Hepatitis B viral transactivator HBx alleviates p53-mediated repression of alpha-fetoprotein gene expression

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SUMMARY

Chronic infection with Hepatitis B Virus (HBV) is associated with development of hepatocellular carcinoma (HCC). The exact mechanism by which chronic infection with HBV contributes to onset of HCC is unknown. However, previous studies have implicated the HBV transactivator protein, HBx, in progression of HCC through its ability to bind the human tumor suppressor protein, p53. In this study, we have examined the ability of HBx to modify p53 regulation of the HCC tumor marker gene, alpha-fetoprotein (AFP). By utilizing \textit{in vitro} chromatin assembly of DNA templates prior to transcription analysis, we have demonstrated that HBx functionally disrupts p53-mediated repression of AFP transcription through protein-protein interaction. HBx modification of p53 gene regulation is both tissue-specific and dependent upon the p53 binding element. Our data suggest that the mechanism by which HBx alleviates p53 repression of AFP transcription is through an association with DNA-bound p53, resulting in a loss of p53 interaction with liver specific transcriptional co-repressors.
Introduction

Chronic infection with Hepatitis B Virus (HBV) is a predominant risk factor associated with development of hepatocellular carcinoma (HCC). Multiple lines of evidence support the relationship between chronic HBV infection and HCC: geographic correlation exists between global distribution of HCC and the prevalence of HBV carrier states; a high incidence of HBV markers in blood and tissue samples is detected in HCC patients; 30% of all virally induced human tumors involve HBV infection \([1, 2]\). Based on epidemiological studies involving chronic HBV infection, it is estimated that the relative risk of developing HCC may be between 100 to 200-fold higher for HBV carriers than for non-carriers \([2, 3]\).

The most likely scenario for HBV’s role in HCC predisposition is by modification of host gene regulation \([4-6]\). Integration of viral DNA into the host genome can mediate host gene deregulation by a variety of mechanisms: integration of viral promoters can activate and/or mutate neighboring host genes \([6]\); integration of viral DNA encoding the HBV transactivator X protein (HBx) enhances HBx expression and subsequent interaction with cellular genes and regulatory proteins \([2, 7, 8]\). Although HBx has not been reported to bind double-stranded DNA, it can activate transcription of both viral and cellular genes through interaction with a variety of host DNA binding proteins \([\text{reviewed in } 4]\). HBx association with cellular transcriptional activators and general transcription factors such as C/EBPα, TBP and TFIIH enhances gene activation. In contrast, HBx binding to human p53 protein antagonizes p53 mediated transcriptional activation \([9, 10]\), and p53-mediated apoptosis \([11, 12]\). p53 is a classical tumor suppressor with a diverse range of functions in transcriptional activation, cell cycle arrest, DNA damage repair,
apoptosis [13, 14] and, as recently demonstrated, transcriptional repression by sequence-specific DNA binding [15, 16].

While p53 mutation or inactivation is detected in over 60% of human cancers, p53 mutations are rare in the early stages of HCC [17]. However, p53-HBx interaction may disrupt p53 function, leading to genomic instability and accumulation of p53 mutations. Multiple studies support HBx-mediated p53 dysfunction, but the exact mechanism by which HBx inactivates p53 remains unclear. Strong support for HBx disruption of p53 function during development of HCC was demonstrated by a transgenic mouse study in which mice expressing HBx developed liver tumors with 80 to 90% penetrance within four months of birth [18, 19]. In these studies, HBx protein and murine p53 protein were found sequestered in the cytoplasm. These studies suggested that HBx-p53 interaction prevented entry into the nucleus. However, more recent studies demonstrated nuclear localization of HBx [reviewed in 20], where it could potentially disrupt the ability of nuclear p53 to bind DNA [9], communicate with general transcription factors or form stable tetramers [10].

We have shown recently that p53 represses transcription of the alpha-fetoprotein (AFP) gene by sequence-specific DNA binding [15]. This repression is both tissue- and developmental-specific, and contributes to the developmental regulation pattern of AFP expression. AFP is secreted by the visceral endoderm of the yolk sac, and is the predominant fetal serum protein synthesized by the developing liver [21, 22]. At birth, AFP expression is silenced: its mRNA level is down-regulated approximately 10,000-fold. This nearly undetectable expression is maintained throughout adult life, except in
cases of liver regeneration and/or HCC where AFP expression is reactivated in 75-80% of hepatocarcinomas [23, 24].

In these studies we have utilized regulation of AFP expression in a cell-free assay system as a marker for HBx-mediated disruption of p53 function. By employing fractionated Xenopus egg extracts to assemble AFP templates into chromatin prior to in vitro transcription analysis, we have examined regulated AFP expression devoid of effects mediated by non-specific transcriptional squelching. Our results demonstrate that HBx can destroy the ability of p53 to regulate AFP in a tissue-specific manner. Additionally, the data demonstrate that the mechanism by which HBx alleviates p53-mediated repression of AFP transcription is by physically interacting with p53, blocking interaction with hepatic-specific proteins that may act in transcription repression.
Experimental Procedures

Plasmids. The AFP/lacZ template contains 3.8 kb of the AFP 5’ flanking sequence, including the previously defined distal and proximal promoter elements and Enhancer I, fused to the β-galactosidase coding region [25, 26]. The AFP/DelA template was prepared from the AFPmut5 plasmid previously described [15] by 3 step PCR [27]. The 10 base pair deletion within the p53 binding site, located at –853 relative to the AFP transcriptional start site, was created through PCR amplification using the AFP/mut5 template and the following primers:

A. 5’ CCTCCATTTTATGAGTACACTATA 3’
B. 5’ GTGTCTTAAGCGTTGCTAAGG 3’
C. 5’CGAGGGGAAAATAGGTGGTTGCGCG 3’
D. 5’ CCTTAGCAACGCTTAAGACAC 3’

A and B primers were used in the 5’ amplification step, with C and D primers used in the 3’ amplification step. Primers A and D were used for final amplification. The PCR product containing the mutated p53 binding site was subcloned into the TA vector, pCR2.1 (Invitrogen) and recovered by BamHI and HindIII restriction digest and gel isolation. The fragment was subcloned into an AFP(-1.0)/lacZ template lacking Enhancer I [25, 26]. The –3.8 to –1 kb Enhancer I region was subsequently cloned as a BamHI fragment into this intermediate plasmid to yield AFP/DelA.

The β-globin template contains the chick β-globin upstream promoter, structural gene and enhancer. Its construction has been previously described [28].

Protein expression. Recombinant histidine-tagged p53Δ30 protein [29], lacking 30 amino acids from the C-terminus of the protein, was prepared from the
pET23bp53Δ30 plasmid as previously described for HNF-3α protein residing in inclusion bodies [30], or as described below for the soluble p53 protein fraction. Briefly, 500 ml cultures were grown at 37°C to an A600 of 0.4. Protein production was induced by addition of IPTG to a 1mM final concentration. Cultures were grown for an additional two hours, then collected, pelleted and resuspended in 20 ml 1X binding buffer (5 mM Imidazole, 0.5 M NaCl, 20 mM Tris-HCl, pH 7.9). Cells were sonicated on ice for 40 seconds. The crude protein extract was collected by centrifugation at 24,000 x g for 10 minutes at 4°C. Soluble p53 was purified from the crude fraction by affinity chromatography over a Ni2+-NTA agarose column (Qiagen). Bound protein was washed and eluted as described [30]. Purified p53 was dialyzed against dialysis buffer (20 mM Tris-HCl, pH 8.0, 0.5 mM EDTA, pH 8.0, 100 mM KCl, 20% glycerol, 1.0 mM PMSF, 0.5 mM DTT).

Recombinant histidine-tagged HBx was prepared from the pRSETc::X plasmid as previously described [31].

Cell extracts. HepG2 whole cell extracts were prepared as previously described, [32] [33]. HeLa cell nuclear extracts were prepared exactly as described in Current Protocols in Molecular Biology [34]. Xenopus egg extracts were prepared exactly as previously described [35, 36]. High speed supernatant (HSS) soluble fractions used for chromatin assembly had protein concentrations ranging from 50 µg/ul to 100 µg/ul.

In vitro transcription. In vitro transcription analysis of templates as chromatin-free (naked) and chromatin-assembled DNA was performed as previously described [33], with minor modifications. For naked DNA transcriptions, recombinant p53 and HBx proteins were added to 500 ng supercoiled DNA templates in transcription reaction mix
and allowed to bind for five minutes at room temperature prior to addition of transcribing extract. RNA products were purified and analyzed by primer extension.

Solid-phase DNA templates for chromatin transcriptions were prepared as described [33, 35]. Briefly, AFP DNA was digested with EcoRI and Clal, then biotinylated with Biotin-21 dUTP and Biotin-14 dATP and Klenow fragment DNA polymerase (Gibco) prior to coupling to streptavidin coated, paramagnetic beads (Dynal). In chromatin transcription reactions, p53 and HBx were added to 500 ng solid-phase DNA templates during a 20 minute pre-incubation in HepG2 or HeLa cellular extracts prior to chromatin assembly. After 1 hour chromatin assembly in fractionated Xenopus egg extract, solid-phase DNA templates were washed three times in modified nuclear dialysis buffer (mNDB) (20 mM Hepes, pH 7.9, 50 mM KCl, 0.2 mM EDTA, 10% glycerol, 1 mM DTT) plus 0.01% NP-40, then transcribed in HeLa extract and analyzed as above.

**p53 immunoprecipitation and western blot analysis.** Immunoprecipitations were performed in HepG2 whole cell extract or HeLa nuclear extract diluted to a total protein concentration of 8 µg/ul in mNDB. Recombinant p53 (approximately 600 ng) and HBx proteins (approximately 300 ng) were added to 25 ul of cellular extract and incubated at 4°C for 20 minutes. Anti-p53 antibody (Santa Cruz pAB 240) was added to the reaction mix and bound for 1 hour in the presence of 1% NP-40 with gentle rocking at 4°C. Immuno-complexes were collected with Protein A/Protein G+ agarose beads (Santa Cruz) equilibrated in mNDB plus 1% NP-40. Immuno-complexes were washed three times with IP wash buffer (100 mM Tris, 1 M NaCl, 0.3% SDS), then resuspended in sample buffer (0.06 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 5% β-
mercaptoethanol, 0.001% Bromophenol Blue). Additional wash buffers used included: AC wash buffer 1 (100 mM Tris pH 8.0, 1 M NaCl); AC wash buffer 2 (100 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% NP-40, 0.3% SDS); AC wash buffer 3 (10 mM Tris pH 8.0, 0.1% SDS); mNDB wash buffer (mNDB + 1% NP-40). Samples were analyzed by SDS-PAGE and Western blot. Western blots were performed as previously described [37], with minor modifications. Membranes were probed with the appropriate antibody (anti-p53 pAB 240, Santa Cruz, and anti-HBx 11/121/52, gift of Dr. Claus H. Schroder, Virus-Host Interactions, German Cancer Center [38]). Binding was visualized by ECL Western Blot Analysis System (Amersham).

**p53 electromobility shift assay (EMSA).** EMSA was performed using the double-stranded p53 regulatory element from the AFP distal promoter (bases –862 through -830) 5’GATCCTTAGCAAACATGTCTGGACCTCTAGAC as previously described [15], with protein-DNA binding carried out for 30 minutes at 30° C. Protein binding assays contained 7 µg of HepG2 or HeLa cell extract and approximately 1 µg purified p53 protein and 1 µg purified HBx protein, except as indicated.

**Solid-phase DNA-protein purification.** Solid-phase DNA oligomers were generated by annealing 5’ biotinylated p53 regulatory element (5’ Bio-GATCCTTAGCAAACATGTCTGGACCTCTAGAC) (Gibco) to complementary strand prior to coupling to streptavidin-coated paramagnetic beads (Dynal). Control reactions to assess protein binding specificity were performed in parallel with AFP site –1007 (5’Bio-GATCCAAATATCCTCTCTCAG) solid-phase DNA oligomers prepared in the same way. Approximately 200 ng p53 regulatory element or –1007 solid-phase oligos were washed in 1x PBS/1% BSA prior to incubation with 1 µg p53 in the presence or absence of HBx.
(1 μg) and/or 70 μg (total protein) HepG2 or HeLa cell extract. Binding reactions proceeded for 30 minutes at 22°C. DNA-bound protein complexes were collected by magnetic concentration and washed two times in 1X PBS/1% NP-40 and once in wash buffer (100 mM NaCl, 50 mM Bis Tris-HCl, 10 mM MgCl₂, 1 mM DTT, pH 6.0) prior to elution. DNA-associated proteins were eluted for 10 minutes at 37°C in urea elution buffer (5M urea, 10 mM Tris pH 8.0, 100 mM NaH₂PO₄, 1% β-mercaptoethanol). Eluted proteins were analyzed by gel electrophoresis and silver stain or western blot.

**Quantitation.** Image analysis was performed by use of ImageQuant 5.0 software (Molecular Dynamics).
Results

**HBx disrupts p53-mediated repression of AFP transcription.** In order to examine the regulatory consequences of HBx transactivator expression on a hepatic-expressed cellular gene, we performed *in vitro* transcription analysis of AFP DNA templates in the presence of HBx and p53 proteins. Transcription extracts isolated from the human hepatoma cell line HepG2, which actively expresses AFP but does not carry integrated HBV DNA, were used for *in vitro* transcription [33]. We utilized a constitutively activated p53 protein harboring a C-terminal truncation to examine the ability of p53 to regulate AFP transcription independently of post-translational modifications within the protein C-terminus, which activate p53 for DNA binding [13]. Addition of recombinant p53 protein resulted in 2-3 fold repression of AFP transcribed as naked (chromatin-free) DNA in HepG2 extract (Fig 1A, lanes 2 and 3 compared to lane 1). Addition of recombinant HBx protein to p53-repressed transcription reactions alleviated p53-mediated repression (Fig 1A, compare lanes 3 and 4). The level of AFP transcription detected upon addition of HBx was derepressed 4-fold to a level slightly higher than transcription in the absence of p53 (Fig 1A, compare lanes 1 and 4). Addition of HBx without p53 resulted in modest activation (less than 2-fold) of AFP transcription (Fig 1A, compare lanes 1 and 5), suggesting that the observed activation of AFP is dependent primarily upon HBx effects on p53, rather than HBx association with hepatoma-enriched transcriptional activators.

**Effects of p53-HBx interaction are tissue-specific.** AFP is expressed in the fetus by endoderm-derived cells of the yolk sac, liver and gut [39, 40]. We have previously shown by cell culture transfection studies that p53 repression of AFP is tissue-
specific [15]. To determine if the observed effects of HBx on p53-regulated AFP expression were also tissue-specific, we performed *in vitro* transcription analysis in cervical cancer-derived HeLa cell extract. In contrast to the observed repression of AFP transcription following addition of p53 to HepG2 reactions, p53 introduction to HeLa transcription reactions resulted in modest activation of AFP expression (less than 2-fold) (Fig 1B, compare lane 1 with lanes 2 and 3). HBx addition to transcription reactions in the presence of p53 slightly augmented this activation to a level of 3-fold over basal expression (Fig 1B, lanes 4 and 5). This is in sharp contrast to results observed in the hepatoma extract where HBx addition strongly reversed p53 effects on AFP transcription.

Addition of low concentrations of HBx protein, in the absence of p53, activated AFP expression approximately 2-fold (Fig 1B, compare lane 1 with lanes 6 and 7). This level of HBx-mediated activation, in the absence of p53, is comparable to that observed in hepatoma extracts, again supporting the hypothesis that HBx-mediated activation of AFP is due primarily to a modification of p53 regulation. Interestingly, increasing concentrations of HBx protein in the absence of p53 did not activate AFP transcription, but diminished transcription to basal levels (Fig 1B, compare lanes 1 and 8). This result could be due to apparent squelching by HBx through self-oligomerization or transcription factor binding. HBx has not previously been demonstrated to squelch transcription; however, because of its ability to associate with multiple transcriptional activators and general transcription factors (GTF’s) [reviewed in 4], addition of high concentrations of HBx in the absence of p53 may promote nonfunctional HBx-protein interactions.

To determine if derepression of p53-regulated AFP transcription was due to a direct interaction between HBx and p53 that occurs only in a hepatoma extract, we
performed immunoprecipitations with anti-p53 antibody (pAB 240, Santa Cruz) in both HepG2 and HeLa transcription extracts. p53 and HBx proteins incubated in HepG2 extract formed a complex, as shown by immunoprecipitation and Western blot analysis with anti-p53 (pAB 240) and anti-HBx (11/121/52) antibodies (Fig 1C, lane 3). Additionally, p53 and HBx interacted in both HeLa extract and extract buffer (Figure 1C, lanes 6 and 8), demonstrating that p53 and HBx proteins form a stable complex in the absence of DNA or additional proteins present in hepatoma cell extracts. Taken together, these data demonstrate that the tissue-specific effects of HBx on p53-regulated AFP transcription are not due to an inability of the proteins to form a stable complex in a non-hepatic cell extract, but rather are likely due to tissue-specificity of p53 transcriptional repression.

**HBx does not disrupt p53-mediated squelching of transcription.** The ability of p53 to repress *in vitro* transcription of AFP templates could be explained by a number of mechanisms. Multiple regions of p53 protein interact with and bind a wide range of proteins mediating, in part, the pleiotropic functions of the tumor suppressor [13, 14]. p53 can interact with GTF’s including TFIID subunits TBP, TAF 31 and TAF70, and TFIIH subunits p62, XPB and XPD. The ability of p53 to squelch transcription through interactions with GTFs, particularly TBP, is well documented [41-43]. If p53-mediated AFP repression was due in part to p53 squelching of TBP in the hepatoma extract, the apparent derepression upon HBx addition could be due to HBx disruption of p53-TBP or p53-GTF interactions.

To determine if HBx could reverse p53-mediated transcriptional squelching, *in vitro* transcription analysis was performed using chick β-globin DNA as template. β-
globin DNA has no p53 binding site and is not directly regulated by p53 (data not shown). In the presence of high levels of p53 protein, apparent transcriptional repression can be attributed, most likely, to p53-mediated squelching of basal transcription factors. As demonstrated in Figure 2, addition of increasing amounts of p53 to β-globin in vitro transcription reactions resulted in greater than 5-fold squelching of transcription (compare lane 1 with lanes 2 through 4). Addition of HBx resulted in very little (1.5-fold) reactivation of squelched transcription (Fig 2, compare lanes 5 and 6 with lane 4). Addition of HBx in the absence of p53 resulted in approximately 2-fold repression of transcription, potentially due to HBx-mediated squelching, as discussed above (Fig 2, compare lanes 1 and 7). Because HBx reactivation of p53-squelched β-globin transcription was much lower than the reactivation of p53-repressed AFP in vitro transcription, we suspected that HBx alleviation of p53-mediated AFP repression was not due simply to reversal of p53 squelching. This inability of HBx to activate transcription from a promoter lacking a p53-binding site suggested that HBx must be targeted to DNA by promoter-bound p53 in order to render its activating effects.

**HBx disrupts p53 regulation of AFP chromatin transcription.** In order to demonstrate conclusively that HBx-mediated derepression of AFP expression in our in vitro transcription system was not an effect on squelching, we utilized in vitro chromatin assembly of AFP DNA templates prior to transcription analysis. Solid-phase AFP DNA templates were prepared by coupling biotinylated AFP DNA to paramagnetic beads as previously described [44]. Chromatin assembly was achieved by incubating the solid-phase DNA templates in fractionated *Xenopus* egg extracts [33]. In this system DNA templates are pre-incubated with cell extracts to allow activating and/or repressive
proteins to bind DNA prior to chromatin assembly. An iso-osmotic wash is performed following assembly and prior to transcription, to remove factors that do not directly or indirectly bind the DNA templates. Washing away excess cellular factors prior to in vitro transcription prevents effects mediated by non-specific squelching.

Chromatin assembly mediates general repression of basal AFP transcription in the absence of activating proteins present in hepatoma cell extracts (Fig 3, compare lanes 1 and 2) [33]. Upon addition of HepG2 whole cell extract, transcription of chromatin assembled AFP templates was activated 50-fold (compare lanes 2 and 3). Titration of p53 into the reaction resulted in greater than 7-fold repression of AFP transcription (lanes 4 and 5), demonstrating that p53 repression of AFP transcription is indeed a specific event, and not simply an effect of p53 squelching GTF’s necessary for functional transcription complexes. Addition of HBx during pre-incubation yielded greater than 6-fold derepression of p53-regulated AFP transcription, almost to levels achieved in hepatoma extract with no added p53 (compare lanes 3 and 6). The ability of HBx to alleviate p53 repression of AFP chromatin transcription, as well as a relative inability to reverse p53-mediated squelching of β-globin transcription, further supports a likely requirement for p53 DNA binding in order for HBx to render its effects.

**p53 DNA binding is required for HBx re-activation of AFP transcription.** In order to determine if HBx-mediated derepression of AFP transcription is dependent upon p53 DNA binding, we performed in vitro transcription analysis with an AFP DNA template containing a mutation within the p53 binding site located at –853 (DelA), relative to the transcriptional start site (Fig 4A). The DelA template contains a 10 base-pair deletion of the 5’ p53-binding half site, as well as sequence mutations in the 3’ half
site, that prevent binding of p53 to its consensus site within the AFP promoter (unpublished data, K. Lee). AFP DelA transcription as naked DNA in both HepG2 and HeLa cell extracts (Fig 4B) and as chromatin assembled (Fig 4C) DNA was unaffected by addition of p53: less than 1.2-fold repression in both naked DNA and chromatin assembled DNA in vitro transcriptions (Fig 4B, compare lanes 1 with lanes 2-4 and lane 7 with lanes 8-10; Fig 4C, compare lane 3 with lanes 4-6). Addition of HBx to p53-containing reactions in either hepatoma or HeLa cell extracts maintained a basal level of transcription (Fig 4B, compare lane 4 with lanes 5 and 6; lane 10 with 11-13; Fig 4C, compare lanes 6 and 7). Addition of HBx in the absence of p53 had negligible effects on DelA naked (Fig 4B, lane 14) and chromatin transcriptions (less than 1.2-fold repression) (Fig 4C, compare lanes 3 and 8). These data further demonstrate that HBx derepression of p53-silenced AFP in a hepatoma background requires p53 binding to its DNA regulatory element.

**Tissue specificity of HBx-mediated disruption of p53 function is maintained with chromatin assembly.** To further examine the apparent tissue-specific effects of the p53-HBx interaction on regulation of AFP transcription, we chromatin assembled AFP DNA templates in the presence of HeLa cell nuclear extract in the presence or absence of increasing amounts of p53 or p53 plus HBx prior to in vitro transcription.

We have shown previously that chromatin assembly confers tissue-specificity on AFP transcription in vitro [33]. Though capable of robust transcription of chromatin-free AFP DNA templates (Figs 1 and 4 and [33]), HeLa extract cannot establish efficient chromatin transcription. We have found that tissue-specific, activated AFP chromatin transcription is highly dependent upon function of a liver-enriched transcriptional
activator, HNF3α [33]. Pre-incubation of AFP DNA templates in HeLa extract prior to chromatin assembly does not program the gene for establishment of activated, open chromatin, and transcription is silenced (Fig 5, compare lanes 2 and 3). Addition of p53 to HeLa extract during pre-incubation resulted in up to 7-fold activation of AFP transcription (Fig 5, compare lanes 4-6 with lane 3). As was observed by transcription of chromatin-free AFP DNA templates, addition of HBx in the presence of p53 slightly augmented this expression in HeLa extract (1.2-fold, compare lanes 6 and 7). Interestingly, addition of HBx to HeLa pre-incubation in the absence of p53 resulted in 11-fold activation over HeLa alone (compare lanes 3 and 8), suggesting that, in the absence of p53, HBx may associate with a non-hepatic derived DNA binding protein in order to activate AFP expression by a chromatin-dependent mechanism. This possibility is currently under investigation in our laboratory.

Thus far, our data demonstrated that HBx interference with p53-mediated regulation of transcription was both hepatoma-specific and required targeting by DNA-bound p53. Therefore, we hypothesized that HBx might be interacting with DNA-bound p53 to potentially dislodge putative tissue specific co-repressors of transcription. An association with DNA-bound p53 in a non-hepatic derived cell extract, where p53 acts in the absence of putative co-repressors as an activator of AFP transcription, would then allow augmentation of activation.

**HBx associates with DNA-bound p53.** In order to assess the effect of HBx on p53 DNA binding and to determine if HBx can associate with DNA-bound p53, we performed a series of electromobility shift assays (EMSA) with both purified proteins and cellular extracts. In examining the ability of purified p53 to bind to its AFP regulatory
element in the presence of HBx, we found that addition of HBx resulted in both a super shift of bound probe (Fig 6A, lanes 3-8), signaling formation of a potential p53-HBx complex, in addition to an enhancement of p53 association with the AFP regulatory element (lanes 7 and 8). HBx did not associate with the p53 regulatory element in the absence of p53 (lane 9).

To assess the possibility of a tissue-specific effect by HBx on p53 DNA binding in hepatic and non-hepatic derived cell extracts, we performed EMSA with the p53 regulatory element from the AFP distal promoter with both HepG2 and HeLa transcription extracts in the presence and absence of p53 and HBx (Fig 6B). Addition of both HepG2 and HeLa transcription extracts to binding reactions with the p53 regulatory element resulted in the appearance of a single predominant band, labeled complex 1 (lanes 2 and 11). Addition of p53 to HepG2 and HeLa binding reactions resulted in a mobility shift similar to that of p53 alone (lane 7 and compare lanes 2 with 3 and 11 with 12). Addition of HBx in the presence of p53 and extract had little to no effect on either of the shifted bands, but did appear to result in a super shift of probe into the well (SS2) (compare lanes 3 with 4 and 12 with 13), as was observed with the purified recombinant proteins (Fig 6A and 6B lane 8). These results suggest complex formation between HBx and DNA-bound p53. Addition of p53 antibody (FL 393, Santa Cruz) in the presence of p53 and either the HepG2 or HeLa transcription extracts resulted in slight decrease of the p53 band concomitant with an increase in probe shifted into the well (compare lanes 3 with 5 and 12 with 14). Addition of p53 antibody to purified p53 protein resulted in appearance of a distinct super shift, SS1 (lane 9). Incubation of HepG2 and HeLa extracts with a regulatory element probe harboring a 10 basepair deletion and 4 point
mutations within the p53 binding site (Del A) resulted in a shift of complex 1, but did not display a p53-specific shift (data not shown).

Because it appeared that HBx-p53 interaction did not modify p53’s ability to bind DNA in either the hepatic or non-hepatic cell extract, we wanted to determine if tissue-specific co-factors binding with p53 to its regulatory site could be affected by HBx addition. To this end, we performed solid-phase DNA-protein pull downs. Incubation of biotinylated, p53 regulatory element oligomers coupled to streptavidin paramagnetic beads (solid-phase DNA) with purified p53 protein in the absence (Fig 6C, lane 1) or presence of HBx (lanes 2 and 3) resulted in p53 association with the DNA template, as evidenced by silver stain (Fig 6C) and western blot (data not shown). Additionally, HBx co-purified with DNA bound p53 (lanes 2 and 3), confirming that HBx is capable of associating with DNA-bound p53. p53 DNA binding was also detected upon incubation of the solid-phase p53 binding element with HepG2 or HeLa extract in the absence (lanes 4 and 6) or presence (lanes 5 and 7) of HBx. Also, as with the purified proteins, HBx was found to co-purify with DNA bound p53 in both the HepG2 (lane 5) and HeLa (lane 7) cell extracts. Two hepatoma-specific differences were revealed in these analyses. One is that p53 incubated in HepG2 appeared to be post-translationally modified, generating a slower migrating band in lanes 4 and 5. The second difference is two proteins co-purifying with p53 from the HepG2 extract were greatly reduced upon addition of HBx (indicated by asterisks, compare lanes 4 and 5). This loss of protein binding was not observed in the HeLa extract (compare lanes 6 and 7). These protein bands may represent liver-specific p53 co-repressors, and their lack of association with DNA-bound p53 in the presence of HBx could contribute to the tissue-specific functional effects of p53-HBx
interaction. These tissue-specific co-repressors are currently under investigation in our laboratory.

To serve as control, p53, HBx and extract were incubated with a non-specific oligo (-1007) lacking any defined p53 binding element. p53 and HBx association with the –1007 oligo could not be detected by silver stain (Fig 6C, lanes 8 and 9), demonstrating that p53 and HBx association with the p53 regulatory binding element is specific, and dependent upon p53 DNA binding.
Discussion

Regulation of AFP gene expression is a complex process mediated, in part, by a number of transcriptional activators and repressors including HNF-1, retinoic acid receptor, C/EBP and other factors binding to sites within the AFP gene [45-48]. Tissue and developmental specific regulation of AFP expression is controlled in part by a developmental repressor region located between –1000 and –200 in the AFP promoter [49-53]. We have previously characterized an overlapping p53/HNF-3 binding site within the developmental repressor region that mediates opposing regulatory signals. HNF-3 is a potent activator of AFP transcription, while p53 acts to repress AFP transcription [15, 33].

Aberrant activation of AFP is a hallmark of hepatocellular carcinoma, and exemplifies a loss in regulated gene expression that is common to numerous cancers. The exact mechanism by which AFP is reactivated in the diseased liver is unknown, but likely is due to transcriptional activator and/or repressor dysfunction. One such transcription factor, p53, which is mutated or modified in over 60% of human cancers [13, 54], is a target of the Hepatitis B Virus-encoded X protein. Using cell-free transcription systems, we show that HBx destroys p53-mediated regulation of AFP expression allowing transcriptional derepression of the tumor marker gene. Evidence of HBx transactivation of viral and cellular genes as a result of its binding to cellular transcriptional activators has been well documented [reviewed in 4]. However, to our knowledge, this is the first account of HBx binding specifically to a transcriptional repressor to derepress a silenced, cellular gene.
p53 association with viral proteins is widespread: p53 forms complexes with the E6 protein from human papillomavirus, the large T antigen of SV40, the BZLF1 protein of Epstein-Barr virus, the E1b protein of adenovirus, and co-localizes with the ICP8 protein of Herpes Simplex Virus-1 \[55-59\]. In most cases, binding of p53 to viral proteins or DNA has a repressive effect on viral replication. As a result, viral protein-mediated antagonism of p53 function has evolved to overcome the general repressive effects of p53. For example, HIV-1 Tat protein disrupts p53-mediated repression of HIV-1 LTR promoter activity and HIV replication \[60\]; human papillomavirus E6 protein interaction targets p53 for degradation \[61\]; and, as demonstrated here and elsewhere, HBx association with p53 can inhibit p53 function and/or lead to cytoplasmic sequestration of the p53 protein [reviewed in 3, 4].

HBx binding to p53 likely evolved as a consequence of p53-mediated disruption of HBV replication in hepatic cells \[62-64\]. In conjunction with a complex of liver specific co-factors, p53 can bind and repress activation from HBV Enhancer II, the enhancer responsible for the tissue-specific replication of the virus. The p53-containing complex acts on HBV Enhancer II to block transcription from promoters under its control, resulting in loss of active HBV replication. HBx expression overcomes this hindrance by HBx binding to p53, decreasing the negative effects of the p53-containing complex on Enhancer II \[65\]. The exact mechanism by which HBx overcomes p53-mediated repression of HBV replication has not been established, but could be due to HBx disruption of p53 association with liver-specific cofactors, as we believe to be the case with derepression of AFP transcription.
An understanding of how HBV modifies the expression pattern of AFP is significant in that changes in AFP expression can be detected before onset of HCC in HBV-infected individuals. A study examining AFP expression in hepatocytes and oval cells in HBV-infected patients demonstrated high levels of AFP reactivation prior to the onset of HCC [66, 67]. Recent evidence also indicates that AFP secretion stimulates human hepatoma tumor cell growth and proliferation [68]. Because the majority of cells losing the ability to silence AFP have a disease phenotype, it is likely that the mechanism by which AFP is re-activated is one way that HBV contributes to the development and progression of HCC. Our demonstration that HBx can derepress AFP through its interaction with p53 lends additional support to the hypothesis that HBx is the primary factor by which HBV contributes to the development of HCC, and that its interaction with p53 is an integral step in the process.

In conclusion, we have shown that HBx overcomes p53-mediated repression of a liver-specific, tumor marker gene by disrupting DNA-bound p53 interaction with potential liver-specific co-repressors. Modification of p53 interaction or communication with protein partners by HBx may be a global mechanism affecting the ability of p53 to regulate multiple genes, potentially contributing to development of HCC in infected individuals. Future studies will include examination of p53 protein partners and additional liver-specific transcriptional repressors and activators of AFP that may contribute to development of HBV-associated HCC.
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Figure legends

Figure 1: HBx and p53 interaction results in loss of p53-mediated repression of AFP transcription. (A) p53 mediated AFP transcriptional repression is lost upon addition of HBx. AFP templates were in vitro transcribed in HepG2 whole cell extract (25 ul, approximately 100 ug total protein) in the presence of increasing amounts of p53 (600 ng, lane 2; 1.4 µg, lanes 3 and 4) and/or HBx (450 ng, lanes 4 and 5). Transcripts were detected by primer extension. Radiolabeled φX 174 DNA digested with Hae III (Gibco/BRL) was used as a molecular weight marker (MW). The 84 base pair AFP primer extension product is indicated by an arrow. (B) Effects of p53 and HBx on AFP transcription are tissue-specific. AFP templates were in vitro transcribed in HeLa nuclear extract (15 ul) in the presence of increasing amounts of p53 (700 ng, lane 2; 1.8 µg, lanes 3, 4 and 5) and/or HBx (250 ng, lanes 4 and 6; 1 µg, lanes 5 and 8; and 625 ng, lane 7). Transcripts were detected by primer extension. Radiolabeled φX 174 DNA digested with Hae III (Gibco/BRL) was used as a molecular weight marker (MW). AFP primer extension products are indicated. (C) p53 and HBx interact in HepG2 and HeLa transcription extracts. Recombinant p53 ((600 ng), lanes 2-8) and HBx ((300 ng), lanes 3, 4, 6 and 8) were added to 25 ul of HepG2 whole cell extract (approximately 200 µg total protein, lanes 1-4), buffer (lanes 5 and 6) or HeLa extract (approximately 200 µg total protein, lanes 7 and 8). p53 complexes were immunoprecipitated with anti-p53 (Santa Cruz pAB 240), subjected to SDS-PAGE, and immuno-blotted with anti-p53 and anti-HBx, as indicated.
Figure 2: p53-mediated squelching of β-globin transcription is not alleviated by HBx. β-globin DNA templates were *in vitro* transcribed in 15 ul of HepG2 whole cell extract (approx. 100 µg total protein) with increasing amounts of p53 protein (450 ng, lane 2; 1.1 µg, lane 3; 2.25 µg, lanes 4-6) in the absence or presence of HBx (500 ng, lane 5; 1.0 µg, lanes 6 and 7). Transcripts were detected by primer extension. β-globin primer extension products are indicated.

Figure 3: HBx alleviates p53 mediated repression of chromatin assembled AFP DNA. Immobilized AFP templates were incubated with nuclear extract buffer (lanes 1 and 2) or HepG2 whole cell extract (20 ul: approx. 200 µg total protein; lanes 3-6) prior to 1h. chromatin assembly in fractionated *Xenopus* egg extract. Reactions were supplemented with recombinant p53 protein (370 ng, lane 4; 1.8 µg, lane 5) or recombinant p53 (1.8 µg) plus recombinant HBx (470 ng, lane 6). Chromatin-assembled templates were washed in nuclear extract buffer and *in vitro* transcribed in HeLa nuclear extract. AFP primer extension products are indicated.

Figure 4: p53 repression and HBx re-activation of AFP transcription are dependent upon p53-DNA binding. (A) AFP-DelA template contains a 10 basepair deletion within the p53 binding site. AFP and DelA templates are diagramed to show the modifications existing within the DelA template. DelA contains a 10 base pair deletion within the consensus p53 binding site, and four single base point mutations within the p53 half site, as indicated. (B) Effects of p53-HBx interaction on AFP transcription require p53-DNA binding. DelA DNA templates were *in vitro* transcribed in HepG2 extract.
whole cell extract (approximately 100 µg total protein, lanes 1-6) or HeLa nuclear extract (approximately 100 µg total protein, lanes 7-14) in the presence of increasing amounts of p53 (560 ng, lanes 2 and 8; 1.1 µg, lanes 3, 6 and 9; 2.25 µg, lanes 4, 5 and 10-13) with and without recombinant HBx (400 ng, lanes 5, 6 and 11; 600 ng, lane 12; 1 µg, lanes 13 and 14). DelA primer extension products are indicated. **(C) p53-HBx binding has no effect on chromatin transcription of AFP-DelA.** Immobilized DelA templates were incubated with extract buffer (lane 2) or HepG2 whole cell extract (approximately 200 µg total protein) in the presence of increasing amounts of p53 (560 ng, lane 4; 1.1 µg, lane 5; 2.25 µg, lanes 6 and 7) and/or recombinant HBx (400 ng, lanes 7 and 8) prior to 1 hour chromatin assembly in fractionated *Xenopus* egg extract. Chromatin-assembled templates were washed in nuclear extract buffer and *in vitro* transcribed in HeLa nuclear extract. DelA primer extension products are indicated.

**Figure 5: Tissue specificity of p53-HBx effect on AFP transcription is maintained in chromatin.** Immobilized AFP DNA templates were incubated in nuclear extract buffer (lane 2) or HeLa nuclear extract (100 µg total protein, lanes 3-8) in the absence (lane 3) or presence of p53 (370 ng, lane 4; 900 ng, lane 5; 1.8 µg, lanes 6 and 7) and HBx (approx. 1 µg, lanes 7 and 8) prior to 1 hour chromatin assembly in fractionated *Xenopus* egg extract. Chromatin-assembled templates were washed in nuclear extract buffer and *in vitro* transcribed in HeLa nuclear extract. AFP primer extension products are indicated.

**Figure 6: HBx associates with DNA-bound p53. (A) p53-DNA binding is maintained upon HBx association.** p53 (approx. 500 ng) was incubated with radio-labeled double
stranded p53 regulatory element probe in the absence (lane 2) or presence of increasing amounts of HBx (approx. 200 ng, lane 3; 400 ng, lane 4; 600 ng, lane 5; 1 µg, lane 6; 1.6 µg, lane 7 and 2 µg, lane 8). Lane 9 contains HBx (approx. 2 µg) and labeled p53 regulatory element probe. Reactions were incubated for 30 minutes at 30°C. Shifted DNA-protein complexes are indicated. (B) HBx associates with DNA-bound p53 in both HepG2 and HeLa cell extracts. Labeled p53 regulatory element probe was incubated with purified p53 protein (approx. 1 µg, lanes 7-9) or p53 (approx. 1 µg) plus HepG2 (approx. 7 µg, lanes 2-6) or HeLa (approx. 7 µg, lanes 11-15) extract in the absence or presence of HBx (approx 1 µg, lanes 4, 8, 13 and 15). p53 antibody (FL 393, Santa Cruz) was included in extract or purified protein binding reactions as indicated (lanes 6, 9, 14). Binding reactions were incubated for 30 minutes at 30°C. Shifted complexes are indicated. (C) HBx co-purifies with DNA-bound p53. Immobilized p53 regulatory element or –1007 DNA templates were incubated with p53 (approx 1.5 µg, lanes 1-9) in the absence or presence of HBx (approx 1 µg, lanes 2, 5, 7-9; 2 µg, lanes 2) and/or cell extract (HepG2 extract, approx. 70 µg, lanes 4, 5 and 8; HeLa extract, approx. 70 µg, lanes 6, 7 and 9). Binding reactions were allowed to proceed for 30 minutes at 22°C. Complexes were eluted and analyzed by SDS-PAGE and silver stain. p53, HBx and putative co-factors are indicated (asterisk denotes putative p53 co-repressor).
Fig 1

A

|       | MW | 1  | 2  | 3  | 4  | 5  |
|-------|----|----|----|----|----|----|
| p53   | -  | +  | ++ | ++ | -  |    |
| HBX   | -  | -  | -  | +  | +  |    |

supercoiled DNA

pre-inc. 5 min

transcription

transcription extract

p53 ± HBx or buffer

1 hour

Primer extension of RNA products

AFP
### Fig 1

**B**

|   | p53  |   |   |   |   |   |   |           |
|---|------|---|---|---|---|---|---|------------|
|   | -    | + | ++| ++| ++| -  | -  | -          |
| HBX| -    | - | - | + | +++| +  | ++| +++        |

|   |   |   |   |   |   |   |   |   |   |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | MW         |

![Image of gel with bands labeled AFP]
Fig 1

C

|       | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  |
|-------|----|----|----|----|----|----|----|----|
| Hela  | -  | -  | -  | -  | -  | -  | +  | +  |
| HepG2 | +  | +  | +  | +  | -  | -  | -  | -  |
| p53   | -  | +  | +  | +  | +  | +  | +  | +  |
| HBX   | -  | -  | +  | +  | -  | +  | -  | +  |
| anti-p53 | +  | +  | +  | -  | +  | +  | +  | +  |
| anti-p53 probe |   |   |   |   |   |   |   |   |
| anti-HBX probe |   |   |   |   |   |   |   |   |
|    | p53 | -   | +   | ++  | +++ | +++ | +++ | -   |
|----|-----|-----|-----|-----|-----|-----|-----|-----|
| HBX|     | -   | -   | -   | +   | ++  | +++ | +   |

Fig 2

β-globin
|     | p53 | -   | -   | +   | ++  | ++  |
|-----|-----|-----|-----|-----|-----|-----|
| HBX | -   | -   | -   | -   | -   | +   |
| HepG2 | -   | -   | +   | +   | +   | +   |
| chromatin | -   | +   | +   | +   | +   | +   |

**Fig 3**

- **AFP**
- **extract or buffer ± p53 ± HBx**
- **wash**
- **bead-DNA**
- **pre-inc.**
- **chromatin assembly**
- **20 min**
- **1 hr HSS**
- **transcription**
A

Enhancer I  Developmental Repressor Region  lacZ

-2.5 kb   -1.0 kb   -.2 kb

-860 GCCTTAGCAAAACATGTCTGGACCTCTAGACA CGGAATCGTTTGTTACAGACCTGGAGATCTGT  -830 AFP

-830 p53 p53

-860 p53 p53

GCCTTAGCAAAACATGTCTGGACCTCTAGACA CGGAATCGTTT

GCCTTAGCAAAACATGTCTGGACCTCTAGACA CGGAATTTCTGT DelA
### Fig 4

#### Table B

|         | HepG2       | HBx | p53 | Hela       |
|---------|-------------|-----|-----|------------|
| 1       | -           | -   | -   | -          |
| 2       | +           | -   | +   | +          |
| 3       | ++          | -   | +++ | ++         |
| 4       | +++         | -   | +++ | +++        |
| 5       | +++         | -   | +++ | +++        |
| 6       | +++         | -   | +++ | +++        |
| 7       | -           | -   | +   | ++         |
| 8       | -           | -   | +++ | +++        |
| 9       | -           | -   | +++ | +++        |
| 10      | -           | -   | +++ | +++        |
| 11      | -           | -   | +++ | +++        |
| 12      | -           | -   | +++ | +++        |
| 13      | -           | -   | +++ | +++        |
| 14      | -           | -   | +++ | +++        |

**B**

![Image of gel electrophoresis with bands labeled DelA](image)

*Note: The gel electrophoresis image shows bands labeled DelA, with lanes 1-6 representing HepG2 and lanes 7-14 representing Hela. The intensities of the bands are indicated by the symbols (+, ++, +++).*
Fig 4

C

|       | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | MW |
|-------|---|---|---|---|---|---|---|---|----|
| p53   | - | - | - | + | ++| +++| +++| + | -  |
| HBX   | - | - | - | - | - | - | + | + | +  |
| HepG2 | - | - | + | + | ++| ++| ++| + | +  |
| chromatin | - | + | + | + | + | + | + | + | +  |

extract or buffer ± p53 ± HBx

pre-inc.

bead-DNA

1 hr HSS

transcription

wash

DelA →
Fig 5

|        | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|--------|---|---|---|---|---|---|---|---|
| p53    | - | - | - | + | ++ | +++ | +++ | - |
| HBX    | - | - | - | - | -  | -  | +  | +  |
| Hela   | - | - | + | + | +  | +  | +  | +  |
| chromatin | - | + | + | + | +  | +  | +  | +  |

extract or buffer ± p53 ± HBx

bead-DNA

pre-inc. 20 min chromatin assembly 1 hr HSS

wast

transcription
Fig 6

A

No protein

HBx

1 2 3 4 5 6 7 8

p53

9

SS

p53

Free probe
Hepatitis B viral transactivator HBx alleviates p53-mediated repression of alpha-fetoprotein gene expression

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