



HBV transcription repression in response to genotoxic stress is p53-dependent and abrogated by pX

Gilad Doitsh¹ and Yosef Shaul^{*.1}

¹Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel

Transcription of hepatitis B Virus (HBV), an important risk factor of hepatocellular carcinoma (HCC), is controlled by cellular transcription activators including some of the cellular signaling targets. Consequently, HBV transcription rate changes in response to the cellular physiological conditions. In this report we investigated HBV gene expression and the role of physiological levels of the viral X protein (pX) under cisplatin induced genotoxic stress. We show that under these conditions the RNA level of an HBV mutant which does not express pX is sharply reduced. Studies revealed that transcription repression is responsible for the observed reduction in HBV RNA level. Repression of HBV transcription was obtained only in the p53 proficient cells. Furthermore, HBV transcription rate is recovered by the cotransfected p53 dominant negative plasmid, indicating that p53 is directly responsible for HBV transcription repression. Unexpectedly, p73, the recent p53 homologue, does not repress but rather activates HBV transcription. Interestingly, pX produced either by the HBV genome or by a cotransfected plasmid, relieves the p53 mediated repression. Collectively, these results attribute a physiological role to p53-inactivation by pX, and explain how pX may support HCC development.

Keywords: HBV transcription; DNA-damage; cisplatin; HCC; pX and p53; HBV RNA half life

Introduction

HBV is the prototype of the hepadnaviruses. HBV is a primarily hepatotropic, enveloped DNA virus bearing a tiny genome that replicates via reverse-transcription. HBV transcription is largely regulated at the level of initiation. RNA processing seems to play a minor role. The viral genome contains enhancer elements that regulate viral gene expression in liver cells (Shaul *et al.*, 1985; Jameel and Siddiqui 1986; Honigwachs *et al.*, 1989; Yee 1989). Upon infection or DNA transfection, four major viral transcripts are detected. The largest 3.5 Kb RNA is composed of two preCore (pcRNA) and preGenomic (pgRNA) mRNAs, that program the synthesis of HBeAg and HBcAg, respectively. pgRNA also serves as a template for reverse-transcription to synthesize the viral DNA genome. Two other transcripts, 2.3 to 2.1 Kb mRNAs direct the production of the S, PreS1, PreS2 surface antigens. The

smallest known transcript is the 0.7 Kb X mRNA, believed to be responsible for X protein production.

Due to the lack of HBV infection system, the study of HBV transcription regulation is in large incomplete. A partial solution to this technical problem came from the study of HBV transgenic mice, active in HBV transcription and replication (Guidotti *et al.*, 1995). These animals express the same set of transcripts detected in infected individuals. However, this experimental approach can not answer the questions of 'if' and 'how' the different HBV transcripts are differentially regulated along the virus life cycle. Furthermore, as in HBV transgenic animals, when the active viral DNA template is integrated into the mouse genome, the pattern of gene expression only partially resembles the genuine viral infection, where the DNA template is mainly episomal. In this study we employed the transient transfection technique to follow HBV gene expression. This experimental approach, although not mimicking natural infection processes, does give rise to the production of the expected viral transcripts from an episomal DNA template.

Transcription of the HBV genes is regulated by multiple cellular transcription factors and activators. Some of these factors are known to be activated by the cellular signaling and therefore, are likely to support HBV transcription under unstable cellular external milieu (Faktor *et al.*, 1990; Ohno *et al.*, 1997; Raney *et al.*, 1997). In addition pX, the viral regulator protein, plays a role in stimulating some of the signaling pathways (Luber *et al.*, 1993; Natoli *et al.*, 1994; Doria *et al.*, 1995; Benn *et al.*, 1996; Su and Schneider 1996; Klein and Schneider 1997). HBV gene expression is also highly affected by cytokines (Guidotti *et al.*, 1996) possibly to escape the host immune surveillance. Thus, one promising way to investigate the mechanisms of HBV gene expression and the role of pX in HBV life cycle is to expose the host-cells to different stimuli and to monitor the production of the HBV transcripts. In this report we investigated HBV behavior under stress condition induced by a genotoxic drug.

Exposing mammalian cells to DNA-damaging agents often causes blocks in cell growth and sometime leads to cell death. The mammalian cellular response to genotoxic stress is a complex process involving many known and probably many as yet unknown genes. Cisplatin is a genotoxic agent that is widely used in the treatment of a variety of human tumors. Cisplatin treatment of cells leads to DNA damage by the formation of DNA interstrand cross-links (Roberts and Friedlos, 1981). Genotoxic treatment, including cisplatin, induces p53 over production (Fritsche *et al.*, 1993). Previously, we have reported that p53 binds, in a sequence specific manner, to the 5' portion of the HBV enhancer and represses its activity

*Correspondence: Y Shaul
Received 2 June 1999; revised 7 September 1999; accepted 7 September 1999

(Ori *et al.*, 1998). This fact and the reported interplay between p53 and pX, suggested an HBV specific response to genotoxic stress. Here we show that HBV transcription is repressed in cisplatin treated cells in a p53-dependent manner. Furthermore, we found that the viral encoded transcription coactivator, pX, can abrogate this p53-mediated repression. These findings attribute possible roles to pX in HBV life cycle and in HCC development.

Results

Repression of HBV gene expression upon genotoxic stress

To test the behavior of HBV under genotoxic stress, cells were transfected with HBV-DNA and treated with cisplatin, a DNA damaging agent. For these experiments HepG2 cells were used as they are known to contain wt p53 (Bressac *et al.*, 1990; Hosono *et al.*, 1991) and to support both HBV gene expression and

replication (Sells *et al.*, 1987; Sureau *et al.*, 1986). The expected HBV different transcripts are easily detected in cells transfected with a plasmid containing a head to tail dimer of HBV DNA (Figure 1a). To investigate the role of pX under these conditions we constructed the X-KO HBV mutant. This HBV mutant contains a stop codon at position 27 of the X ORF (Ori *et al.*, 1998), and does not produce pX (confirmed by Western blotting, data not shown). Cells were transfected with either wt or mutant HBV-DNA and were cisplatin treated in a time course manner, and RNA and protein fractions were extracted. RNA samples were analysed by Northern blotting using an HBV DNA probe. Interestingly, a significant reduction in the steady state level of the HBV mutant transcripts was observed in the cisplatin treated cells (Figure 1b, lanes 4–6). This reduction is specific and was not observed with the control GAPDH RNA. To determine the p53 level we analysed the protein extracts of the same transfected cells. As expected Western analysis revealed that upon cisplatin treatment, p53 was specifically accumulated, but not the β -tubulin control (Figure 1b, lower panels).

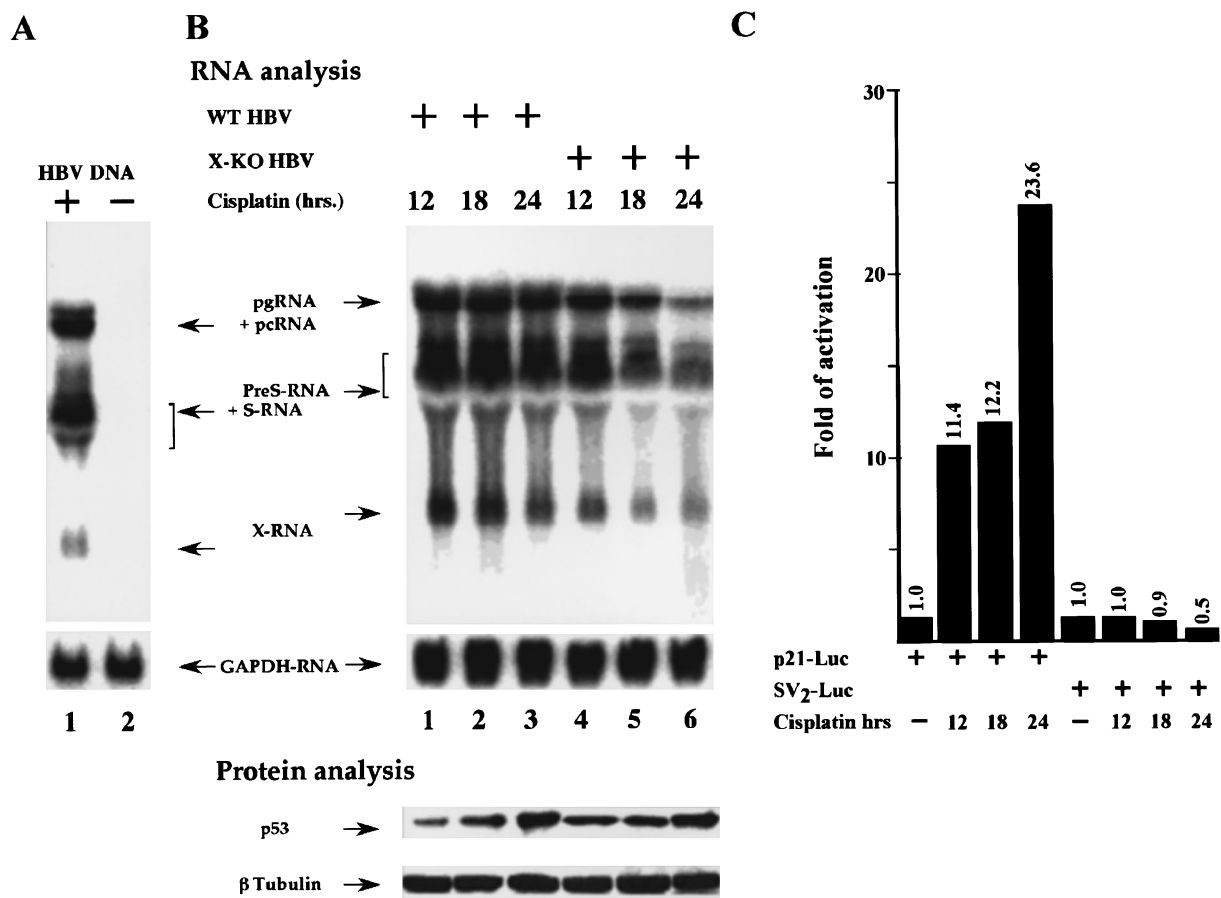


Figure 1 The level of X-KO HBV transcripts is reduced in cisplatin treated cells. (a) Detection of HBV transcripts in HepG2 cells that were transfected with a plasmid that contains two tandem HBV DNA (lane 1) but not a control plasmid (lane 2). (b) Transfected cells either with wild type HBV DNA or a mutant with a stop codon at position 27 of the X gene open reading frame (X-KO HBV), were treated with 2.5 μ g/ml cisplatin for the indicated time points before harvesting. RNA and proteins were extracted, separated on formaldehyde-agarose gel and SDS-PAGE, respectively and analysed. 32 P HBV DNA probe was used to detect the known viral transcripts and GAPDH probe to quantify RNA in each lane. For Western analysis anti human p53 (1801+DO-1) and anti β -tubulin (clone no. TUB2.1 Sigma) antibodies were used. The known viral transcripts are indicated by arrows. (c) Cisplatin activates the p21 but not SV40 early promoter in HepG2 cells. Cells were transfected with 2 μ g of either p21-luciferase or SV40-luciferase (Promega) reporter plasmids and after 24 h were treated with cisplatin for the indicated time points. Cell extracts were analysed for luciferase activity. With each of the reporter plasmids the activity in the untreated cells was taken as 100% for calculation of fold of activation

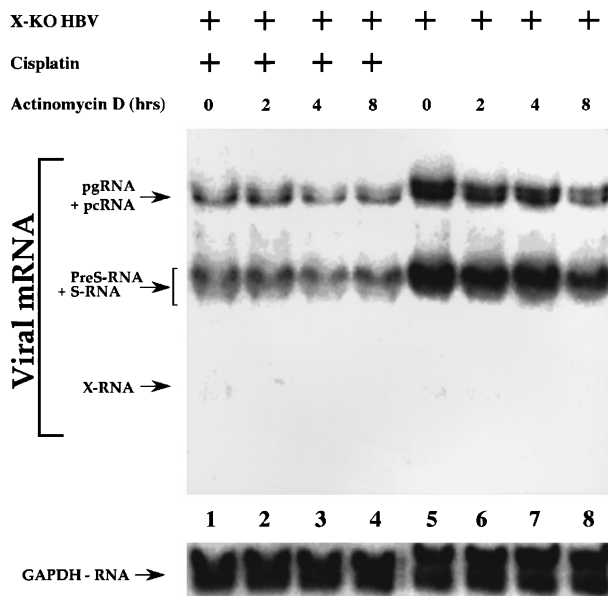
RNA analysis


Figure 2 Determination of the HBV RNA half life. HepG2 cells were transfected and cisplatin treated for 18 h as in Figure 1, but before harvesting Actinomycin D (5 μ g/ml culture media) was added for the indicated time. Cells were harvested and RNA was extracted at the indicated time points after actinomycin D treatment. The treatment protocol was as such to harvest all the cells at the same time point

The fact that p53 was also accumulated in the wt HBV transfected cells, that show only a mild reduction in the RNA level, strongly argues for specific response of the HBV mutant. Furthermore, under similar conditions the expected activation of p21 promoter was obtained, but not a control plasmid (Figure 1c), suggesting that the accumulated p53 is functional in transcription activation.

Cisplatin represses X-KO HBV transcription

The cisplatin dependent reduction in the steady-state level of X-KO HBV RNA molecules might be the result of either an increased rate of RNA degradation or a decrease in HBV transcription rate. To address this point we measured the HBV RNA half life under different conditions, using actinomycin D (Shaul *et al.*, 1981). Transfected HepG2 cells were either cisplatin treated for 18 h or left untreated before actinomycin D was added to block RNA synthesis. RNA was harvested at the indicated time points and analysed by Northern blotting. The cisplatin treated cells, although as expected poorly expressing HBV RNA (Figure 2, lanes 1–4), displayed a reduction in RNA level that is not significantly different from untreated cells (lanes 5–8). Thus, the reduction in the HBV RNA level under cisplatin treatment is likely to be due to transcription repression and not to enhanced RNA degradation.

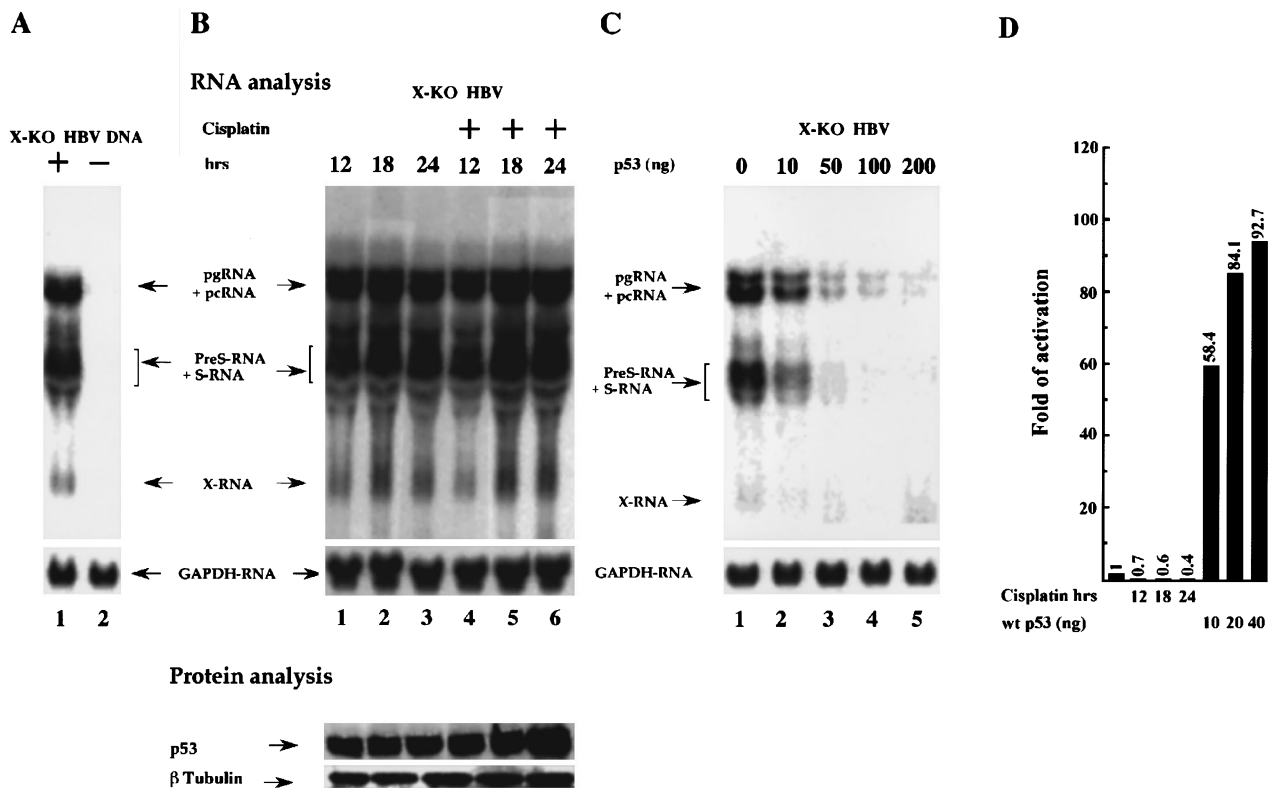


Figure 3 The effect of cisplatin and p53 on HBV transcription in Huh7 cells. (a) Detection of HBV transcripts in Huh7 cells that were transfected with a plasmid that contains two tandem HBV DNA (lane 1) but not a control plasmid (lane 2). (b) Cisplatin does not repress HBV transcription in Huh7 cells. Cells were transfected with a dimer of X-KO HBV DNA plasmid and cisplatin treated for the indicated time (hours), according to the protocol described in Figure 1. The extracted RNA and proteins were analysed (a, lower panels). (c) Cotransfected wt p53 represses HBV transcription in Huh7 cells. Cells were cotransfected with X-KO HBV DNA together with increasing amounts of a plasmid that directs the expression of p53. The ng amounts of the p53 DNA plasmid used in each lane are shown. (d) Cisplatin does not activate the p21 promoter in Huh7 cells. Cells were transfected with 2 μ g of p21-luciferase and either treated with cisplatin or cotransfected with wt p53 plasmid and analysed as detailed in Figure 1c

Cisplatin does not repress X-KO HBV transcription in Huh7 cells that bear a mutated p53 gene

To investigate the molecular mechanisms of genotoxic stress dependent HBV transcription repression, we used the Huh7 differentiated hepatocellular cell line, which harbors a mutated p53 gene (Bressac *et al.*, 1990). These cells show HBV RNA production only when transfected with HBV but not with a control DNA (Figure 3a). Interestingly, and in contrast to the HepG2 cells, no transcription repression was noticed upon treating the X-KO HBV transfected cells with cisplatin up to 24 h (Figure 3b). However, repression of X-KO HBV transcription in Huh7 cells could be obtained by cotransfection with a very low amount (10 ng) of the wt p53 expression plasmid (Figure 3c), suggesting that these cells contain all the components required to elicit p53-dependent HBV transcription repression. Furthermore, a p21-luciferase reporter plasmid was employed to show that in contrast to the HepG2 cells, cisplatin does not activate this promoter in Huh7 cells whereas low amounts of the cotransfected p53 plasmid can do so (Figure 3d). The inability of cisplatin to repress HBV transcription in Huh7 cells, therefore, is clearly due to the absence of wt p53.

Repression of X-KO HBV transcription by cisplatin is blocked by a p53 dominant negative truncated protein

To further substantiate the possibility that the transcription repression of HBV obtained under

genotoxic stress is p53 mediated, we employed a plasmid that expresses the production of the C-terminus oligomerization region of p53 (DD-plasmid). This plasmid was previously shown to sustain a p53 specific dominant negative activity (Shaulian *et al.*, 1995). Here again, the transcription of X-KO HBV in HepG2 cells was repressed upon exposing the cells to cisplatin (Figure 4, lanes 1–3). Interestingly, the cotransfected DD-plasmid induced a significant increase in the level of HBV transcripts in every single tested time point (Figure 4, lanes 4–6). These results suggest that in the cisplatin treated HepG2 cells, the X-KO HBV transcription repression is mostly mediated by p53 accumulation.

pX abrogates the p53-mediated repression of HBV transcription

The finding that cisplatin had a more dramatic effect on X-KO HBV (Figure 1) prompted us to investigate the role of pX in this process. Since we found that the effect of pX is more dramatic at early time post-transfection (data not shown), we repeated the experiments but this time the RNA was harvested earlier (35 h). Under these conditions wt HBV was barely affected by cisplatin whereas the X-KO virus was more responsive and displayed sharp reduction in transcription (Figure 5a,b, compare lanes 4–6 to 10–12). These results illustrate the differential behavior of the two HBV DNA genomes and substantiate the possible role of pX in both supporting HBV transcription and abrogating the p53-mediated repression effect. These findings were also confirmed by cotransfection experiments. When the X-KO HBV plasmid was cotransfected together with an X-expressor plasmid (SV₂-X), an increase in the level of HBV transcription was obtained, which was only partially affected by cisplatin (Figure 5b, compare lanes 4–6 to 7–9). Western analysis revealed that cisplatin treatment was effective in inducing the accumulation of the endogenous p53 level (Figure 5b lower panels). Therefore, we concluded that pX assures efficient HBV transcription under genotoxic stress by antagonizing the negative effect of p53 on HBV gene expression.

p73 the recent p53 homologue activates HBV transcription

p73 is a recent member of the p53 family, with similar transcriptional activation, DNA binding, and oligomerization domains (Kaghad *et al.*, 1997). Functionally, both p53 and p73 can induce apoptosis and transcription from promoters containing a p53/p73-response element (Jost *et al.*, 1997; Kaghad *et al.*, 1997), raising the possibility that this protein might bind HBV enhancer containing a p53 binding site (Ori *et al.*, 1998). To investigate the effect of p73 on HBV transcription, HepG2 cells were cotransfected with either p53 or p73. Interestingly, unlike p53 that repressed HBV transcription (Figure 6a, lane 2), p73 activated it to a certain level (Figure 6, lane 3). To substantiate this point we repeated the experiment by cotransfecting increasing amounts of p73 plasmid in the range of ng DNA, stopping before reaching squelching conditions. Clearly, p73 activates HBV

RNA analysis

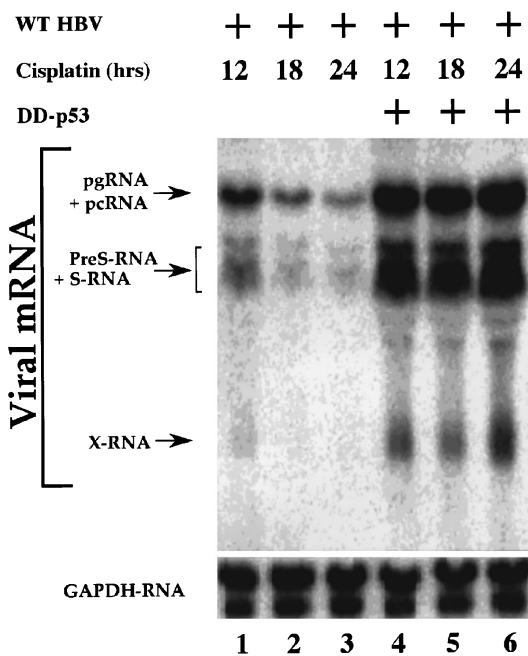


Figure 4 A dominant negative p53 plasmid recovers HBV transcription under genotoxic stress. HepG2 cells were transfected with a plasmid that harbors a dimer of X-KO HBV DNA either alone (lanes 1–3) or together with 7 μg DD-p53 plasmid (lanes 4–6) that expresses the production of p53 truncated dominant negative protein (Shaulian *et al.*, 1995). Cells were cisplatin treated for the indicated time (hours) and extracted according to the protocol described in Figure 1

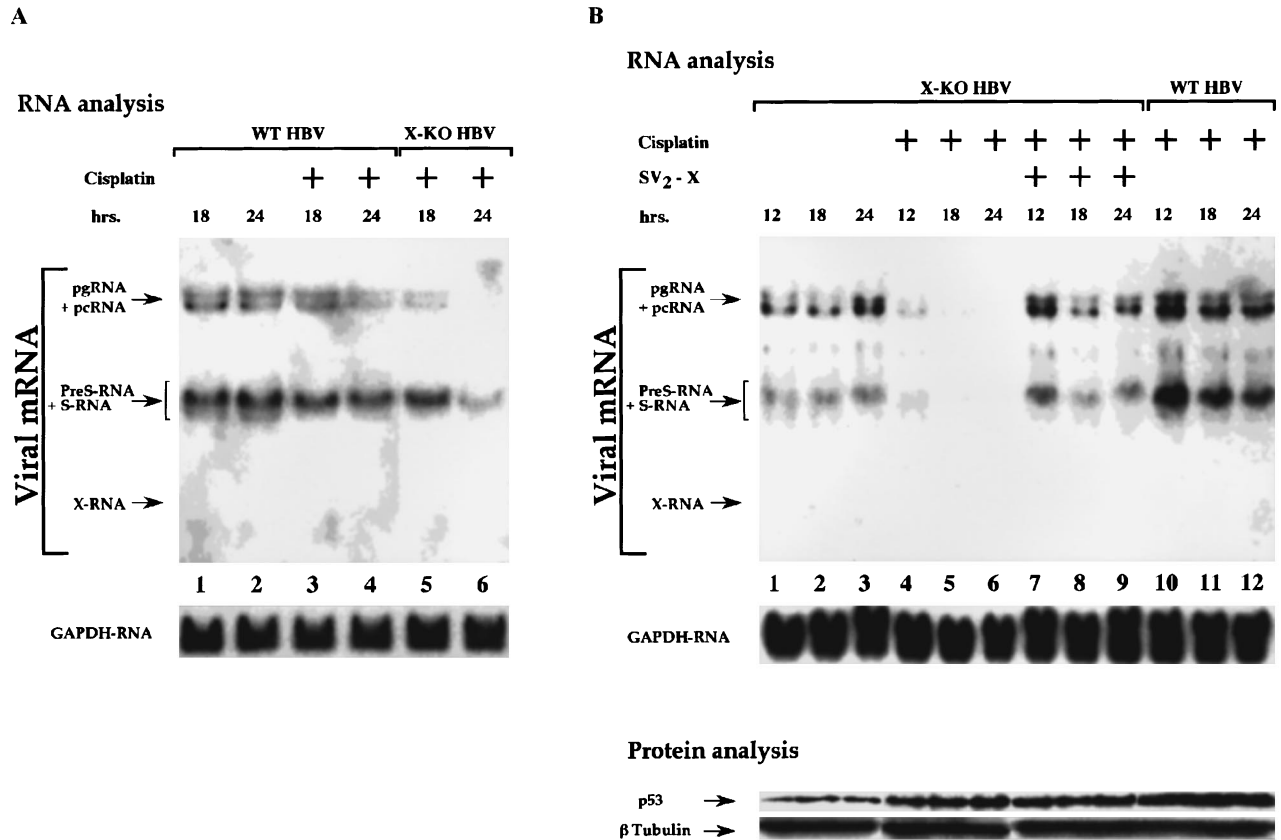


Figure 5 pX abrogates the p53-mediated repression of X-KO HBV transcription. (a, b) HepG2 cells were transfected either with a plasmid that harbors a dimer of either X-KO HBV DNA or with a wt HBV DNA. In (b) lanes 7–9 cells were cotransfected with 2 μ g SV2-X plasmid (Haviv *et al.*, 1998a) that expresses pX production. Cells were cisplatin treated (lanes 4–12) for the indicated time points (hours). The extracted RNA and proteins were analysed (b, lower panel) according to the Figure 1 protocol

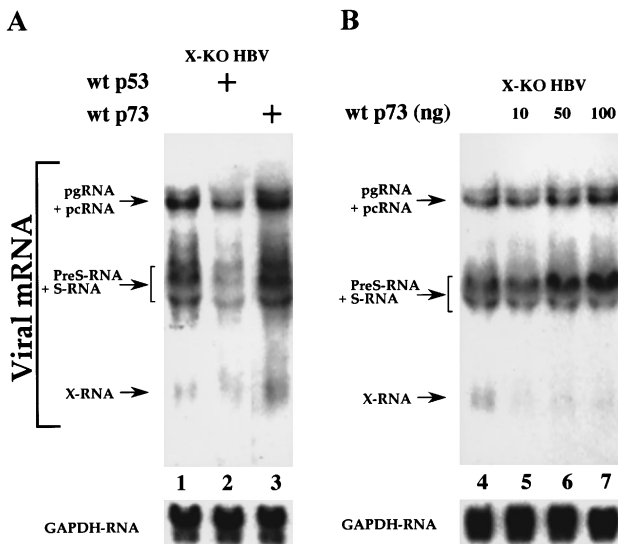


Figure 6 p73 α stimulates HBV transcription. (a) The HBV X mutant plasmid (X-KO HBV) was used to transfect HepG2 cells either alone or together with 100 ng plasmid that expresses either p53 or p73 α , as indicated. (b) The same HBV mutant in (a) was used to cotransfect HepG2 cells together with an increasing amounts of p73 α plasmid

transcription in a dose-dependent manner (Figure 6b). Thus, unlike p53, p73 plays a positive role in regulating the HBV enhancers/promoters activity.

Discussion

In this study we report a dramatic reduction in the HBV RNA level under genotoxic stress in the absence of an intact X open reading frame. Genotoxic drugs induce either cell-cycle arrest or apoptosis. Although not directly measured, we do not think that the observed reduction in HBV transcription in response to genotoxic drug treatment, is the result of apoptosis. First, cells were cisplatin treated for relatively short periods of time (from 12 to 24 h), which are not long enough to induce effective apoptosis and/or cell cycle arrest. Second, the level of total extracted RNA (data not shown) and GAPDH control mRNA were not reduced upon cisplatin treatment. Also, no reduction in β -tubulin protein along the different treatments was observed, ruling out a reduction in cell number due to cell death. Third, despite our attempts we did not see any changes in cell morphology along cisplatin treatments (data not shown).

Cells exposed to various types of stress, in particular those that lead to DNA damage, exhibit an increase in the cellular level of p53 (reviewed in Haffner and Oren 1995; Ko and Prives 1996; Gottlieb and Oren 1996). Accordingly, we show that the cisplatin mediated HBV transcription repression is mostly p53 dependent. First, there is good correlation between p53 level and repression of HBV transcription; second, HBV transcription repression was not detected in a cell line that contains a mutant p53 gene; third, a cotransfected

p53 dominant negative mutant eliminated the cisplatin-dependent repression effect on HBV transcription. Furthermore, the obtained repression is abrogated by a cotransfected papilloma E6 gene (data not shown) whose product selectively enhances p53 degradation (Scheffner *et al.*, 1990). Collectively, these data strongly suggest that p53 is an important upstream effector in repressing HBV transcription.

An intimate and unusual interaction between p53 and the HBV genome has been reported. Previously we reported that p53 binds to the 5' portion of the HBV enhancer in a sequence specific manner and represses its activity (Ori *et al.*, 1998). This was an unexpected finding, since DNA-bound p53 is known to stimulate transcription. Although the underlying repression mechanism is unknown, it might repress to a certain level HBV transcription in p53 proficient cells. The obtained dramatic increase in HBV transcription by the p53 dominant negative mutant (Figure 4) is in accordance with this possibility. The p53-mediated repression activity is an intrinsic behavior of the HBV enhancer and depends on an intact enhancer EP element (Ori *et al.*, 1998). The EP-DNA element of the HBV enhancer binds RFX1 and c-Abl (Agami and Shaul 1998; Dikstein *et al.*, 1992; Siegrist *et al.*, 1993). Interestingly, genotoxic drugs were reported to induce interaction of the c-Abl tyrosine kinase and p53 (Yuan *et al.*, 1996), therefore, HBV enhancer is designed to effectively respond to genotoxic stress. This is not so puzzling considering the fact that the liver cells, the HBV natural host cells, are often exposed to toxic agents absorbed via the digestive tract. Furthermore, at least in HBV transgenic mouse model, chronic active hepatitis displays greatly increased hepatic oxidative DNA damage (Hagen *et al.*, 1994). Thus, the p53 HBV DNA interplay illuminates a molecular mechanism of virus-host adaptation.

Our data also suggest that an autoregulatory loop regulates X gene transcription. The X gene promoter is positioned next to the viral enhancer that binds p53 (Ori *et al.*, 1998), therefore, the level of pX production depends on the level of p53, but p53 effect on the enhancer is pX dependent. This pX mode of action is compatible with its role ensuring optimal viral gene expression under a wide range of physiological conditions. A similar scenario was observed with papilloma viruses. Treatment with genotoxic agent led to strong repression of E6/E7 expression in HPV16- and HPV18-positive cervical carcinoma cell lines (Butz *et al.*, 1996) possibly via p53. However, E6 in turn enhances p53 degradation (Scheffner *et al.*, 1990).

In contrast to p53, the p73 protein level is not elevated by genotoxic stresses (Kaghad *et al.*, 1997), therefore it is unlikely that p73 is responsible for the observed HBV transcription repression under these conditions. Given the facts that HBV genome contains a functional p53 binding site (Ori *et al.*, 1998), and that p73 binds DNA with the same sequence specificity (Zhu *et al.*, 1998), it is not surprising that p73 also regulates HBV transcription. Remarkably, we found that p73 activates rather than represses HBV gene expression. To our knowledge this is the first example of these two proteins playing opposite roles. As in the differentiated liver cells the level of p53 is likely to be low, it is not too speculative to assume that under

normal conditions p73 is the more accessible factor to interact and hence to support HBV transcription. This trend will be shifted to the opposite direction while exposing the cells to conditions that give rise to p53 accumulation, such as genotoxic stress. Thus, the mode of interplay between p73, p53 and the HBV pX protein described here seems to be designed to assure not only HBV transcription activation but also repression, the latter possibly activated under unfavorable conditions for viral transcription and replication. This might explain how HBV sustains a long term and persistent infection in concert with the host-cell physiology without inducing noticeable cytopathic effects.

We also report here that physiological levels of pX that is expressed from the HBV genome and antagonizes the p53 dependent HBV transcription repression. The relationship between p53 and pX was investigated by a number of groups under different artificial systems (Chirillo *et al.*, 1997; Feitelson *et al.*, 1993; Lin *et al.*, 1997b; Schaefer *et al.*, 1998; Takada *et al.*, 1996; Truant *et al.*, 1995; Ueda *et al.*, 1995; Wang *et al.*, 1994). Different models were proposed to explain the antagonistic effect observed between these two proteins. For example, some suggested that pX changes the p53 compartmentalization by localizing it predominantly in the cytoplasm (Ueda *et al.*, 1995; Takada *et al.*, 1997), while others proposed physical and interfering interaction between the two proteins (Wang *et al.*, 1994; Lin *et al.*, 1997a; Truant *et al.*, 1995). However, the activation domain of p53 when fused to the Gal4 DNA-binding domain, is coactivated and not inhibited by pX (Haviv *et al.*, 1998a). Therefore, the site of interference between the two proteins localized outside the p53 activation domain. Furthermore, the p53 activation domain targets TAFs in transcription machinery (Thut *et al.*, 1995; Farmer *et al.*, 1996a), whereas pX targets TFIIB and polymerase II (Cheong *et al.*, 1995; Haviv *et al.*, 1998b; Lin *et al.*, 1997a). The mechanism of transcription repression by p53 is unknown, but since it can be alleviated by coexpression of the TAF proteins (Farmer *et al.*, 1996a), it was suggested that p53 represses transcription via TAF sequestration (Farmer *et al.*, 1996b; Liu and Berk 1995). In contrast, pX supports transcription in the absence of TAFs both *in vitro* and *in vivo* (Haviv *et al.*, 1998a; 1996). Therefore, pX is expected to activate transcription under conditions whereby TAFs are inactive due to their sequestration by p53. This likely possibility suggests a novel way of functional inactivation of p53 by pX that does not require their physical interaction.

HBV is an important risk factor of chronic liver diseases, including HCC. It has been speculated that pX, the HBV regulatory protein, is a major player in this process. Functional inactivation of p53 by pX was demonstrated so far only under artificial contexts, i.e., overproduction of either pX or p53, and therefore their relevance remained questionable. We show that physiologically activated p53 represses HBV transcription. Furthermore, this repression is abrogated by physiological levels of pX that is produced in the context of an intact HBV genome. Thus, it is not too speculative to propose that in the natural route of HBV infection hepatocytes experience p53 functional inactivation and therefore become more prone to transformation.

Materials and methods

Cell culture, DNA transfection and treatments

HepG2 and Huh7 cells were cultured in Dulbecco modified Eagle's minimal essential medium (GIBCO Laboratories) containing 100 U of penicillin and 100 µg of streptomycin per ml, supplemented with 8% fetal bovine serum. Transfection was carried out by the CaPi method as previously described (Haviv *et al.*, 1995). Each transfection reaction contained a constant amount of 25 µg DNA per 10 cm dish, consisting of 15 µg HBV plasmid. The latter contains two tandem copies of HBV full-length DNA (subtype adw), ligated via the unique *EcoRI* site. The HBV mutant (X-KO HBV) was constructed by generating a stop codon at position 27 of the X gene (Ori *et al.*, 1998). WT HBV or X-KO HBV dimers were inserted into pGEM-3Z in a head to tail orientation, which confirmed by restriction enzyme analysis. For cisplatin treatment, HepG2 and Huh7 cells were treated with 2.5 µg/ml cisplatin about 26 h after transfection. For analysis of HBV RNA half-life, HepG2 cells were treated with 2.5 µg/ml cisplatin for 18 h and then 5 µg/ml Actinomycin D (A4262, Sigma) was added for the indicated time points.

RNA analysis

Northern analysis was carried out by agarose-formaldehyde (1%) method according to published protocols (Sambrook *et al.*, 1989). RNA quality and quantity was monitored by UV-absorption and by ethidium-bromide staining. Radioactive probes were prepared by random priming protocol, using either a full length HBV DNA or GAPDH cDNA templates and ³²PdCTP (Amersham, 3000 Ci/mmol). Total RNA was extracted from transfected cells at various time points after transfection by TRI REAGENT (MRC, INC.). The RNA samples were treated with RNase free DNaseI (Boehringer) for 15 min at 37°C and stored until used. For Northern blot analysis 25 µg of total RNA per sample was separated on 1% formaldehyde-agarose gel and blotted to a Hybond-N nylon membrane (Amersham). Membranes were prehybridized and

hybridized according to the manufacturer instructions. About 10⁶ c.p.m. (10 ng DNA) labeled either 3.2 Kb HBV or 1.3 Kb GAPDH DNA fragments was used per 1 ml hybridization buffer. After hybridization the membrane was washed for 60 min at 65°C in a 0.1% SSC 0.1% SDS buffer and exposed to an X-ray film for autoradiography. Densitometry was performed by a Fujix Bas 2500 phosphorimager (Fuji).

Protein analysis

Proteins were extracted from cells by TRI-REAGENT (MRC, INC.) according to the manufacturer's instructions and solubilized for 30 min at 50°C in Laemmli sample buffer containing 4 M urea. Soluble proteins were boiled for 10 min and subsequently fractionated on 10% SDS-polyacrylamide gel. For Western blot analysis, gels were electroblotted to a nitrocellulose membrane for 1 h at 200 mA. Membrane filters were stained after blotting by Ponceau S, soaked for 2 h at RT in a blocking solution [Phosphate buffer saline (PBS) containing 4% w/v dried non-fat-milk powder and 0.01% v/v tween-20 (Sigma)]. All further incubation steps were performed in the same solution. Filters were incubated for 1–2 h at RT in the presence of either anti human p53 (1801+DO-1) or anti β-tubulin (clone no. TUB2.1, Sigma) antibodies, and washed three times with PBS+0.01% tween-20. Protein A horse radish peroxidase (HRP) conjugated (ICN laboratories) was added (diluted 1:10 000 in blocking solution) and incubation allowed to proceed for an additional hour followed by three rounds of washes. Antibody-antigen complexes were visualized by the ECL chemiluminescent detection system (Pierce) according to the manufacturer instructions, on an X-ray film.

Acknowledgements

We thank Dr M Oren for p53 and DD plasmids and for anti p53 antibodies. This work was supported by a grant from the national council for research and development. Israel and the Deutsches Krebsforschungszentrum (DKFZ).

References

- Agami R and Shaul Y. (1998). *Oncogene*, **16**, 1779–1788.
- Benn J, Su F, Doria M and Schneider RJ. (1996). *J. Virol.*, **70**, 4978–4985.
- Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR and Ozturk M. (1990). *Proc. Natl. Acad. Sci. USA*, **87**, 1973–1977.
- Butz K, Geisen C, Ullmann A, Spitkovsky D and Hoppe SF. (1996). *Int. J. Cancer*, **68**, 506–513.
- Cheong JH, Yi M, Lin Y and Murakami S. (1995). *EMBO J.*, **14**, 142–150.
- Chirillo P, Pagano S, Natoli G, Puri PL, Burgio VL, Balsano C and Levrero M. (1997). *Proc. Natl. Acad. Sci. USA*, **94**, 8162–8167.
- Dikstein R, Heffetz D, Ben-Neriah Y and Shaul Y. (1992). *Cell*, **69**, 751–757.
- Doria M, Klein N, Lucito R and Schneider RJ. (1995). *EMBO J.*, **14**, 4747–4757.
- Faktor O, Budlovsky S, Ben-Levy R and Shaul Y. (1990). *J. Virol.*, **64**, 1861–1863.
- Farmer G, Colgan J, Nakatani Y, Manley JL and Prives C. (1996a). *Mol. Cell Biol.*, **16**, 4295–4304.
- Farmer G, Friedlander P, Colgan J, Manley JL and Prives C. (1996b). *Nucleic Acids Res.*, **24**, 4281–4288.
- Feitelson MA, Zhu M, Duan LX and London WT. (1993). *Oncogene*, **8**, 1109–1117.
- Fritsche M, Haessler C and Brandner G. (1993). *Oncogene*, **8**, 307–318.
- Gottlieb TM and Oren M. (1996). *Biochim. Biophys. Acta*, **1287**, 77–102.
- Guidotti LG, Ishikawa T, Hobbs MV, Matzke B, Schreiber R and Chisari FV. (1996). *Immunity*, **4**, 25–36.
- Guidotti LG, Matzke B, Schaller H and Chisari FV. (1995). *J. Virol.*, **69**, 6158–6169.
- Haffner R and Oren M. (1995). *Curr. Opin. Genet. Dev.*, **5**, 84–90.
- Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN and Chisari FV. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 12808–12812.
- Haviv I, Matza Y and Shaul Y. (1998a). *Genes Dev.*, **12**, 1217–1226.
- Haviv I, Shamay M, Doitsh G and Shaul Y. (1998b). *Mol. Cell Biol.*, **18**, 1562–1569.
- Haviv I, Vaizel D and Shaul Y. (1995). *Mol. Cell Biol.*, **15**, 1079–1085.
- Haviv I, Vaizel D and Shaul Y. (1996). *EMBO J.*, **15**, 3413–3420.
- Honigwachs J, Faktor O, Dikstein R, Shaul Y and Laub O. (1989). *J. Virol.*, **63**, 919–924.
- Hosono S, Lee CS, Chou MJ, Yang CS and Shih CH. (1991). *Oncogene*, **6**, 237–243.
- Jameel S and Siddiqui A. (1986). *Mol. Cell Biol.*, **6**, 710–715.
- Jost CA, Marin MC and Kaelin WJ. (1997). *Nature*, **389**, 191–194.
- Kaghad M, Bonnet H, Yang A, Creancier L, Biscan JC, Valent A, Minty A, Chalou P, Lelias JM, Dumont X, Ferrara P, McKeon F and Caput D. (1997). *Cell*, **90**, 809–819.

- Klein NP and Schneider RJ. (1997). *Mol. Cell Biol.*, **17**, 6427–6436.
- Ko LJ and Prives C. (1996). *Genes Dev.*, **10**, 1054–1072.
- Lin Y, Nomura T, Cheong J, Dorjsuren D, Iida K and Murakami S. (1997a). *J. Biol. Chem.*, **272**, 7132–7139.
- Lin Y, Nomura T, Yamashita T, Dorjsuren D, Tang H and Murakami S. (1997b). *Cancer Res.*, **57**, 5137–5142.
- Liu X and Berk AJ. (1995). *Mol. Cell Biol.*, **15**, 6474–6478.
- Luber B, Lauer U, Weiss L, Hohne M, Hofschneider PH and Kekule AS. (1993). *Res. Virol.*, **144**, 311–321.
- Natoli G, Avantaggiati ML, Chirillo P, Puri PL, Ianni A, Balsano C and Levrero M. (1994). *Oncogene*, **9**, 2837–4283.
- Ohno H, Kaneko S, Kobayashi K and Murakami S. (1997). *J. Med. Virol.*, **52**, 413–418.
- Ori A, Zauberman A, Doitsh G, Paran N, Oren M and Shaul Y. (1998). *EMBO J.*, **17**, 544–553.
- Raney AK, Johnson JL, Palmer CN and McLachlan A. (1997). *J. Virol.*, **71**, 1058–1071.
- Roberts JJ and Friedlos F. (1981). *Biochim. Biophys. Acta*, **655**, 146–151.
- Sambrook J, Fritsch EF and Maniatis T. (1989). *Molecular Cloning, a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York.
- Schaefer S, Seifer M, Grimmsmann T, Fink L, Wenderhold S, Hohne MW and Gerlich WH. (1998). *J. Gen. Virol.*, **79**, 767–777.
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ and Howley PM. (1990). *Cell*, **63**, 1129–1136.
- Sells MA, Chen ML and Acs G. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 1005–1009.
- Shaul Y, Kaminchik J and Aviv H. (1981). *Eur. J. Biochem.*, **116**, 461–466.
- Shaul Y, Rutter WJ and Laub O. (1985). *EMBO J.*, **4**, 427–340.
- Shaulian E, Haviv I, Shaul Y and Oren M. (1995). *Oncogene*, **10**, 671–680.
- Siegrist CA, Durand B, Emery P, David E, Hearing P, Mach B and Reith W. (1993). *Mol. Cell Biol.*, **13**, 6375–6384.
- Su F and Schneider RJ. (1996). *J. Virol.*, **70**, 4558–4566.
- Sureau C, Romet LJ, Mullins JI and Essex M. (1986). *Cell*, **47**, 37–47.
- Takada S, Kaneniwa N, Tsuchida N and Koike K. (1996). *Virology*, **216**, 80–89.
- Takada S, Kaneniwa N, Tsuchida N and Koike K. (1997). *Oncogene*, **15**, 1895–1901.
- Thut CJ, Chen JL, Klemm R and Tjian R. (1995). *Science*, **267**, 100–104.
- Truant R, Antunovic J, Greenblatt J, Prives C and Cromlish JA. (1995). *J. Virol.*, **69**, 1851–1859.
- Ueda H, Ullrich SJ, Gangemi JD, Kappel CA, Ngo L, Feitelson MA and Jay G. (1995). *Nat. Genet.*, **9**, 41–47.
- Wang XW, Forrester K, Yeh H, Feitelson MA, Gu JR and Harris CC. (1994). *Proc. Natl. Acad. Sci. USA*, **91**, 2230–2234.
- Yee JK. (1989). *Science*, **246**, 658–661.
- Yuan ZM, Huang Y, Fan MM, Sawyers C, Kharbanda S and Kufe D. (1996). *J. Biol. Chem.*, **271**, 26457–26460.
- Zhu J, Jiang J, Zhou W and Chen X. (1998). *Cancer Res.*, **58**, 5061–5065.