Src kinases involved in hepatitis B virus replication

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Chronic infection by hepatitis B virus is a leading cause of human liver cancer and liver disease. The hepatitis B virus HBx protein is a regulatory factor that is essential for virus infection in mammals and is implicated in development of liver cancer and liver disease. Among the reported activities of HBx is the ability to stimulate Src tyrosine kinases, Ras-GTPases and transcriptional activation. We now demonstrate that HBx activation of Src tyrosine kinases, but not Ras, promotes a high level of viral replication in cell culture. HBx is shown to stimulate reverse transcription of the viral pregenomic mRNA into genomic DNA through a Src-mediated pathway in tissue culture cells. Targeted inhibition of Src tyrosine kinase activity, mutational inactivation of the HBx gene or retargeting of HBx to the nucleus to abolish cytoplasmic signal transduction activity, are shown to impair viral reverse transcription strongly. These studies implicate HBx stimulation of the Src family of tyrosine kinases in stimulation of viral polymerase activity.

Keywords: HBx protein/hepatitis B virus/liver disease/
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Introduction

Hepatitis B viruses (HBVs) are hepadnaviruses that preferentially infect the liver of their mammalian and avian hosts, often establishing chronic infection. Chronic infection is strongly associated with development of liver disease and liver cancer in mammals (hepatocellular carcinoma, HCC), and is a leading cause of human cancer worldwide (Wands and Blum, 1991). Prototypic members of the HBV family include human hepatitis B virus (HBV), woodchuck hepatitis B virus (WHV) and duck hepatitis B virus (DHBV). HBVs are distantly related to retroviruses, since they undergo reverse transcription of the viral mRNA pregenome into genomic DNA, which is packaged as a partially double-stranded DNA (dsDNA) circular genome within virus particles (Ganem and Varmus, 1987; Nassal and Schaller, 1993). Upon infection, the HBV dsDNA

genome is converted to a covalently closed circular (ccc) DNA episome that remains within the nucleus of the infected hepatocyte, where it serves as the template for transcription of viral mRNAs (Newbold *et al.*, 1995). Transcripts consist of the pregenomic mRNA which encodes the viral reverse transcriptase (Pol protein), and the core protein (HBcAg), a component of the virus particle that also participates in reverse transcription and replication of the viral genome (Hatton *et al.*, 1992; Nassal, 1992). Other mRNAs are synthesized that encode precore protein, the three envelope proteins (surface antigens, HBsAg) and the regulatory protein HBx (Nassal and Schaller, 1993). Reverse transcription of the pregenomic mRNA and synthesis of the viral dsDNA genome occurs within core protein particles in the cytoplasm (Summers and Mason, 1982), which are subsequently enveloped by HBsAg and exported from the cell.

Mammalian HBVs are all highly related and are the only HBVs that encode HBx protein. Studies in the woodchuck model system have shown that expression of WHV HBx (WHx) is essential to establish infection, in that direct injection of WHV genomes into the livers of animals establishes infection only if WHx is expressed (Chen *et al.*, 1993; Zoulim *et al.*, 1994). WHx expression is also strongly correlated with active centers of viral replication in the liver during chronic WHV infection (Dandri *et al.*, 1996), and therefore it is thought to be essential for human HBV replication as well. HBx and WHx are highly related and appear to possess similar functional properties. Although HBx and WHx do not bind DNA (Twu and Schloemer, 1987; Spandau and Lee, 1988; Zahm *et al.*, 1988; Yen, 1996), they stimulate a variety of cellular and viral transcriptional elements (Yen, 1996), including RNA polymerase II- and III-dependent promoters (Aufiero and Schneider, 1990; Kwee *et al.*, 1992; Wang *et al.*, 1995). HBx and WHx are predominantly cytoplasmic proteins, but may also be found in small amounts in the nucleus (Doria *et al.*, 1995; Dandri *et al.*, 1998). *In vitro*, HBx binds several transcription factors (Maguire *et al.*, 1991; Cheong *et al.*, 1995; Haviv *et al.*, 1995; Williams and Andrisani, 1995; Haviv *et al.*, 1996; Qadri *et al.*, 1996; Lin *et al.*, 1997), and can stimulate transcription if tethered to a DNA binding domain (Haviv *et al.*, 1995) or experimentally directed to the nucleus (Doria *et al.*, 1995). HBx also binds the DNA repair protein UV-DDB and possibly impairs cellular DNA repair (Lee *et al.*, 1995b; Sitterlin *et al.*, 1997; Becker *et al.*, 1998).

A number of studies have shown that HBx and WHx are *in vivo* cytoplasmic activators of signal transduction pathways in transfected cells (Cross *et al.*, 1993; Kekule *et al.*, 1993; Lucito and Schneider, 1993; Benn and Schneider, 1994, 1995; Natoli *et al.*, 1994; Doria *et al.*, 1995; Wang *et al.*, 1995; Benn *et al.*, 1996; Chirillo *et al.*,

1996; Su and Schneider, 1996, 1997; Cong *et al.*, 1997; Klein and Schneider, 1997; Wang *et al.*, 1997, 1998). The ability to influence cellular signal transduction pathways might be important for HBV and WHV infection and replication, which is the subject of this report. HBx and WHx activate the Ras-Raf-MEK-mitogen activated protein kinase (MAPK) signaling pathway, which is essential for many HBx transcriptional activities, including activation of transcription factor AP1/Fos-Jun and NF- κ B (Cross *et al.*, 1993; Benn and Schneider, 1994; Natoli *et al.*, 1994; Doria *et al.*, 1995; Benn *et al.*, 1996; Chirillo *et al.*, 1996; Su and Schneider, 1996; Klein and Schneider, 1997), and RNA polymerase III-directed transcription (Wang *et al.*, 1997). HBx also modestly stimulates deregulation of early cell cycle checkpoint controls (Koike *et al.*, 1994; Benn and Schneider, 1995) in a manner that involves stimulation of signal transduction pathways (Benn and Schneider, 1995). We have recently demonstrated that HBx and WHx activate Ras signaling by stimulating the Src family of protein tyrosine kinases (Klein and Schneider, 1997).

Here we investigated whether activation of Src or Ras by HBx and WHx proteins is required for viral replication in cultured cells. We show that activation of the Src family of tyrosine kinases by both HBx and WHx promotes strong viral replication by enhancing reverse transcription of packaged viral pregenomic mRNAs. Inhibition of HBx or WHx activation of Src signaling did not strongly impair the formation of viral core particles that contain pregenomic mRNA, and only modestly reduced the level of viral transcription in the short time frame of these studies. Instead, HBx activation of Src signaling was found to stimulate reverse transcription of viral pregenomic mRNA and second-strand genomic DNA synthesis. These results imply a specific requirement for Src-type kinases in mammalian hepadnavirus replication.

Results

HBx and WHx induce activation of Src-Ras signaling during viral replication

Cultured cells cannot be efficiently infected by WHV or HBV, which is thought to result from lack of expression of the viral receptor in cultured cell lines. However, HBV replication in cultured cells can be established by transfection of plasmid cDNA copies of viral RNA genomes (Seeger and Maragos, 1989; Zoulim *et al.*, 1994), or a head-to-tail dimer of viral genomic DNA (Blum *et al.*, 1983). Both templates initiate synthesis of viral mRNAs and polypeptides that reverse transcribe, replicate and package viral genomic DNAs within cytoplasmic viral particles. HBV and WHV replicate to levels 2- to 10-fold less in the absence of HBx or WHx during transient studies in many cell lines (Yaginuma *et al.*, 1987; Colgrove *et al.*, 1989; Nakatake *et al.*, 1993; Zoulim *et al.*, 1994; Lee *et al.*, 1995a; Melegari *et al.*, 1998). Here we show that HBx and WHx activate Src and Ras signaling during the course of viral replication in cultured cells. Chang cells or HepG2 cells were transfected with plasmids encoding wild-type (wt) or HBx(-) forms of HBV or WHV genomes. Initial transfection efficiencies were similar for the different constructs (~30% in Chang cells, 5% in HepG2 cells) based on cotransfection of a green fluorescent

Fig. 1. Prolonged expression of HBx or WHx activates Ras and Src signaling. (A) Chang cells were transfected with head-to-tail dimer genomic forms of HBV encoding wtHBV or a mutant that fails to synthesize HBx, X-21HBV; or pregenomic WHV DNA corresponding to wtWHV or the mutant, CWHV(WHx-). (B) HepG2 cells were transfected with pregenomic forms. Cells were passaged when confluent, then serum-starved for 1 day before analysis of MAPK activity, at 2 weeks post-transfection. Equal amounts of cell lysate were immunoprecipitated with anti-Erk2 antibody, and MAPK activity was measured by *in vitro* kinase assay using the substrate MBP and [γ - 32 P]ATP. Labeled MBP was resolved by 15% SDS-PAGE and visualized by autoradiography. Control vector refers to transfection with plasmid pGEM3 devoid of viral sequences. Results are typical of at least three independent experiments and were quantified by digital densitometry. In the control lane cells were transfected with vector alone.

protein (GFP) reporter (data not shown). Cells were maintained for up to 3 weeks, passaged when confluent and placed in low serum (0.5%) for 1 day before assay of Ras-Raf-MAPK signaling. There was no evidence for cell killing following transfection (data not shown). Activation of immunoprecipitated MAPK Erk2 was assayed to measure HBx or WHx stimulation of the Ras-Raf-MAPK pathway, using *in vitro* phosphorylation of the MAPK substrate myelin basic protein (MBP) with [γ - 32 P]ATP (Klein and Schneider, 1997). As shown for the 2-week samples, Erk2 activity was stimulated ~3- to 4-fold only in Chang cells expressing wtWHV or wtHBV viral genomes, and not in cells containing the HBx(-) virus genomes, CWHV or X21-HBV, nor in control cells (Figure 1A). Examination of Erk2 activity at 1 and 3 weeks (the last time point tested) provided identical results (data not shown). Similar but slightly less pronounced results were observed for activation of Erk2 in HepG2 cells containing wtWHV (Figure 1B). Stimulation of Ras by HBx and WHx was previously shown to result from Src family tyrosine kinase activation (Klein and Schneider, 1997). It was therefore determined whether WHx stimulates Src kinases in the context of WHV gene expression.

Fig. 2. Prolonged expression of WHx stimulates Src kinases. Chang and HepG2 cells were transfected with cDNA forms of pregenomic wtWHV or CWHV(WHx-) plasmid, and a plasmid encoding Csk or dominant inhibitor RasN17. Four days post-transfection, cells were serum-starved and Erk2 or c-Src were immunoprecipitated from equal amounts of cell lysate and their activity measured by *in vitro* phosphorylation of MBP (A) or enolase (B and C), respectively, using [γ - 32 P]ATP. Immunoblots are shown for immunoprecipitated Erk2 or c-Src protein levels. In the control lane cells were transfected with vector alone. Control vector as in Figure 1 legend. Results are typical of at least three independent experiments and were quantified by digital densitometry.

Chang cells and HepG2 cells were transfected with plasmids expressing wt or WHx(-) CWHV genomes, with and without co-expression of the Csk tyrosine kinase. Csk specifically phosphorylates the C-terminus of the family of Src kinases and blocks their activity (Sabe *et al.*, 1992; Nada *et al.*, 1993). Cells were also transfected with the dominant-inhibiting N17 form of p21 Ras (Feig and Cooper, 1988) to block Ras activation. MAPK Erk2 activity was elevated by 3- to 4-fold only in cells containing replicating wtWHV(WHx+), and was inhibited by over-expression of Csk, which blocks activation of Src kinases, or by inhibition of Ras with the N17 inhibitor (Figure 2A). These results implicate HBx stimulation of Src

signaling in stimulation of the Ras-Raf-MAPK pathway in the context of virus gene expression. Activation of c-Src kinase was shown directly by the ability of immunoprecipitated c-Src to phosphorylate the substrate enolase *in vitro* with [γ - 32 P]ATP in Chang cells (Figure 2B) and HepG2 cells (Figure 2C). WHx stimulated Src kinase activity 2.5- to 4-fold, which was blocked by co-expression with the Csk inhibitor of Src. Coexpression of WHx and the N17 Ras inhibitor had no effect, demonstrating that HBx stimulation of Src kinases lies upstream of Ras stimulation. Thus, HBx and WHx proteins moderately stimulate Src kinases and Ras-Raf-MAPK signaling in the context of virus replication in cultured cells (as shown next).

HBx or WHx activation of Src family kinases stimulates viral replication

It was determined whether activation of Src kinases or Ras constitutes a relevant activity of HBx or WHx that might be involved in mammalian hepadnavirus replication. Chang and HepG2 cells were transfected with plasmids encoding wtWHV(WHx+), or the WHx(-) mutant CWHV. The effect of inhibition of Ras or Src kinases on WHV replication in Chang cells was examined by cotransfecting plasmids encoding the RasN17 inhibitor or the Src family inhibitory kinase, Csk. Viral core protein particles (core particles) were isolated from equal numbers of cells at 4 days post-transfection. Core particles contain either viral pregenomic mRNA, or reverse-transcribed viral genomic DNA. Viral DNA genomes range from single-stranded (ss) DNAs to mature circular double-stranded (ds) forms, and hence the population migrates electrophoretically as a smear. The amount of replicated viral dsDNA within core particles was examined by gel electrophoresis and Southern blot analysis. Replication of WHV DNA was reduced 10- to 15-fold in Chang cells by the absence of WHx expression (Figure 3A, compare wt and WHx(-) genomes, lanes 2 and 5). Surprisingly, cotransfection of the Ras N17 dominant inhibitor had little negative effect on WHV replication (Figure 3A, compare lanes 2 and 3). The Ras N17 inhibitor was shown to ablate Ras activity under these conditions (Figure 2). In contrast, transfection of Chang cells with a Csk expression vector strongly impaired WHV replication, reducing it 10- to 15-fold to the low level of replication observed for the CWHV(WHx-) virus (Figure 3A). The expression of Csk was shown to inhibit Src family tyrosine kinase activity under these conditions (Figure 2). The absence of WHx gene expression was also found to impair WHV replication in HepG2 cells. The levels of core particle-associated viral genomic DNA was reduced by ~4- to 5-fold in the absence of WHx (Figure 3B). It is not known why the effect of WHx gene expression on viral DNA replication was somewhat less pronounced in HepG2 cells than in Chang cells. Nevertheless, the ability of WHx to stimulate viral replication, as observed in Chang cells, was demonstrated in HepG2 cells. As the response to WHx was better in Chang cells they were used for the remainder of this study.

Control studies next demonstrated that the poor replication of the CWHV(WHx-) genomes resulted from the lack of WHx gene expression and stimulation of cytoplasmic Src family signal transduction, and not from a mutation of the WHV genome unrelated to WHx activity.

Fig. 3. Activation of Src family kinases is involved in replication of WHV. (A) Chang cells or (B) HepG2 cells were transfected with pregenomic forms of the WHx(-) mutant CWHV. Chang cells were also cotransfected with wtWHV and Csk or the dominant inhibitor RasN17. Four days post-transfection viral core particles were purified from equal numbers of cells, encapsidated viral DNA was extracted and analyzed by Southern blot hybridization. Control refers to cells transfected with vector alone.

Chang cells were transfected with a wild-type or CWHV(WHx-) mutant genome and either wtHBx or an HBx containing a potent nuclear localization signal (HBx-NLS). Previous studies showed that HBx-NLS is relocalized exclusively to the nucleus, which abolishes cytoplasmic activation of signal transduction but not certain nuclear transcriptional activities (Doria *et al.*, 1995; Klein and Schneider, 1997). Viral core particles were isolated 4 days post-transfection, genomic WHV DNA was purified and resolved by Southern analysis. Human wtHBx complemented CWHV replication *in trans* whereas the HBx-NLS mutant did not (Figure 4). Thus, the poor replication of the CWHV(WHx-) genome resulted specifically from the lack of WHx expression, and stimulation of cytoplasmic (Src family kinase) signal transduction.

WHx stimulates reverse transcription of pregenomic mRNA and second-strand DNA synthesis by Pol

Studies were carried out to determine the step at which viral replication was impaired by inhibition or absence of

Fig. 4. Expression of HBx *in trans* complements lack of WHx in WHV replication. Chang cells were transfected with plasmids encoding wtWHV pregenomic viral DNA, the pregenomic CWHV (WHx-) form or CWHV and 10 µg of pCMV-HBx, which expresses the human HBV HBx protein, or pCMV-HBx-NLS, which expresses a variant containing the SV40 T-antigen nuclear localization signal. Four days post-transfection, intracellular viral core particles were isolated, viral DNA was purified and analyzed by Southern blot hybridization. No transf. refers to cells transfected with pCMV vector alone.

Fig. 5. Role of WHx in WHV replication in cell culture. Chang cells were transfected as described for Figure 3. (A) Total cellular RNA was isolated 4 days post-transfection, equal amounts (based on levels of 18S and 28S rRNA, not shown) were resolved by formaldehyde-agarose gel electrophoresis, transferred to nylon membrane and identified by Northern blot hybridization to a ³²P-labeled full-length WHV probe. mRNAs were identified by electrophoretic mobilities relative to standards. Control refers to cells transfected with vector alone. (B) Equal amounts of total cellular protein were resolved by SDS-PAGE and Western immunoblotted with antisera to WHV core protein.

WHx. The dependence of viral transcription on WHx was determined by Northern blot analysis. Equal amounts of whole cytoplasmic RNA were prepared from Chang cells at 4 days post-transfection with wtWHV or the CWHV(WHx-) mutant, with or without cotransfection of plasmids encoding the Ras N17 dominant inhibitor, or the Src kinase inhibitor, Csk (Figure 5A). Steady-state levels of viral pregenomic and preS/S antigen mRNAs were enhanced only 2- to 4-fold in cells containing wtWHV compared with the CWHV(WHx-) genome. These results are consistent with previous reports demonstrating only moderate reduction in viral mRNA levels for HBx mutants of WHV, GSHV and HBV (Yaginuma *et al.*, 1987; Colgrove *et al.*, 1989; Nakatake *et al.*, 1993; Zoulim *et al.*, 1994; H.Lee *et al.*, 1995; Melegari *et al.*, 1998). Inhibition of Ras signaling had little or no effect on viral mRNA levels, while inhibition of the Src family of kinases

decreased the abundance of WHV mRNAs between 2- and 4-fold, to the same extent as in WHx(-) samples. Control studies showed that inhibition of Src kinases by Csk only reduced the activity of the CMV promoter, which drives pregenomic mRNA transcription, by 50% in Chang cells (Klein and Schneider, 1997). Inhibition of Src family kinases therefore does not strongly block synthesis of pregenomic mRNA and cannot account for the stronger inhibition of virus replication in these short-term replication assays. Sustained inhibition of viral DNA replication would ultimately be expected to reduce viral mRNA and protein levels severely as a result of depletion of genome templates for transcription. Immunoblot analysis of cell extracts showed a 2- to 3-fold stimulation of core protein levels resulting from WHx expression during WHV replication (Figure 5B). These results are consistent with core and pregenomic mRNA levels. Thus, the modest reduction in viral mRNA levels, or the absence of WHx, cannot account for the much greater decrease in viral DNA replication observed during the short (3–4 day) period of Src kinase inhibition.

These results indicate that WHx either (i) increases the abundance of core protein particles that package viral genomes, (ii) enhances the packaging of viral pregenomic mRNA into viral core particles, or (iii) stimulates the reverse transcription of viral pregenomic mRNA into genomic DNA. To discriminate between these possibilities, we examined the level of viral core particles and encapsidated pregenomic mRNA. Chang cells were transfected with wtWHV or CWHV pregenomic cDNA for 4 days. WHV core protein particles were purified from equal numbers of cells by sedimentation, resolved by SDS-PAGE and immunoblotted for core protein (Figure 6A). WHx stimulated formation of core protein particles 2-fold compared with Csk inhibition of its ability to stimulate Src kinases. The increase in core particles is consistent with the abundance of core protein (Figure 5B). The significant effect of WHx on WHV replication does not result from a significantly increased abundance of core protein or core protein particles. The level of encapsidated pregenomic WHV mRNA was therefore determined. Core particles were purified at 4 days post-transfection from equal numbers of cells expressing wtWHV or CWHV(WHx-) genomes, or from cells cotransfected with wtWHV and the Csk expression vector to inhibit Src kinases. DNA was exhaustively degraded with DNase I, pregenomic mRNA was extracted, and hybridized to a radiolabeled riboprobe complementary to the 5' end of pregenomic mRNA, and the products resistant to RNase A were resolved by denaturing urea-gel electrophoresis (Figure 6B). Approximately 2-fold higher levels of WHV pregenomic mRNA were evident in core particles obtained from cells expressing wtWHV, compared with CWHV(WHx-) or wtWHV with Csk. RNase A treatment of samples prior to hybridization eliminated protected fragments (no RNA sample), demonstrating that the pregenomic mRNA 5' end was specifically detected. As an independent measure of pregenomic mRNA packaging, the amount of pregenomic mRNA within core particles was determined by semi-quantitative RT-PCR (Figure 6C). Reverse transcription of the 5' end of pregenomic mRNA and PCR amplification showed similar levels of viral pregenomic mRNA in core particles obtained from

Fig. 6. Core-associated pregenomic mRNA levels and reverse transcription. (A–C) Chang cells were transfected for 4 days with wtWHV or CWHV pregenomic cDNA expression vectors and plasmid pCsk, and core protein particles were purified from equal numbers of cells by sedimentation. (A) Proteins were resolved by SDS-PAGE and immunoblotted with WHV core-specific antisera. Mock refers to cells transfected with vector alone. (B and C) Purified core particles were disrupted, core-associated genomic DNA was degraded with RNase-free DNase I, and encapsidated pregenomic mRNA was extracted. Amounts of encapsidated viral pregenomic mRNA were determined by (B) specific RNase A protection and resolved by urea-gel electrophoresis, or (C) semi-quantitative RT-PCR in comparison with internal controls, as shown. No RNA refers to RNase A digestion of samples prior to RNase A protection or RT-PCR assay. (D) Chang cells were transfected with pWHV or pCWHV, or pCWHV and pSrc^{F527}, which expresses a constitutively active form of human c-Src (Cooper and MacAuley, 1988). Core particles were purified from equal numbers of cells and used for analysis of endogenous viral polymerase activity by incubation with [α -³²P]dATP and unlabeled dTTP, dCTP and dGTP. Products of reverse transcription and second-strand DNA synthesis were resolved by electrophoresis in 1% agarose gels containing SDS and visualized by autoradiography.

cells expressing wtWHV, wtWHV with Csk or CWHV(WHx-). Extracts were exhaustively digested with DNase I prior to RT-PCR analysis, to exclude contamination by viral genomic DNA, as demonstrated in the PCR-only samples. RT-PCR was performed semi-quantitatively, as shown by three independent but consistent levels of amplified ribosomal S15 reporter mRNA that was included. The range of experimental error for RT-PCR is inclusive of the 2- to 3-fold change derived from RNase protection, which probably explains the failure to detect differences in packaged pregenomic mRNA by this approach. These results are consistent with WHx promotion of reverse transcription of viral pregenomic mRNA into viral genomic DNA.

To investigate directly whether HBx stimulates Pol activity for reverse transcription and (+) strand DNA synthesis, cytoplasmic core particles were purified from cells transfected for 3 days with either wtWHV, CWHV or CWHV and a plasmid expressing a constitutively activated c-Src kinase (Src^{F527}) (Cooper and MacAuley, 1988). Endogenous viral polymerase activity within core particles was examined by incubating particles with [α -³²P]dNTPs, thereby measuring the activity of endogenous

viral polymerase for reverse transcription and/or second-strand DNA synthesis (Nassal, 1992). Products were resolved by gel electrophoresis and visualized by autoradiography (Figure 6D). Wild-type WHV generated predominantly products of ~3 kb in length descending to ~2 kb, consistent with relaxed circular DNA and linear duplex molecules, respectively (Nassal, 1992). In the absence of WHx protein (CWHV), only very low levels of endogenous polymerase activity were detected, consistent with the reduced levels of core-associated viral genomic DNA. Importantly, a constitutively active c-Src kinase (Src^{F527}) partially (~30%) but not fully stimulated endogenous polymerase activity in CWHV samples. Transfection of increased amounts of Src^{F527} did not further increase polymerase activity (data not shown). Studies have also excluded the possibility that Src kinase activity decreased the rate of virus particle secretion, thereby increasing virus export in the WHx-deficient samples. Although the level of WHV secreted into medium is very low, much higher levels were detected in the presence of WHx expression (data not shown). These results therefore indicate that the stimulation of Src family kinases by HBx or WHx is an important but not sufficient activity for viral replication.

Discussion

This study demonstrated that activation of Src signal transduction by HBx or WHx protein stimulates WHV replication in cultured cells. These results are consistent with those from infection of woodchucks with a WHV deleted of WHx (Chen *et al.*, 1993; Zoulim *et al.*, 1994). In animal studies, infection of hepatocytes in young woodchucks could not be established by the direct injection into the liver of WHx(-) CWHV genomes, whereas injection of wtWHV genomes led to successful infection. The augmentation of WHV replication by WHx or HBx in cultured cells is therefore consistent with animal infection studies in which WHx was essential, but which were not open to the molecular analysis carried out here.

A key result of these studies was the demonstration that WHx strongly enhances viral replication (Figure 3), but only modestly stimulates viral transcription (Figure 5). The increase in viral mRNA abundance by WHx is consistent with observations of others (Yaginuma *et al.*, 1987; Colgrove *et al.*, 1989; Nakatake *et al.*, 1993; Zoulim *et al.*, 1994; Lee *et al.*, 1995a; Melegari *et al.*, 1998), and contrasts with its more pronounced stimulation of viral reverse transcription and DNA replication. These results therefore indicate that in tissue culture WHx promotes the replication phase of the mammalian hepadnavirus life cycle, which is distinct from stimulation of viral transcription. It is important to note, however, that the primary block to viral DNA replication by inhibition of HBx should ultimately reduce viral mRNA levels as well as a secondary consequence. Studies in woodchucks were interpreted similarly when it was noted that in the absence of WHx protein, neither a low level of virus production nor viremia could be detected (Zoulim *et al.*, 1994). Had WHx functioned primarily as an activator of viral transcription, a low level of virus would have been evident. Moreover, the expression of WHx is closely associated with active WHV replication in the infected liver (Dandri

et al., 1996), but it cannot be detected in mature virus particles (Dandri *et al.*, 1996), indicating that WHx indirectly promotes viral replication. Whether a fraction of HBx protein might also function by binding to the DNA repair protein UV-DDB (T.-H.Lee *et al.*, 1995; Becker *et al.*, 1998) has not been examined in our study. It is possible that HBx might also act as a component of the HBV replication complex that is not detectable in viruses. Nevertheless, it is apparent from our results that WHx promotes, but is not essential, for WHV replication in cultured cells. The ability of WHx or HBx to stimulate the Src family of tyrosine kinases was found to be important in HBx stimulation of WHV replication. The fact that HBx or WHx activation of Ras signaling had no effect on viral replication demonstrates that it is Src activation that is specifically involved, rather than the effects of a general increase in cell signal transduction. Because core particles and encapsidated pregenomic viral mRNAs were detected at only 2- to 3-fold higher levels if HBx or WHx were expressed, we conclude that in cultured cells WHx and HBx promote reverse transcription of the packaged viral pregenomic mRNA into DNA. It is not clear why a strong increase in HBx-stimulated reverse transcription is not reflected by a corresponding decrease in pregenomic mRNA levels in core particles. It is possible that packaging of pregenomic mRNA into core particles is not a rate limiting step in this system compared with reverse transcription, thereby accounting for the fairly similar levels of accumulation of core particles containing pregenomic mRNA. Importantly, the HBx stimulation of Src kinases is unlikely to account fully for the role of HBx in viral replication, since WHx could only be partially substituted by a constitutively active c-Src.

At present it is not known how stimulation of Src kinases by HBx or WHx promotes WHV reverse transcription. However, it is interesting to note that the phosphorylation of the C-terminus of the viral core protein is strongly associated with reverse transcription of viral pregenomic mRNA (Hatton *et al.*, 1992; Nassal, 1992). Although it has not been definitively addressed, C-terminal core protein phosphorylation defines several cyclin-dependent protein kinase sites (unpublished results). This is supported by studies that showed the ability of cdc2 kinase to phosphorylate the C-terminus of core protein *in vitro* (Liao and Ou, 1995). Activation of Src kinases can lead to stimulation of cdk activity (Courtneidge *et al.*, 1993; Erpel and Courtneidge, 1995), and HBx stimulates Src kinases (Klein and Schneider, 1997; and this report) and cdks (Benn and Schneider, 1995). In unpublished studies, we have found that WHx stimulates phosphorylation of WHV core protein. We are currently attempting to determine whether this provides a link between HBx activity and its ability to stimulate WHV DNA replication.

A requirement for HBx activation of Src kinases in mammalian hepadnavirus replication in cell culture might indicate that mammalian hepadnaviruses replicate optimally in cells that have exited the G₀ phase of the cell cycle. Few studies have addressed this issue in hepadnavirus replication, although DHBV was observed to initiate DNA replication more strongly in S-phase hepatocytes in culture (Turin *et al.*, 1996). Certain DNA tumor viruses, such as cytomegalovirus, force cells to accumulate at the G₁/early S phase of the cell cycle (Jault *et al.*, 1995). In support

of this hypothesis, Src activation is known to promote deregulation of early cell cycle checkpoints (Kypta *et al.*, 1990; Courtneidge *et al.*, 1993; Dikic *et al.*, 1996), and HBx protein can induce quiescent cells to emerge from G₀ to G₁/early S phase (Benn and Schneider, 1994, 1995; Benn *et al.*, 1996). Alternatively, activation of Src might instead stimulate pathways that promote mammalian hepadnavirus replication exclusive of a need for cell cycle progression. Accordingly, the surprising observation that HBx and WHx activation of Ras was not essential for viral replication might instead indicate a strict dependence on Src signaling that is unrelated to the proliferative state of the cell cycle.

Materials and methods

Cell culture and transfection of cells

Chang and HepG2 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS) and 50 µg/ml of gentamicin. Serum starvation of Chang and HepG2 cells was carried out in 0.05% BCS for 18 h. Cells were transfected by calcium phosphate precipitation for 7 h (10 µg of DNA per 10⁶ cells), then medium was replaced with fresh DMEM containing 10% BCS. Transfection efficiencies were 5–10% for HepG2 cells and 30–40% for Chang cells based on inclusion of a GFP expression plasmid. Cells were serum starved, as indicated in the text, before analysis of viral replication or HBx/WHx protein activities.

Antibodies and plasmids

Rabbit anti-Src, anti-Erk2 and anti-Csk were purchased from Santa Cruz, Inc. Rabbit anti-WHcAg (WHV core protein) was the gift of C. Seeger (Fox Chase Cancer Center). Anti-Flag M2 antibodies were from Kodak-IBI. Plasmid pCsk expresses the *Csk* gene under the control of the CMV promoter. Plasmid pCMV-X expresses wild-type HBV HBx protein controlled by the CMV promoter. Plasmid pCMV-Xo is a mutant that does not express HBx protein. Plasmids pwtWHV and pCWHV (Zoulim *et al.*, 1994) were the gift of C. Seeger (Fox Chase Cancer Center). Plasmid pwtWHV contains a wt cDNA of the pregenomic mRNA of WHV linked to the CMV promoter. Plasmid pCWHV is identical but contains a premature stop codon in the C-terminal third of the WHx coding region, generating a non-functional translation product (Zoulim *et al.*, 1994). Plasmids pHBV-HTD and pX-21HBV (HBx-) contain, respectively, a head-to-tail dimer (HTD) of human wtHBV genomic DNA or a mutant possessing an AUG to GUG alteration in the HBx initiation codon (Blum *et al.*, 1992). HBV HTD plasmids were a gift from J. Liang (NIH). Chang and HepG2 cells were transfected with 10 µg per 10⁶ cells of a head-to-tail genomic plasmid dimer of wt or HBx(-) mutant human HBV (pwtHBV-HTD, or pX-21HBV, respectively) (Blum *et al.*, 1992), or a plasmid containing a genomic cDNA copy of wt or WHx(-) genomes (wtWHV or CWHV, respectively) (Zoulim *et al.*, 1994). Plasmid pSrc^{F527}, which expresses a constitutively active mutant of human c-Src containing a tyrosine to phenylalanine substitution at position 527, was a gift from J. Cooper (Cooper and MacAuley, 1988).

Kinase assays, immunoprecipitations and immunoblotting

Assays of MAPK (Erk2) and c-Src were carried out exactly as described previously (Klein and Schneider, 1997) using equal amounts of fresh cell extracts containing [γ-³²P]ATP and myelin basic protein (MBP) for Erk2 or enolase for c-Src. Labeled substrate was resolved by 15% SDS-PAGE, visualized by phosphorimager analysis and quantified by digital densitometry. Immunoprecipitations were carried out with specific antisera, collected with protein A-agarose (Santa Cruz, Inc.), washed and resolved by SDS-PAGE as described previously (Klein and Schneider, 1997). Immunoblots were visualized using an enhanced chemiluminescence (ECL) system (Amersham). Core protein immunoblots were detected using a rabbit anti-WHV core protein antisera.

Southern blot analysis of core-associated DNA

Chang or HepG2 cells (2 × 10 cm plate per sample) were washed twice with cold phosphate-buffered saline (PBS) and disrupted in 500 µl lysis buffer per 10 cm plate for 15 min (20 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40). Lysates were clarified by centrifugation at 12 000 g for 30 min at 4°C, the pellet was discarded

and the supernatant adjusted to 10 mM MgCl₂, 20 U/ml DNase I, and incubated at 37°C for 2 h. Following DNase treatment, lysates were centrifuged for 5 min at 12 000 g, the supernatant was layered onto a 20% sucrose cushion (300 µl of 20% sucrose in 20 mM Tris-HCl pH 8.0, 150 mM NaCl) and centrifuged for 178 000 g for 3 h at 20°C in a tabletop ultracentrifuge (Beckman). The pellet was resuspended in 250 µl of digestion buffer (10 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS, 1 mg/ml proteinase K) and digested for 16–20 h at 37°C. Samples were extracted once with phenol and twice with phenol:CHCl₃, 100 µl sterile distilled water added and core-associated DNAs were precipitated with 0.2 M NaCl/ethanol and resuspended in 20 µl TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). A 10 µl aliquot of each DNA sample was electrophoresed in a 1.2% agarose gel, transferred to Duralon UV membrane (Stratagene) pre-hybridized, and hybridized using ³²P-labeled DNA probes prepared from full-length WHV genomes.

Northern analysis

Cells were washed and total RNA prepared according to the manufacturer's instructions (RNeasy mini kit; Qiagen). Equal amounts of total RNA were resolved by 1.2% formaldehyde-agarose gel electrophoresis, and hybridized to probes prepared as above.

Core particle-associated pregenomic viral mRNA levels

WHV core particles, purified by centrifugation as described above, were treated with 1 µg/ml RNase A for 30 min at 37°C prior to proteolysis and extraction of nucleic acids. Purified nucleic acids were exhaustively digested with 25 U of RNase-free DNase I for 30 min at 37°C to fully degrade encapsidated viral genomic DNA. One-quarter of each sample was subjected to 30 cycles of PCR using the following primers that correspond to the 5' end of the WHV pregenomic mRNA: (RT and PCR downstream primer: 5'-CTGAGCAGCTTGTTAGAG-3'); (PCR upstream primer: 5'-GGACACTGCTACTGCCTTG-3'). Agarose-gel electrophoresis was carried out to demonstrate the absence of contaminating viral genomic DNA. For RT-PCR analysis, one-quarter of each core-associated RNA sample was reverse transcribed using the downstream primer and the 'RETROscript' kit (Ambion Inc.), for first strand cDNA synthesis by m-MLV reverse transcriptase. Products of RT were then subjected to semi-quantitative PCR using upstream and downstream primers for WHV. After 30 cycles of PCR, products were resolved by agarose gel electrophoresis, stained with ethidium bromide and photographed. Quantitative RT-PCR was performed by comparison with an added internal standard of mouse S15 mRNA as described by the manufacturer (Ambion Inc.). RT-PCR was performed three times from independent experiments. Results for each sample did not vary by >50% relative to the internal control mRNA. RNase protection analysis used core-associated WHV pregenomic mRNA, purified as above from equal numbers of cells. A pregenomic mRNA-specific riboprobe was constructed by subcloning the WHV sequence from base-pairs 1920–2191 into plasmid pGEM3Z, linearized with *Eco*RI and *in vitro* transcription performed on the complementary strand with SP6 polymerase (Promega) and [α-³²P]UTP (100 µCi at 3000 Ci/mmol), using the CRPA III RNase protection system (Ambion Inc.). The 335-nucleotide probe was purified by urea-gel electrophoresis, equal amounts hybridized to mRNA samples, digested with RNase A and the products resolved by urea-gel electrophoresis. Quantitation of the 271-nucleotide protected fragment was performed by phosphorimager analysis and digital densitometry. Each analysis was carried out at least three times and typical results are shown.

Endogenous polymerase assay

Assay was performed using core particles purified as above from equal numbers of cells. Cores were incubated with 12.5 µM dCTP, dTTP, dGTP and 50 µM [α-³²P]dATP (3000 Ci/mmol) in detergent-free buffer for 2 h at 37°C, as described (Nassal, 1992). A chase was performed with 12.5 µM dATP, nucleic acid was extracted as above, purified by Sephadex G-100 fractionation and electrophoresed in 1% agarose gels containing SDS (Nassal, 1992). Gels were dried before autoradiography.

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