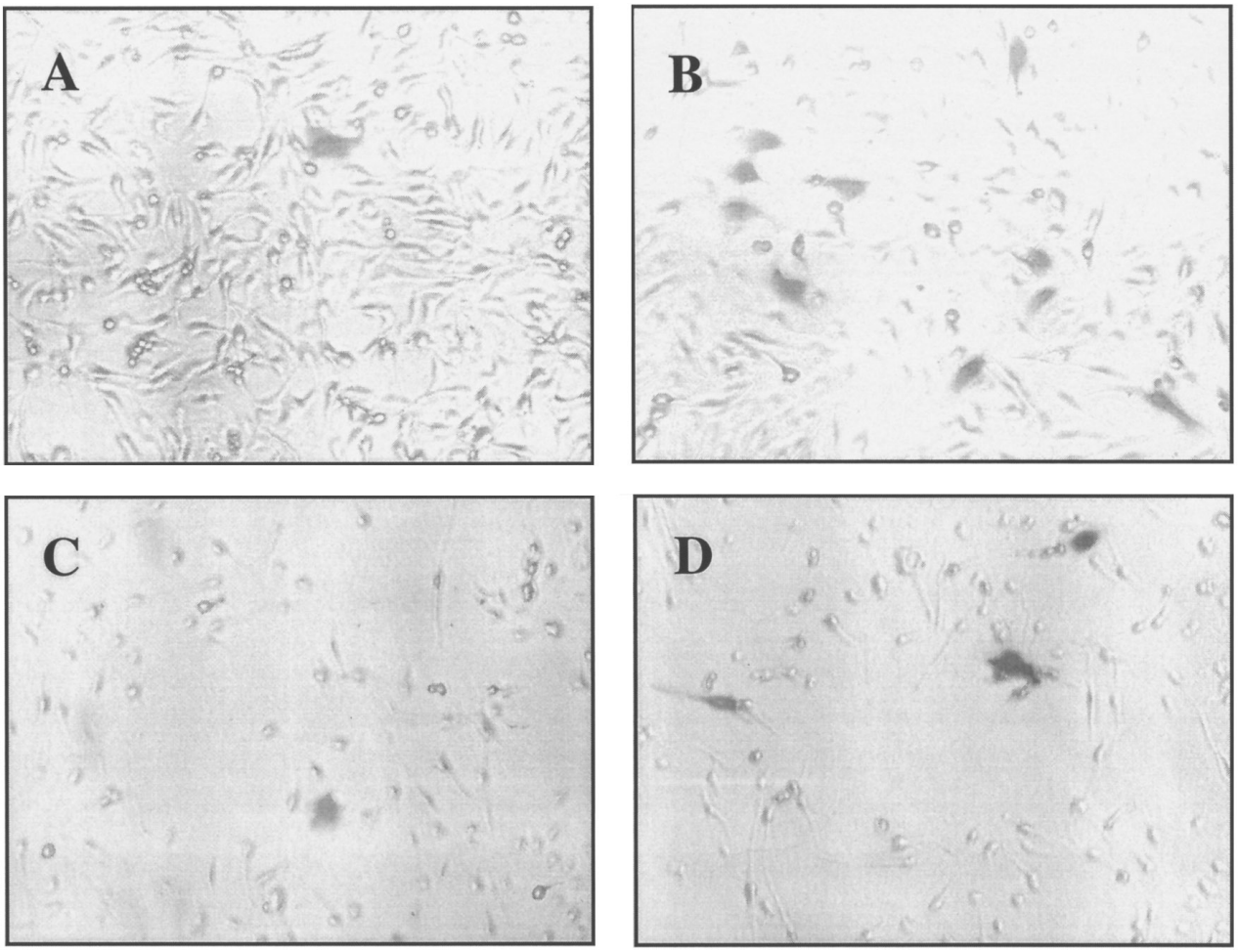

FIG. 3. Representative low power microscopic fields of cells transfected with pβgal and pCIneo-X (panels B and D) or with pβgal and pCIneo (panels A and C). The cells shown in panels A and B were unirradiated while those in panels C and D were exposed to 1000 μJ/cm^2.

bility of HBx expressing cells to the damaging effects of ultraviolet irradiation. The absolute number of β-galactosidase positive cells transfected with pCIneo was similar before and after ultraviolet irradiation (data not shown) indicating that Chang liver cells, originally derived from normal hepatocytes, are able to survive the effects of the ultraviolet irradiation we used in our assay. Our data indicate that hepatocytes expressing HBx are prone to genomic hypermutability which may lead to cell death.

DISCUSSION

There is compelling epidemiological evidence which shows that chronic infection with human HBV is the major risk factor for the development of HCC (1). Several mechanisms have been proposed for HBV induced carcinogenesis. The clonal integration of HBV DNA in the cellular genome of most human HCCs implies that viral insertional mutation plays a role (27,28). However, integration of HBV DNA is varied and appears to take place by a non sequence-specific process of illegitimate recombination which is not essential for the replication of the virus. Moreover, potentially oncogenic viral integration in human HCC has been an exceptional finding (29). Frequently found genetic changes in human HCC, such as point mutations of the anti-oncogene p53 (30,31) bear no apparent direct relationship to HBV integration (29). The spectrum of genetic changes suggests an effect of HBV on the maintenance of hepatocyte genomic integrity rather than a direct effect of HBV integration in causing HCC.

The demonstration of a preferential association between HBx and ultraviolet damaged DNA as well as the previously documented association of HBx with p53 (19-21) and the putative DNA repair protein XAP-1 (12), supports the idea that HBx interacts with the cellular DNA repair machinery. A functional role is

corroborated by our observation of decreased survival of HBx expressing liver cells following low dose ultraviolet irradiation. The lethal effects of ultraviolet irradiation on cells with defective nucleotide excision repair is well documented (32,33). An analagous situation is described here where an exogenous effect of HBx diminishes cell survival following exposure to ultraviolet light. Cells with malfunctioning DNA repair proteins are prone to a variety of mutations and are also more susceptible to maligancy. This has been demonstrated in inherited diseases of defective nucleotide excision repair (32,33) and mismatch repair (34).

The premalignant liver *per se* of patients chronically infected with HBV is thought to predispose to acquiring and propagating hepatocyte mutations (29). The liver of patients chronically infected with HBV is characterised by a regenerative necroinflammatory process (cirrhosis) which is sustained for several years. The occurrence of replication errors or oxidative DNA damage is therefore likely to be increased. The risk of acquiring mutations would be enhanced by compromised cellular DNA repair caused by HBx expression. The mutations which arise would also be rendered irreparable by the sustained stimulus for cell division. According to this hypothesis, exposure to other environmental hepatocarcinogenic risk factors which cause cirrhosis should act synergistically with HBV. This is corroborated by evidence which shows a multiplicative relative risk of hepatocarcinogesis resulting from a combination of chronic HBV infection together with aflatoxin exposure (35) or HCV infection (36).

The basis of the influence of HBx on cellular DNA repair and transcription is not well understood. It is thought that such a process would facilitate the replication of HBV while disrupting the maintenance of hepatocyte genome integrity (13). Following intracellular uncoating of viral particles, partially double stranded non-covalently closed circular DNA is repaired to form covalently closed circular double stranded DNA (cccDNA) which is the template for transcription of viral genes. It has been postulated that HBx recruits DNA repair enzymes to enable the intracellular conversion of the HBV genome to cccDNA and at the same time inhibits enzymatic repair processes that might be deleterious to HBV DNA. The molecular mechanism for such an interaction remains to be established.

In conclusion, our findings argue that HBx may disrupt the normal cellular processes which maintain hepatocyte genomic integrity. The implication of this is that with time, persistent HBV infection and HBx expression may lead to the accumulation of a variety of mutations which would ultimately lead to hepatocarcinogenesis.

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