Regulatory mechanisms of viral hepatitis B and C

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"There is an intrinsic simplicity of nature and the ultimate contribution of Science resides in the discovery of unifying and simplifying generalization, rather than in the description of isolated situation-in the visualization of simple, overall patterns rather than in the analysis of patchworks"

Luria's Credo, 1955

Of all the hepatitis viruses, only the hepatitis B virus (HBV) and hepatitis C virus (HCV) cause chronic hepatitis, which can progress to cirrhosis and hepatocellular carcinoma. In this review, we discuss how these two biologically diverse viruses use common pathways to induce oxidative stress and activation of key transcription factors, known to be involved in inflammatory processes in cells. Activation of NF-κB and STAT-3 most likely contribute to the progression of viral infections to chronic hepatitis and liver oncogenesis associated with HBV and HCV infections. In this review, we focus on the mechanisms of action of HBx and HCV NS5A proteins in inducing intracellular events associated with the viral infections.

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1. Introduction

The human hepatitis B virus (HBV) and hepatitis C virus (HCV) are a major health problem worldwide and cause a wide spectrum of clinical manifestations ranging from apparently healthy carrier state to acute self-limited or fulminant hepatitis and a chronic liver disease (Hoofnagle 1997; Nolt 1997). HBV and HCV are biologically different viruses. HBV contains a DNA genome, which replicates by reverse transcription of an RNA pregenome. HCV is an RNA virus, which replicates on the cellular membrane by RNA replication. Despite their different life cycles and modes of gene expression, they share common characteristics in the mechanisms of chronic liver disease they cause and association of cirrhosis and hepatocellular carcinoma with viral infections. The routes of transmission by HBV and HCV are similar, which raises the opportunities of co-infection and superinfection of hepatocytes (Feray *et al* 1993; Liaw 1995). Chronic hepatitis is frequently associated with the development of liver cirrhosis, which ultimately develops into hepatocellular carcinoma (Haydon *et al* 1997). Hepatocellular carcinoma is the fifth most common cancer in the world (Freeman 2002). The rate of progression from chronic hepatitis through cirrhosis to cancer, in hepatitis C, is dramatically higher than the rate of progression of hepatitis B-induced cirrhosis to cancer (Kew *et al* 1997). The pathogenesis of dual infection and biological interactions between HBV and HCV has not yet been clearly established.

In this review, we discuss our recent observations on the role of oxidative stress in HBV-infected and HCV-

Keywords. ER stress; hepatitis B virus; hepatitis C virus; NF-κB; oxidative stress; STAT-3

Abbreviations used: EOR, Endoplasmic reticulum overload response; ER, endoplasmic reticulum; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; NAC, N-acetyl L-cysteine; NLS, nuclear localization signal; NS5A, nonstructural protein 5A; PDTC, pyrrolidine dithiocarbamate; PKR, double stranded RNA-dependent protein kinase; ROS, reactive oxygen species; RR, ruthenium red; UPR, unfolded protein response; VDAC, voltage dependent anion channel.

infected hepatocytes, and its possible contribution to liver disease pathogenesis. Our studies show that HBV X protein (HBx) and HCV NS5A protein from their cytoplasmic residence induce the generation of reactive oxygen species (ROS), which by an unknown mechanism activate latent transcription factors, NF-κB and STAT-3. These factors upon activation by phosphorylation translocate to the nucleus and regulate gene expression.

2. Hepatitis B virus

Hepatitis B virus is a small DNA virus that is maintained in circular conformation within the virions. HBV replicates via a reverse transcription of an RNA pregenome and predominantly infects human hepatocytes in the liver. This marked hepatotropism is a prominent feature of hepadnaviruses. The majority of individuals that are chronically infected with HBV ultimately experience severe liver disease and are at a high risk of developing hepatocellular carcinoma (Beasley *et al* 1981). The 3·2 kb HBV DNA genome codes for four genes named C, S, P and X (figure 1A). The C gene codes for the core protein and the serum e antigen, the S gene codes for three related viral envelope proteins known as surface antigen, the P gene codes for the viral DNA polymerase, and the X gene codes for a 16·5 kDa protein. The viral transcription is governed by four promoters and two enhancer elements that are located in the HBV genome. To investigate the regulatory mechanisms that contribute to the hepatotropic nature of this virus, a considerable amount of work has focused on liver-specific aspects of HBV gene expression (Yen 1993; Kosovsky *et al* 1996). The compact nature of this small genome necessitates an extensive overlapping arrangement of the genetic information. Therefore, it is likely that a number of complex mechanisms regulate the temporal and differential expression of the viral RNAs. This regulation is mediated by the HBV promoters and interactions between *trans*-acting cellular factors and enhancer element 1, located at a

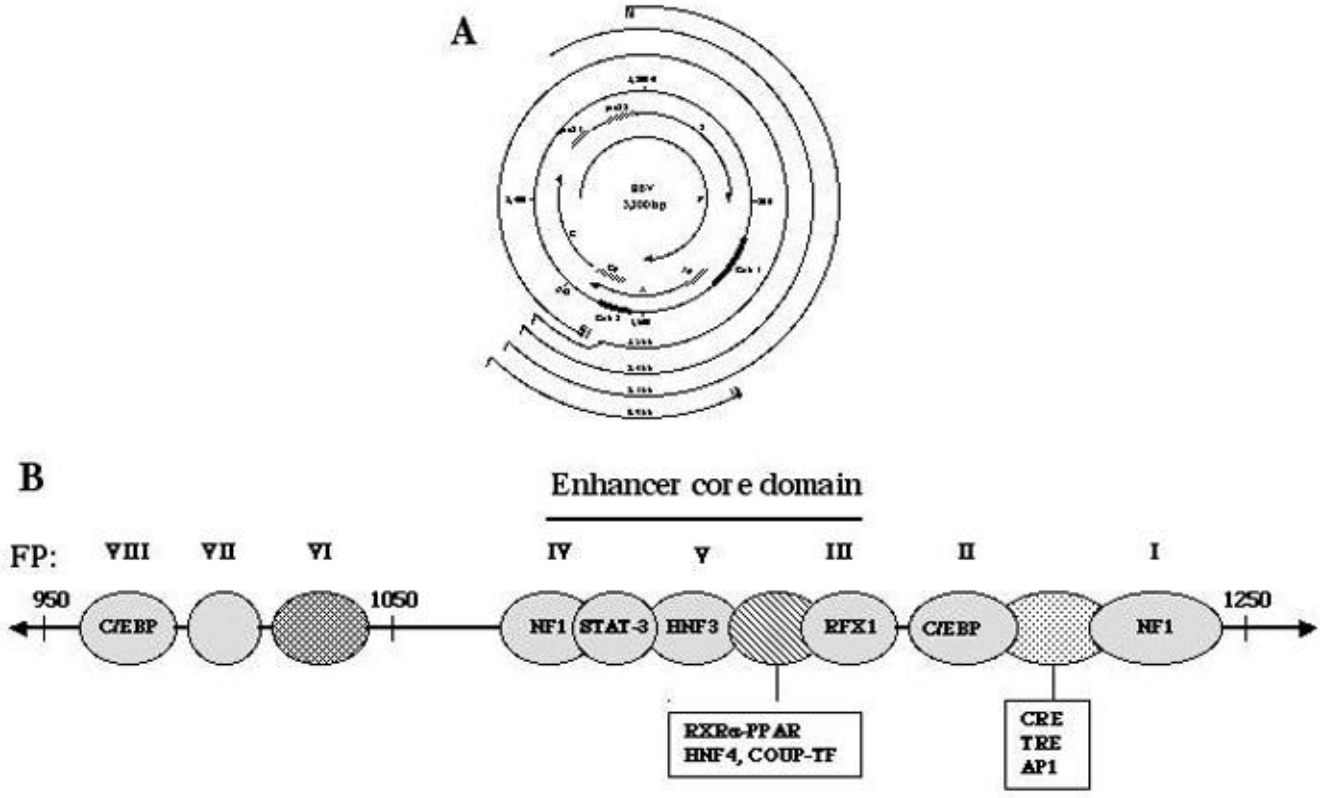

Figure 1. HBV genome. (**A**) The viral genome is numbered (0 to 3,200 bp) according to the adw2 subtype of HBV. S, C, P, and X represent the viral genes encoding the surface antigen, core or e antigen, DNA polymerase, and X proteins, respectively. The HBV promoters (preS1, preS2, Cp and Xp) and enhancers (Enh1 and Enh2) are indicated. Multiple transcription initiation sites are indicated at the 5′ end of the 3·5–2·1, and 0·9 kb RNAs. (**B**) Protein binding sites on the enahncer 1 element are shown and footprint designations are indicated above the corresponding sites.

strategic site within the viral genome (figure 1B). The enhancer function in the hepatocytes results from the combinatorial action of both ubiquitous and liver-enriched transcriptional factors and has been shown to play an important role in the overall regulation of HBV gene expression in a liver-specific manner (Shaul *et al* 1985; Jameel and Siddiqui 1986; Antonucci and Rutter 1989; Kosovsky *et al* 1996). Regulation of viral gene expression also occurs at the level of translation. The pregenome serves as the mRNA not only for the viral core protein but also for the viral polymerase, which initiates from an AUG located several hundred nucleotides from the 5′ end of the RNA molecule and is read in a different reading frame from that of core (Chang *et al* 1989; Ou *et al* 1990).

Among the proteins encoded by the HBV genome, the X gene product, termed HBx, which is essential for productive infection of the mammalians HBV (Chen *et al* 1993; Zoulim *et al* 1994), has drawn considerable attention due to its pleiotropic functions. HBx does not directly bind DNA but functions via protein–protein interaction (Maguire *et al* 1991). HBx has been shown to function as transcriptional transactivator via several motifs including, NF-κB, ATF/CREB, NF-AT, AP-1, C/EBP, p53 and Egr-1, STAT-3 (Siddiqui *et al* 1989; Twu *et al* 1989; Maguire *et al* 1991; Lucito and Schneider 1992; Kekule *et al* 1993; Wang *et al* 1994a; Becker *et al* 1998; Waris *et al* 2001). HBx can also activate cellular signalling pathways including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK) and Src tyrosine kinases (Benn and Schneider 1994; Klein and Schneider 1997). HBx activation of Src is important for HBV DNA replication. Recently, it has been shown that HBx activated cytosolic calcium-dependent proline-rich tyrosine kinase-2 (Pyk2), a Src kinase activator (Bouchard *et al* 2001). Increased cytosolic calcium activates Pyk2, leading to its autophosphorylation at tyrosine 402, which creates a binding site for Src kinase and activates it. Additional properties associated with HBx, include its binding to proteosome and UVDBP (Huang *et al* 1996; Becker *et al* 1998).

3. Hepatitis C virus

Based on genomic organization, biochemical properties, and molecular features, HCV has been classified as hepacivirus of *Flaviviridae* family (Miller and Purcell 1990). HCV infection leads to chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Saito *et al* 1990; Plagemann 1991). Over 80% of the HCV infections lead to chronic hepatitis. About 20–30% of these patients develop cirrhosis. The viral genome is composed of 9·6 kb positive-sense single stranded RNA containing a 5′ noncoding region (NCR), a large single open reading frame, and 3′ NCR (figure 2). The 3′ NCR has been divided in to three regions: a variable sequence of approximately 40 bases, a variable length of poly-UC rich tract, and a highly conserved 98 base region (Kolykhalov *et al* 1996; Bartenshlager and Lohmann 2000). The 5′ NCR, which represents a highly conserved region among HCV isolates, contains an internal ribosome entry site (IRES) which regulates the translation of viral polyprotein (Brown *et al* 1992; Tsukiyama-kohara *et al* 1992; Wang *et al* 1994a,b). The HCV polyprotein precursor is co- and post-translationally cleaved by viral proteases and host cell signal peptidases, resulting in at least three structural (core, E1, and E2) and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B) (Kolykhalov *et al* 1994; Tanji *et al* 1994). It is presumed that transcriptional initiation by the RNA-dependent RNA polymerase in coordination with other virally encoded and host proteins occurs in both the 3′ and 5′ NCRs, using positive- and negative-stranded viral RNAs as templates. Several host proteins have been shown to bind 3′ and 5′ HCV NCR, including PTB, La autoantigen (Ali and Siddiqui 1995, 1997; Spangberg *et al* 2001). Although there have been several reports demonstrating low level and intermittent replication of the intact HCV genome in a variety of cell culture systems, efficient *in vitro* replication has not been observed (Yoo *et al* 1995; Dash *et al* 1997). The development of sub-genomic HCV RNA replicons capable of stable and high level expression represents a major breakthrough in the field (Lohmann *et al* 1999). The HCV sub-genomic replicon is a bi-cistronic RNA, containing neomycin resistance gene under the translational control of HCV IRES followed by HCV nonstructural proteins including NS3 through NS5B under the translational control of EMCV IRES. These can be maintained as replicating RNAs in the cytoplasm expressing neomycin resistance in cell culture (Huh-7) in the presence of G418. During replication, adaptive mutations arise which contribute to their high efficiency of replication. Some of these mutants were localized in the NS5A gene, implicating an important role of this protein in replication (Lohmann *et al* 1999; Blight *et al* 2000). Recently full-length hepatitis C virus RNA genome-replicon similar in design to those described by Lohmann *et al* (1999) has been developed (Ikeda *et al* 2002; Pietschmann *et al* 2002). The functions of non-structural proteins have been elucidated by a large number of studies and by analogy to related viruses; only NS4B and NS5A have no well–defined functions during translation and replication to date.

HCV nonstructural protein 5A (NS5A) is a serine phosphoprotein, which exists as a polypeptide of p56 or p58 with varying degrees of phosphorylation (Kankeo *et al* 1994; Reed *et al* 1997). The NS5A protein possesses a nuclear localization-like signal (NLS) sequence but is localized to the nuclear periplasmic membrane fraction

(Tanji *et al* 1995). The significance of NLS is not clear at this time. NS5A came into prominence because of its suggested role in interferon (IFN) resistance. NS5A has been shown to bind PKR kinase and inhibit its homodimerization and kinase activity (Gale *et al* 1998). A defined region within NS5A protein termed ISDR (Gale *et al* 1998) was identified as the PKR interactive domain. IFN-resistance resulting from this interaction is controversial. Double stranded RNA-dependent protein kinase (PKR) is one of the major intracellular enzymes that mediates the antiviral action of IFN (Katze 1995). PKR, upon induction by IFN, phosphorylates eIF-2α, attenuating cellular protein synthesis (Meurs *et al* 1990). However, in NS5A expressing cells, eIF-2α remains unphosphorylated leaving protein synthesis unaffected. Among other cellular targets of NS5A, interactions with cellular transcription factor SRCAP, and a membrane fusion protein VAP-30, have been described (Ghosh *et al* 1999; Tu *et al* 1999). NS5A has been shown to bind NS5B (Shirota *et al* 2002). NS5A protein transcriptionally down-regulates the cyclin-dependent kinase inhibitor p21/waf1 gene (Ghosh *et al* 1999) and promotes cell growth (Ghosh *et al* 1999). Functional significance of these interactions remains to be correlated with HCV-associated liver pathogenesis.

4. Oxidative stress

Oxidative stress is characterized by an increase in the intracellular levels of reactive oxygen species (ROS), which is associated with nearly all pathological states, especially those involving inflammatory processes (Schreck *et al* 1991). High doses of ROS are produced during chronic and acute inflammatory diseases or as a result of environmental stress (Schwarz 1996). Reactive oxygen metabolites play a complex role in many diseases and metabolic regulation. Because viruses replicate in living cells, such metabolites influence the growth of viruses in addition to

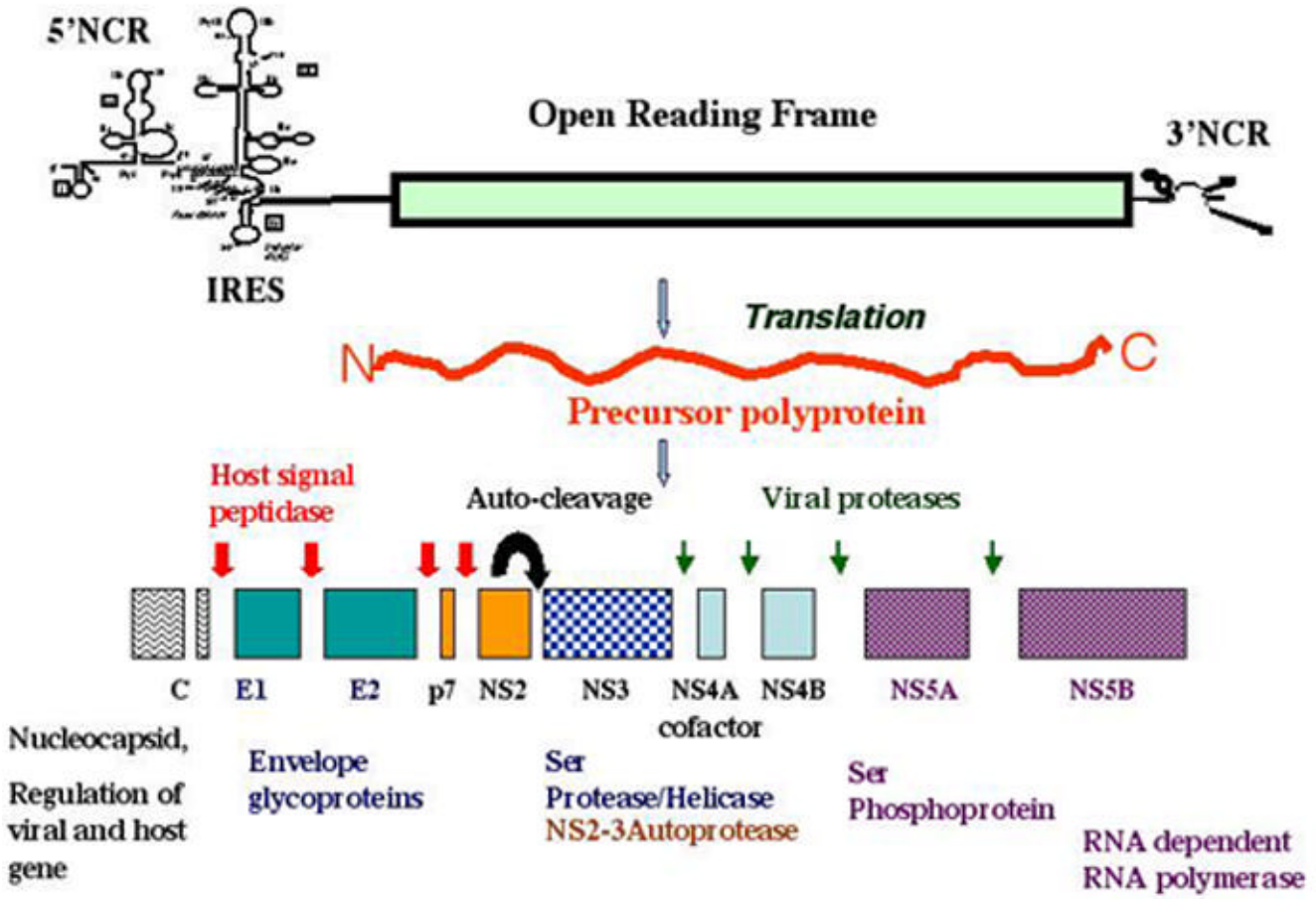

Figure 2. Organization of the HCV RNA genome. The 5′ and 3′NCRs flank the single large open reading frame showing the structural proteins located at the NH_2-terminal portion and the remainder showing the nonstructural proteins (NS2 to NS5B) at the carboxy-terminal portion of the polyprotein.

serving as a host defense mechanism. Chronic HBV and HCV infections are associated with an increased production of ROS within the liver that are responsible for the oxidation of intracellular macromolecules.

ROS can damage DNA – with unrepaired or misrepaired damage – leading to mutations. Mutations caused by oxidative DNA damage include a range of specifically oxidized purines and pyrimidines, as well as single strand breaks formed directly or by repair processes (Dizdaroglu 1994; Breen and Murphy 1995). The rearrangement of DNA sequence, miscoding of DNA lesion, gene duplication and activation of oncogenes are involved in the initiation of various cancers (Du *et al* 1994).

A growing body of evidence suggests an essential role of cellular redox, both in cell survival and cellular signalling pathway. ROS can function as a component of signal transduction cascades by activating transcription factors including STAT-3 and NF-κB (Schreck *et al* 1991; Carballo *et al* 1999; Schoonbroodt *et al* 2000; Waris *et al* 2001). STAT-3 and NF-κB motifs are found in a wide variety of cellular genes whose functions range from growth promotion, to proliferation, to DNA replication, to repair, and to functions involved in cell death and cancer development. Work by Broomberg *et al* (1999) demonstrates STAT-3 as an oncogene.

Both HBx and HCV NS5A proteins have been shown to activate cellular transcription factors via oxidative stress in cells (Meyer *et al* 1992; Gong *et al* 2001; Waris *et al* 2001). While HBx induces oxidative stress via its direct association with mitochondria leading to the elevation of ROS (Rahmani *et al* 2000; Huh and Siddiqui 2002); whereas HCV NS5A induces ER stress, causes disturbance of calcium homeostasis, which triggers the elevation of ROS in mitochondria, leading to the translocation of transcription factors to the nucleus. The downstream events from the ER to the nucleus leading to the activation of STAT-3 and NF-κB are illustrated in figure 3.

The *in vitro* interaction of HBx with components of transcriptional machinery has led to the idea that HBx functions directly in the nucleus (Maguire *et al* 1991; Cheong *et al* 1995; Qadri *et al* 1995, 1996; Williams and Andrisani 1995; Haviv *et al* 1998). While most of the available evidence points to its predominantly cytoplasmic distribution (Siddiqui *et al* 1987; Doria *et al* 1995), we and others have shown that HBx associates with mitochondria (Takada *et al* 1999; Rahmani *et al* 2000; Henkler *et al* 2001). We further characterized this association by showing that HBx directly and physically interacts with an outer mitochondrial voltage-dependent anion channel (VDAC3) and that this association leads to a decrease in the mitochondrial membrane potential and causes the elevation of ROS (Rahmani *et al* 2000; Waris *et al* 2001). A c-terminal HBx deletion mutant, HBxΔ99, which fails to target to mitochondria and bind VDAC3 does not cause the elevation of ROS. Mitochondria is the principal organelle in which ROS are generated in response to stress induced by a variety of conditions, including viral infection (Green and Reed 1998). Meyer *et al* (1992) have shown that both $MHBs^t$, a hepatitis B surface antigen derivative, as well as HBx activated NF-κB and that these activities were sensitive to NAC and PDTC, implicating the involvement of ROS. $MHBs^t$, being a viral envelope glycoprotein may induce ROS via its ER association in a manner similar to HCV NS5A.

HBx induces oxidative stress via its association with mitochondria, which in turn leads to the activation of a series of transcription factors including STAT-3 and NF-κB (Waris *et al* 2001). NF-κB was one of the first HBx-responsive element that was identified (Siddiqui *et al* 1989; Twu *et al* 1989). However, the mechanism by which HBx stimulated transcription via NF-κB motif was not clearly understood. The model presented in figure 3, provides novel insights into the mechanism of transcriptional regulation by HBx from its cytoplasmic location. HBx constitutively activates STAT-3, a transcription factor that is normally activated by cytokines such as epidermal growth factor or IL-6 (Darnell 1997; Waris *et al* 2001). Activated STATs form dimers or multimers through their Src-homology domain II, and are transported into the nucleus, where they bind to the cognate DNA sequences and activate gene expression. Oxidative stress has been shown to trigger STAT-3 tyrosine phosphorylation and nuclear translocation (Carballo *et al* 1999), which correlates with the activation of STAT-3 leading to its DNA binding activity.

As a result of the HCV NS5A-mediated induction of ER stress, calcium effluxes from the ER and is subsequently taken up by mitochondria which triggers the elevation of ROS in mitochondria (figure 4), leading to the activation of transcription factors including STAT-3 and NF-κB (figure 5). The evidence in support of these conclusions is based on the studies in which antioxidants and calcium chelators abrogated ability of NS5A to activate these factors. A similar role of mitochondrial calcium is implicated in the generation of ROS in HBx expressing cells with the use of ruthenium red (RR), an inhibitor of calcium uptake by mitochondria (Waris G and Siddiqui A, unpublished results).

Transcription factor NF-κB induces expression of many genes that are critically involved in cell survival and proliferation and in the regulation of immune and inflammatory responses (Baldwin 2001). Activation of NF-κB occurs via Ser^{32} and Ser^{36} phosphorylation of IκBα for ubiquitination and subsequent degradation by 26S proteosome (Baeuerle and Baltimore 1988; Traenckner *et al* 1995). An alternative mechanism for the activation of NF-κB involves the phosphorylation at Tyr^{42}, and PEST sequences of IκBα under oxidative stress (Schoonbroodt

et al 2000; Livolsi *et al* 2001; Waris G and Siddiqui A, unpublished results). Tyrosine phosphorylation of IκBα has been observed during ischemia/reperfusion of the liver (Zwacka *et al* 1998), and oxidative stress suggesting the functional role in this pathway. Livolsi *et al* (2001) show that tyrosine kinases act at several levels to dissociate IκBα-NF-κB complexes. We have recently observed that HCV NS5A expression as well as the HCV sub-genomic replicon activate NF-κB via tyrosine phosphorylation and calpain-mediated degradation of IκBα (Waris G, Peyron J F and Siddiqui A, unpublished results). Inhibitors of tyrosine kinases abrogate NF-κB activation but IκB kinase inhibitor (Bay 11-7085) does not affect the NF-κB activation induced by NS5A expression.

5. Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is the major signal-transducing organelle within the cell that continuously responds to environmental cues to release calcium (Kaufman 1999). Inside the organelle, proteins destined for secretion or transport to the cell surface are folded and become glycosylated. ER stress can arise from a disturbance in protein folding, leading to an accumulation of un- or misfolded proteins in the organelle (Yoshida *et al* 1998; Kaufman 1999). The ER is exquisitely sensitive to alterations in homeostasis, where, upon a variety of different stimuli, signalls are transduced from the ER to the cytoplasm and the nucleus to eventually result in adaptation for survival or

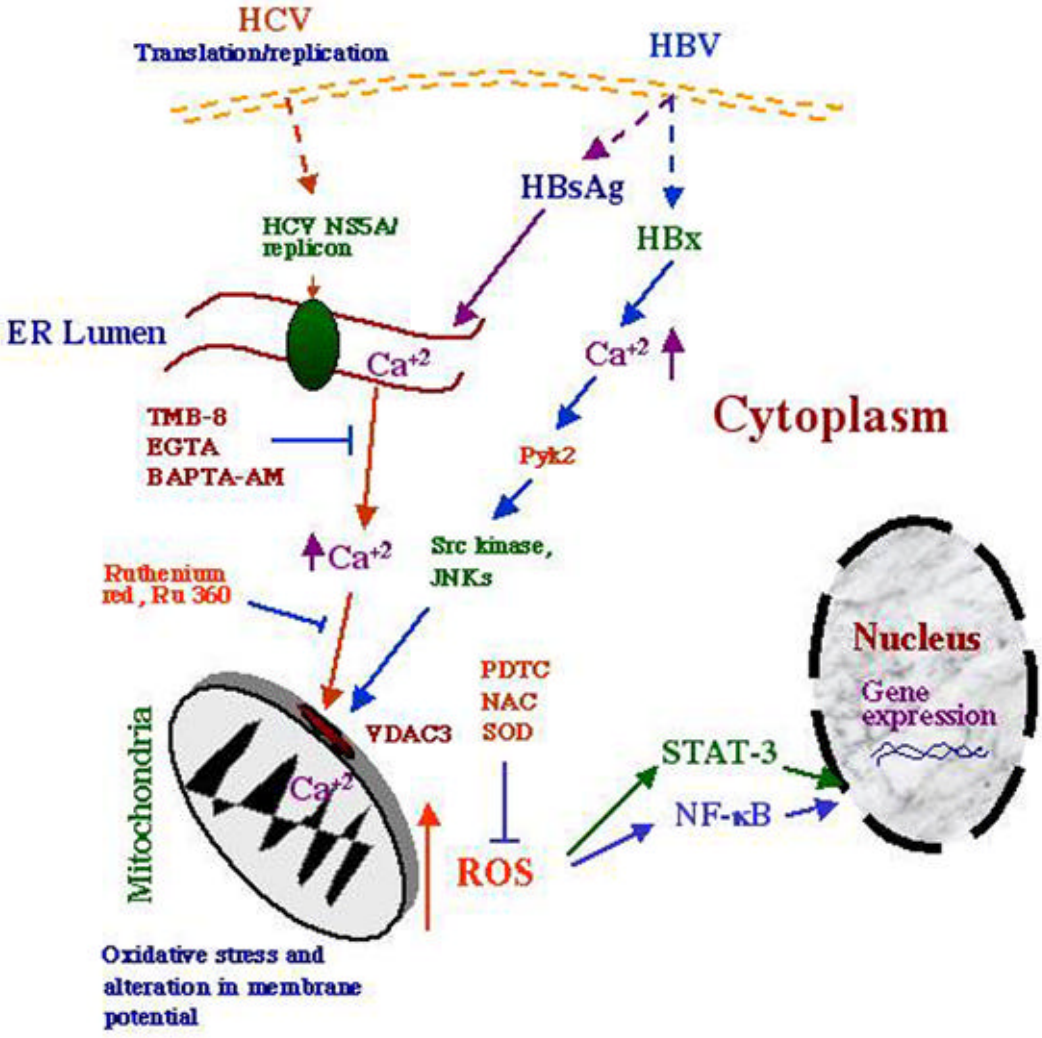

Figure 3. A model illustrating HBx, HBsAg and NS5A-induced activation of NF-κB and STAT-3 via Ca^{+2} signalling and ROS.

induction of apoptosis (Kaufman 1999). ER stress responses can include transcriptional induction, translational attenuation, and protein degradation. HCV induces ER stress in hepatocytes and triggers two functionally distinct ER stress response pathways: the ER-overload response (EOR); and the unfolded protein response (UPR) (Tardiff *et al* 2002).

The HCV nonstructural proteins including NS5A, are associated with the membrane in the reticular network of the ER in the perinuclear area where they form a ribonucleoprotein complex with genomic RNA for RNA replication (Hijikata *et al* 1993; Bartenschlager and Lohmann 2000). The association of the HCV ribonucleoprotein complex with the ER membrane induces ER stress (Tardif *et al* 2002). In response to ER stress, cells expressing HCV replicons activate transcription factor ATF6, by inducing the proteolytic cleavage of a transcriptionally active N-terminal domain of ATF6 from the ER membrane (Haze *et al* 1999; Tardif *et al* 2002). This induction of ATF6 leads to the increased transcription of GRP78 (Bip), an ER luminal chaperon protein. The ER overload response has been documented for other viral proteins including influenza haemagglutinin (Pahl and Baeuerle 1995), adenovirus E3/19K (Pahl *et al* 1996). Meyer *et al* (1992) have shown that hepatitis B virus surface antigen, MHBS^t a c-terminal truncation and an N-glycosylated integral membrane protein localized to the ER and transactivated NF-κB. The hepatitis B virus large surface protein and the HCV E2 envelope protein have been shown to activate the UPR (Xu *et al* 1997; Liberman *et al* 1999). Recently, cells expressing HCV replicons have been shown to activate the UPR in the absence of the HCV structural protein E2 (Tardif *et al* 2002). These results collectively demonstrate that both HBV gene expression, and HCV translation and replication activities in the ER are capable of inducing an ER stress response.

Another intracellular event characteristic of the UPR is translational attenuation. However, cells expressing HCV replicons have lower levels eIF2α phosphorylation, indicating the lack of translation attenuation (Tardif *et al* 2002). This is likely the result of NS5A-mediated inhibition of PKR kinase activity, which is one of the kinases involved in the phosphorylation of eIF2α (Gale *et al* 1997; Kaufman 1999). Furthermore, cap-dependent and HCV IRES and GRP78 IRES mediated cap-independent translation is enhanced in cells expressing HCV replicons

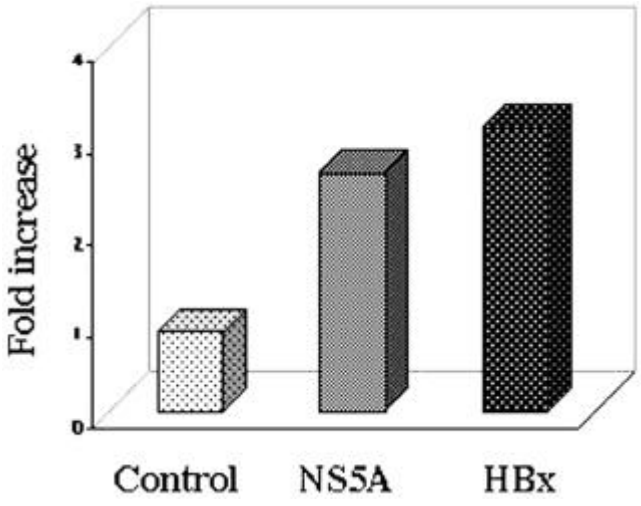

Figure 4. Generation of ROS in Huh-7 cells transfected with HBx and NS5A expression vectors respectively. ROS were measured by flow cytometry. The cells were treated with 4 μM of dihydroethidium (DHE) for 45 min. The bars show the fold increase in oxidized DHE fluorescence. Untransfected Huh-7 cells were used as control.

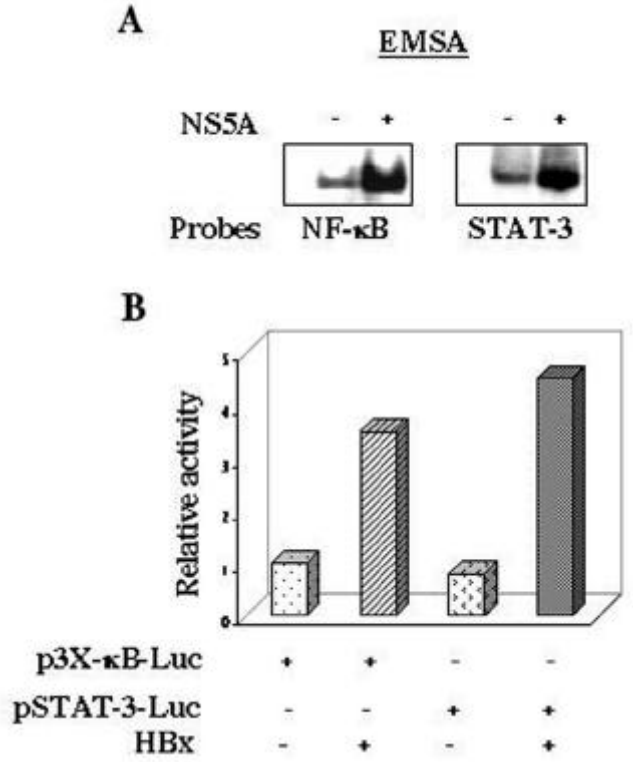

Figure 5. (**A**) Activation of transcription factors by NS5A. Mobility shift gel analysis of nuclear lysates transfected with HCV NS5A was carried out in the presence of [^{32}P]-labelled oligonucleotide probes corresponding to NF-κB and STAT-3. Untransfected Huh-7 cells were used as control. (**B**) HBx stimulates the STAT-3- and NF-κB-dependent transcriptional activation. The reporter plasmids p-STAT-3-Luc, which contained STAT-3-binding sites and p3X-κB-Luc, which contained three NF-κB binding sites were linked to luciferase respectively. These were cotransfected with wild-type HBx expression vector. 36 h posttransfection, cells were harvested to used to assay for luciferase activity.

(Tardif *et al* 2002). These results illustrate how HCV alters the typical course of the UPR to prolong its survival in hepatocytes.

In summary, these observations collectively demonstrate unique schemes by which HCV NS5A and HBx proteins induce oxidative stress in cells. The activation of NF-κB and STAT-3 transcription factors by ROS implies a role of whole host of cellular genes in contributing to the establishment of chronic liver disease in infected hepatocytes. Increased ROS can enhance replication of viruses. This is certainly advantageous to hepatitis viruses, which need to replicate in the hepatocytes that are posed at G_0. This leads to the idea that antioxidants can be utilized to decrease viral replication and the virus-induced oxidant injury. Understanding the complex networking of viral infections, oxidative stress and host response to the viral infections may pave the way toward important advances in the therapeutic design to control viral pathogenesis and oncogenesis.

Both of these viruses are generally noncytopathic. The host immune response is believed to cause the hepatodestruction or apoptosis that is seen in acute and chronic active hepatitis. It was noted that neither HCV NS5A nor HBx proteins directly caused apoptosis. HBx expression did not lead to cytochrome C release and, by tunnel assay, cell death was not observed (Rahmani *et al* 2000; Huh and Siddiqui 2002). In light of these results, it can be argued that NF-κB and/or STAT-3 may regulate gene expression of survival factors to further ensure antiapoptotic environment in the cells, a situation favourable for oncogenesis. This is in keeping with the mild chronic hepatitis in which very little, if any, hepatocytolysis occurs. But the dire consequences of such a scenario is the ultimate assault to the infected hepatocytes, which is the establishment of pre-malignant state leading to the onset of hepatocarcinogenesis.

Acknowledgements

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