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Hepatitis B virus HBx protein deregulates cell cycle checkpoint controls

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ABSTRACT **The human hepatitis B virus (HBV) HBx protein is a small transcriptional activator that is essential for virus infection. HBx is thought to be involved in viral hepatocarcinogenesis because it promotes tumorigenesis in transgenic mice. HBx activates the RAS–RAF–mitogen-activated protein (MAP) kinase signaling cascade, through which it activates transcription factors AP-1 and NF-κB, and stimulates cell DNA synthesis. We show that HBx stimulates cell cycle progression, shortening the emergence of cells from quiescence (G_0) and entry into S phase by at least 12 h, and accelerating transit through checkpoint controls at G_0/G_1 and G_2/M. Compared with serum stimulation, HBx was found to strongly increase the rate and level of activation of the cyclin-dependent kinases CDK2 and CDC2, and their respective active association with cyclins E and A or cyclin B. HBx is also shown to override or greatly reduce serum dependence for cell cycle activation. Both HBx and serum were found to require activation of RAS to stimulate cell cycling, but only HBx could shorten checkpoint intervals. HBx therefore stimulates cell proliferation by activating RAS and a second unknown effector, which may be related to its reported ability to induce prolonged activation of JUN or to interact with cellular p53 protein. These data suggest a molecular mechanism by which HBx likely contributes to viral carcinogenesis. By deregulating checkpoint controls, HBx could participate in the selection of cells that are genetically unstable, some of which would accumulate unrepaired transforming mutations.**

Chronic infection by human hepatitis B virus (HBV) is associated with an increased incidence of human hepatocellular carcinoma (HCC) (1). The HBV HBx protein is a transcriptional activator (2–4) that performs an essential function for viral infection (5, 6). HBx activity has also been linked to liver carcinoma caused by HBV on the basis of its ability to induce liver tumors in transgenic mice in certain genetic contexts (7, 8). HBx is an activator of a variety of RNA polymerase II transcription factors, including AP-1 (9–13), AP-2 (9), NF-κB (14–17), and RNA polymerase III transcription factors (18). The HBx protein stimulates the RAS–RAF–mitogen-activated protein (MAP) kinase cascade, which is required for activation of AP-1 (12, 13) and NF-κB (F. Su and R.J.S., unpublished data). Activation of the RAS–RAF–MAP kinase cascade includes the distantly related extracellular-responsive kinases (ERKs) and c-Jun N-terminal kinases (JNKs), which leads to prolonged expression of c-Jun (unpublished data). Recent studies demonstrate that HBx is a dual function nuclear and cytoplasmic protein, activating specific transcription elements in the nucleus, and RAS-signaling pathways in the cytoplasm (19). In the nucleus, HBx protein might activate transcription directly at the promoter (9, 20), possibly by binding and stimulating transcription factor ATF/CREB (21, 22), the RPB5 subunit of RNA polymerase (23), and TATA-binding protein (24). HBx has also been reported to bind cellular p53 protein *in vitro* (25, 26). It is not known whether HBx stimulation of transcription, activation of RAS (12, 13), or possibly other signaling cascades (11, 27) accounts for its ability to induce focus formation in certain transformation assays (28), promote tumorigenesis when expressed at high levels in transgenic mice (7, 8), or stimulate cellular DNA synthesis (12). We therefore examined the molecular basis for these different HBx activities.

MATERIALS AND METHODS

Flow Cytometry. Chang cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% (vol/vol) calf serum. Cells were made proliferatively quiescent by culture in DMEM containing 0.2% calf serum for 48 h. Quiescent cells were then infected with adenovirus (Ad) vectors Ad-CMV-X or Ad-CMV-X_0 at 25 plaque-forming units per cell (12, 19) and maintained in DMEM containing 10% (vol/vol) calf serum for the indicated times. Cells were lysed in 0.1% Nonidet P-40/50 μg of propidium iodide per ml/100 μg of DNase-free RNase A per ml/5 mM NaCl/10 mM trisodium citrate. Flow cytometry was performed without modification as described (29). Flow cytometry was repeated three times with the variation in results $<20\%$.

Cyclin-Dependent Kinase Assays. Cells were cultured and infected with Ad-X vectors as described in the legend to Fig. 1. Preparation of kinase extracts (extract buffer = 50 mM Hepes, pH 7.0/10 mM $MgCl_2$/1 mM dithiothreitol), immunoprecipitation of CDK2, CDC2, or either kinase in a complex with cyclins A and E or cyclin B, respectively, and assay for kinase activity of immunocomplexes with histone H1 were carried out as described (30). Kinase reactions were performed in a volume of 50 μl containing 5 μCi (1 Ci = 37 GBq) of [γ-^{32}P]ATP, 50 mg of histone H1 per ml, and equal amounts of protein extract. Histone H1 was resolved by SDS/10% PAGE and quantitated by phosphorimage analysis in ImageQuant units (relative kinase activity). Plots represent data averaged for at least two independent experiments. Antibodies to cyclins A, B, and E, and to kinases CDK2 and CDC2 were from Upstate Biotechnology (Lake Placid, NY).

Combined Immunoprecipitation/Immunoblot Analyses. Immunoprecipitation of CDC2 with CDC2-specific antibodies was carried out with equal amounts of cell extracts prepared in kinase buffer as described above. Proteins were resolved by SDS/10% PAGE, transferred to nitrocellulose, and immunoblotted with antibodies to CDC2, cyclin B, or phosphotyrosine. Immunocomplexes resolved by electrophoresis were visualized with the enhanced chemiluminescence system (ECL; Amersham). Results were quantitated by densitometry, and experiments were repeated at least three times.

Transfection of Cells. Chang cells at 20% confluence were starved for 48 h in DMEM containing 0.2% calf serum, then transfected with 10 μg of plasmid DNA per 10-cm plate by the

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Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; Ad, adenovirus.
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calcium phosphate precipitation method as described (12). Cells were then grown in DMEM containing 10% (vol/vol) calf serum. Proliferating cells were nonstarved cells grown to 50% confluency in DMEM containing 10% (vol/vol) calf serum.

RESULTS

HBx Stimulates Proliferation of Quiescent Cells. Flow cytometry was performed to investigate the effect of HBx on cellular DNA synthesis and cell cycle progression. The HBx gene was introduced into quiescent (serum-starved) Chang cells via a replication-defective recombinant Ad vector that expresses HBx in place of Ad genes E1A and E1B (Ad-CMV-X). A control virus (Ad-CMV-X_0) expresses a mutated HBx mRNA lacking all AUG codons (HBx_0). We previously showed that these vectors rapidly express HBx protein (12, 17) and remain largely genetically silent for the duration of these studies (unpublished data). Mammalian cells arrested by serum deprivation at G_0 emerge from quiescence very slowly, when serum is restored, in contrast to cycling cells (31, 32). Compared with cells stimulated by either serum alone or serum plus HBxo (Fig. 1), HBx-expressing cells quickly emerged from G_0/G_1 arrest and entered S phase at least 12 h earlier (12 h vs. 24 h) and to nearly twice the level (38% vs. 25–27% of cells). HBx also accelerated the time of entry and the proportion of cells in G_2/M (32% at 24 h) compared with control cells at 36 h (22%). HBx therefore halved the time of arrest at G_0/G_1 and G_2/M checkpoints and doubled the percentage of proliferating cells. HBx was also found to strongly stimulate cycling of quiescent NIH 3T3 cells (data not shown). Studies could not be conducted in HepG2 cells, a differentiated human hepatocyte cell line, because the extended serum deprivation required for accumulation of quiescent cells resulted in cell death (data not shown). It should be pointed out that not all cells emerged from quiescence, and likely represent a fraction that have undergone terminal withdrawal due to extended growth arrest.

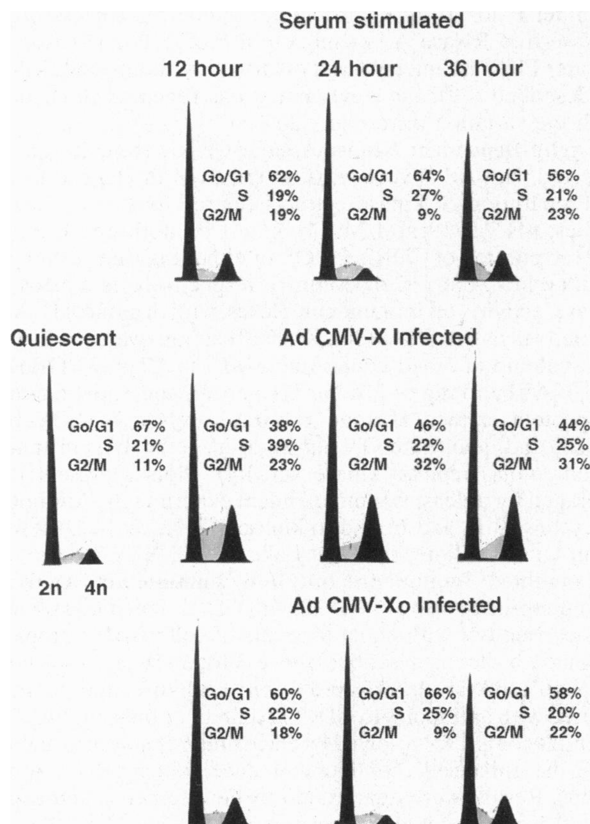

FIG. 1. Effect of HBx on cell cycle kinetics. Flow cytometry histograms of nuclei from quiescent cells expressing HBx or HBx_0 or stimulated with 10% calf serum for 12, 24, or 36 h. DNA content of nuclei was determined within 2 h of cell lysis by flow cytometry using the MODFIT program. Because the nuclear envelope breaks down in M phase, the G_2/M readings reflect G_2 nuclei. Flow cytometry was repeated three times, and the results varied by <20%.

HBx Accelerates Sequential Activation of Cyclin-Dependent Kinases in Quiescent Cells. Emergence of cells from G_0/G_1 into S phase involves sequential activation of CDK2–cyclin E complexes followed by formation of active CDK2–cyclin A complexes (31, 32). The kinetics of activation of cyclins E- or A-CDK2 complexes were examined in uninfected or Ad-X-infected quiescent cells (Fig. 2). Cyclin E- and A-associated kinase activities were determined by immunoprecipitation with cyclin-specific monoclonal antibodies and *in vitro* phosphorylation of the CDK2 substrate, histone H1 (31, 32). Cyclin E-associated kinase was strongly elevated by 3 h in HBx-expressing cells, peaked by 6 h, and declined by 12 h (Fig. 2*A*), consistent with accelerated accumulation of cells in S phase (Fig. 1). In control cells stimulated with 10% serum alone, or with HBx_0 (Fig. 2*A*), cyclin E-associated kinase activity did not appear until 6 h, and then rose only to about half the HBx-stimulated level. Cyclin A-associated kinase activity followed that of cyclin E, and was accelerated 6 h by HBx and rose to a level 2-fold higher than in control cells (Fig. 2*B*). CDK2 kinase activity was directly examined in CDK2 immunoprecipitates by phosphorylation of histone H1 (Fig. 2*C*). HBx induced a strong and rapid (by 6 h) activation of CDK2, as observed for cyclins E- and A-associated activities, 6 h earlier than and about twice the level of control cells. HBx therefore accelerated the emergence of cells from G_0/G_1 and their entry into S phase by facilitating a rapid and strong activation of CDK2 kinase activity.

Entry into mitosis at the G_2/M checkpoint involves stable accumulation of active CDC2–cyclin B complexes (33). Inactive complexes accumulate during S and G_2 phases. Activation in M phase correlates with dephosphorylation of CDC2 at Tyr-15. CDC2 kinase activity was examined in quiescent cells expressing either HBx or HBx_0 or only stimulated with serum by phosphorylation of histone H1 with CDC2 immunocomplexes (Fig. 3*A*), as described for CDK2. HBx induced a rapid and strong increase in CDC2 kinase activity that was not observed in control cells. CDC2 kinase was activated 12 h faster and 2-fold higher by HBx compared with control cells (Fig. 3*A*), consistent with enhanced entry of cells into mitosis (Fig. 1). Activation of CDC2 kinase was also observed when the HBx gene was introduced by transient transfection of cells rather than by infection with recombinant Ad vectors (Table 1). Cells transfected at low confluence were serum starved and then returned to normal growth conditions, and CDC2 kinase activity was measured in immunoprecipitates by phosphorylation of a CDC2 peptide substrate. HBx induced a 3.5-fold higher level of CDC2 activity by 24 h than observed in HBx_0- or serum-stimulated quiescent cells. These data confirm that it is HBx protein which strongly activates CDC2 kinase and not an unexpected activity from the viral vector.

HBx Reduces Serum Dependence for Stimulation of Cycling in Quiescent Cells. Studies were carried out to determine whether HBx induces cell proliferation by overriding serum and RAS dependence. Cells were infected with Ad-X vectors and then returned to low serum (2%) or normal serum (10%), and CDC2 activity was assayed. Quiescent cells returned to 2% serum did not resume proliferation even after 36 h (data not shown). Entry of cells into mitosis was examined by quantitating the activity of CDC2 kinase complexed with cyclin B and the state of CDC2 tyrosine phosphorylation. CDC2 was immunoprecipitated from cell extracts and resolved by gel electrophoresis, and associated cyclin B was detected by immuno-

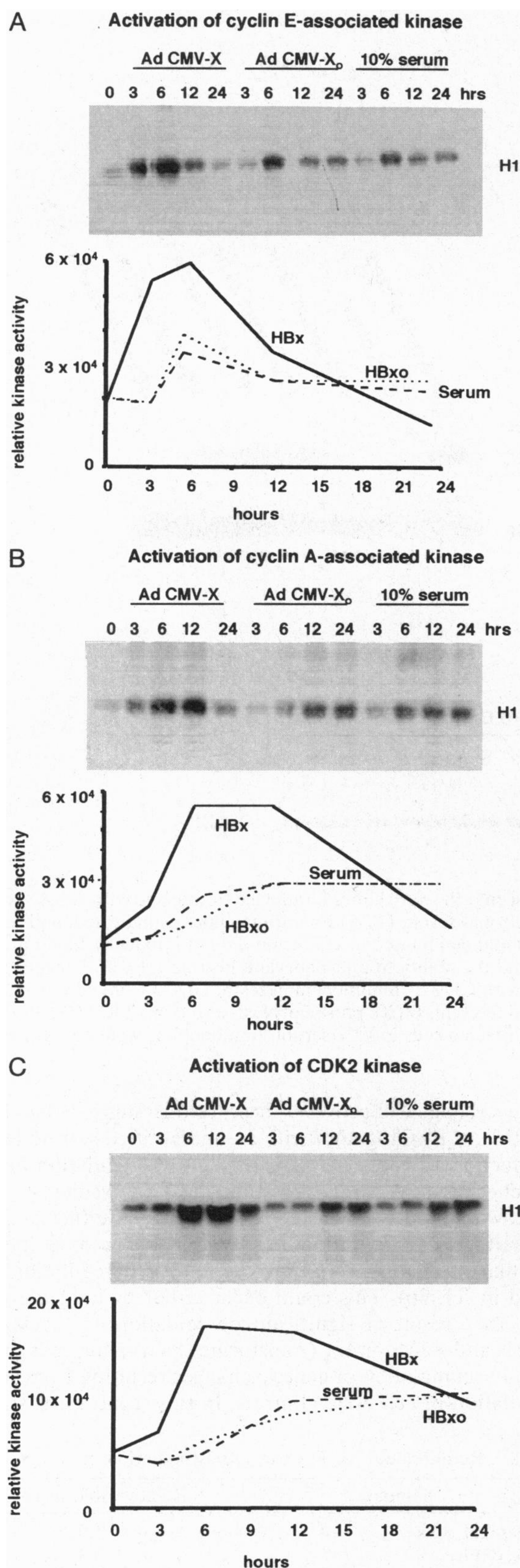

FIG. 2. HBx accelerates rate of induction and magnitude of CDK2 activity. Quiescent Chang cells were stimulated with 10% serum or infected with Ad-CMV-X or Ad-CMV-X_0 vectors and then stimulated with 10% serum. Immunocomplexes of cyclin E (*A*), cyclin A (*B*), or CDK2 (*C*) prepared from equal amounts of cell extracts were assayed for *in vitro* phosphorylation activity of histone H1, a substrate of CDK2. Phosphorylated histone H1 was resolved by gel electrophoresis.

blotting with specific antibodies (Fig. 3*B*). CDC2–cyclin B complexes were detected by 24 h in uninfected cells returned to 10% serum (Fig. 3*B*) but not in HBx_0-expressing cells in low serum. In contrast, cells expressing HBx in 2% serum displayed a striking increase in the rate of formation of CDC2–cyclin B complexes, which were at maximal levels by 12 h. CDC2–cyclin B complexes induced by HBx at 12 h in low serum were found to be active. Activation of the complex at the G_2/M transition requires dephosphorylation of CDC2 at Tyr-15 (33). CDC2 immunoprecipitates were resolved by gel electrophoresis and immunoblotted with anti-phosphotyrosine antibody. In cells expressing HBx in 2% serum, CDC2 was significantly dephosphorylated on tyrosine at 12 and 24 h (Fig. 3*C*). HBx_0-expressing cells in 2% serum did not contain tyrosine-dephosphorylated (active) CDC2, and in uninfected cells in 10% serum slight dephosphorylation was detectable by 24 h. Parallel immunoblots probed with antibodies specific to CDC2 demonstrated equal amounts of the kinase in all samples (Fig. 3*D*). Thus, expedited activation of CDC2 kinase by HBx and movement of cells into mitosis with little requirement for serum provide further evidence that HBx overrides checkpoint controls.

HBx Requires RAS Activation to Stimulate Cycling of Quiescent Cells. To determine whether HBx overrides or requires RAS to activate cell cycling, quiescent cells were first transfected with the Asn-17 RAS dominant-negative mutant, then infected 5 h later with Ad-X or Ad-X_0 vectors. Immunocomplexes of CDC2 were prepared at optimal times for HBx or serum stimulation, and activity of the kinase was determined by *in vitro* phosphorylation of histone H1 (Table 2). The infection of cells shortly after transfection drives the uptake of plasmid into most cells in culture. Both HBx (at 24 h) and HBx_0/10% serum (at 36 h) activated CDC2 kinase compared with unstimulated quiescent cells. Expression of the RAS dominant-negative mutant largely prevented stimulation of CDC2 kinase by HBx or by HBx_0/serum at the respective time points of nontransfected cells. Flow cytometric analysis of cells transfected with the RAS dominant-negative mutant confirmed that HBx and serum require RAS activation to release quiescent cells from G_0/G_1 arrest (data not shown).

DISCUSSION

Several lines of evidence previously implicated HBx protein in HBV-mediated HCC. HBx was shown to induce HCC when expressed at high levels in the livers of transgenic mice (7, 8), to induce foci in cells in certain transformation assays (28), and to stimulate DNA synthesis and proliferation of cultured cells (12, 34). Although the mechanism for carcinogenesis by chronic HBV infection is not known, these studies provide a compelling reason to focus on the HBx gene as a participant in the development of HCC mediated by HBV infection. Moreover, it is now well accepted that, in humans, HBV-mediated oncogenesis does not often involve direct insertional activation of protooncogenes or inactivation of antioncogenes (reviewed in ref. 35). Thus, the ability of HBx to influence cellular signal transduction pathways, particularly those controlled by RAS, offers an attractive alternate possible connection to the mechanism of carcinogenesis.

In this study, we provide a link to molecular carcinogenesis by HBx. We demonstrate that in quiescent cells HBx accelerated sequential activation of cyclin-dependent kinases, causing an inappropriately rapid and high level of activation that was not observed by stimulating resting cells with serum. HBx was found to accelerate transit through checkpoint controls at G_0/G_1 and G_2/M phases of the cell cycle, consistent with accelerated activation of CDK2 and CDC2 kinases and their respective formation of active complexes with cyclins A and E or B. Thus, the ability of HBx to stimulate proliferation of quiescent cells perfectly correlates with deregulation of checkpoint controls by rapid, high-level activation of cyclin-

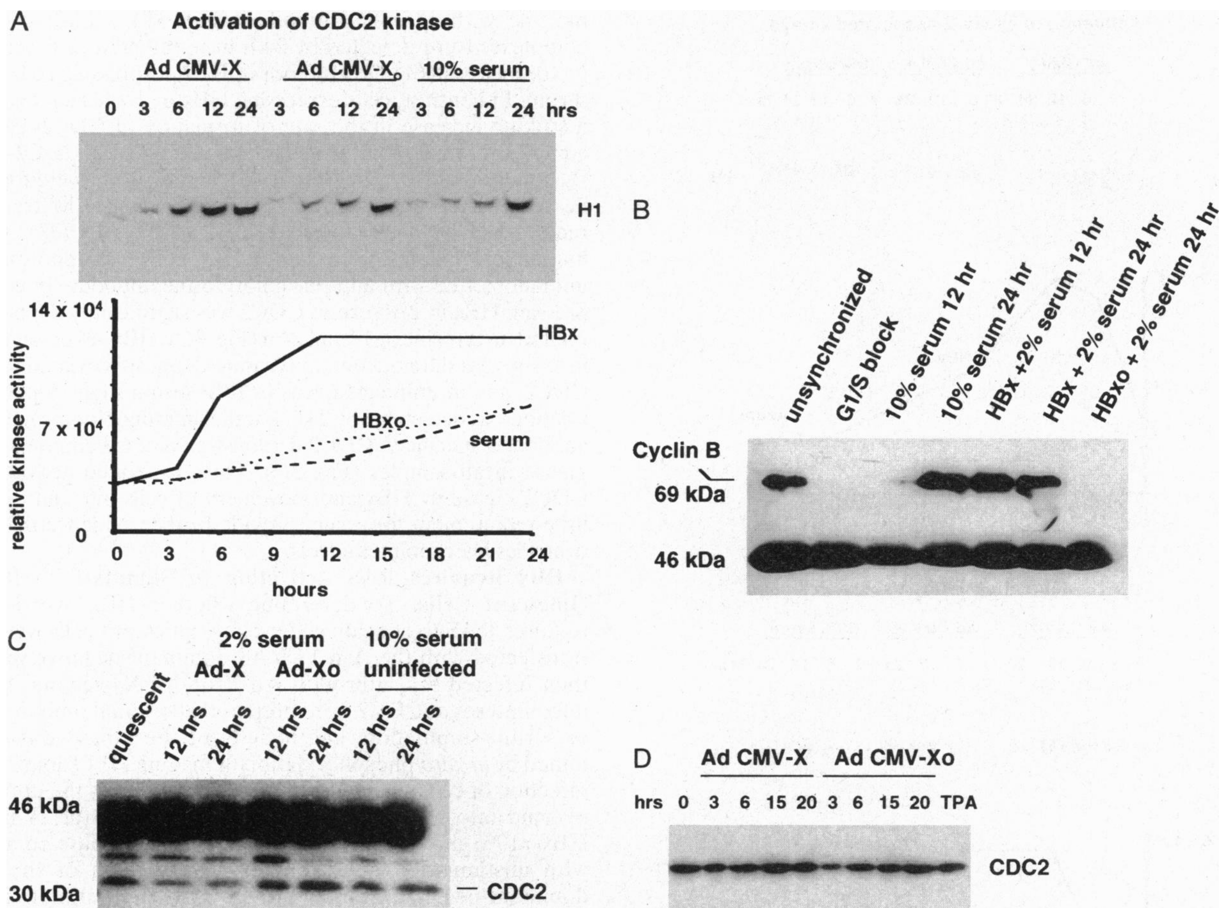

FIG. 3. HBx promotes mitogenesis with little or no requirement for serum. Quiescent cells were either uninfected or infected with Ad-X vectors, as described in the legend to Fig. 1. Cells were maintained in low serum (2%) or normal serum (10%) for various times as indicated, and lysates were prepared as described in the legend to Fig. 2. Uninfected cells made quiescent and maintained in 2% serum did not initiate cycling (data not shown). (*A*) CDC2 was immunoprecipitated from equal amounts of cell extract, and the ability to phosphorylate histone H1 was determined as described in the legend to Fig. 2. (*B–D*) Level and activity of cyclin B associated with CDC2. Immunocomplexes of CDC2 from equal amounts of cell extracts were resolved by PAGE and immunoblotted with antibodies directed to cyclin B (*B*), phosphotyrosine (*C*), or CDC2 (*D*). In *B* and *C*, HBx-expressing cells in 2% serum were compared with HBx$_0$-expressing and uninfected cells in 10% serum. Immunoblots were quantitated by densitometry. Plots represent data averaged from two independent experiments.

dependent kinases. Activation of RAS was essential for stimulation of proliferation in quiescent cells by HBx or serum. However, HBx and serum both stimulate RAS to a similar extent (ref. 8, and unpublished data), but only HBx shortened transit through checkpoint controls. Additionally, HBx could stimulate cycling in quiescent cells in low serum, indicating that HBx reduces the dependence of quiescent cells on serum to initiate cycling. Collectively, these results suggest that HBx activation of Ras, together with an unidentified second effector, which is activated by HBx, mediates deregulation of cell cycle checkpoint controls and stimulates cell cycling.

HBx would ultimately be expected to increase the accumulation of genetic mutations, as described for a variety of molecular mechanisms that involve mitotic deregulation (reviewed in ref. 36). This could occur either by inducing apoptosis, as a result of significant deregulation of checkpoint controls and selection for (transformed) surviving cells, or by gradual accumulation of genetic changes resulting from slight deregulation of cell cycle controls. In this regard, it is signif-

Table 1. Transfection of cells with HBx stimulates CDC2 kinase activity

Sample	Relative fold increase
Quiescent cells	1.0
Proliferating cells	6.0
HBx-transfected cells	3.5
HBx$_0$-transfected cells	1.0

Chang cells at 20% confluency were starved for 48 hours in 0.2% serum, then transfected with plasmids encoding HBx or HBx$_0$ under the control of cytomegalovirus promoter at 10 μg of DNA per 10-cm plate of cells, as described (12). Nontransfected and transfected cells were grown in 10% (vol/vol) serum for 20 h, and immunocomplexes of CDC2 were tested for phosphorylation of histone H1 as described in the legend to Fig. 2. Proliferating cells were nonstarved cells at 50% confluence in 10% (vol/vol) serum. Data represent the average of three trials and were normalized to serum-starved cells (relative fold increase). The minimum phosphorylation of histone H1 for the HBx sample is 10^3 cpm.

Table 2. Requirement for RAS in HBx stimulation of mitogenesis

Sample	Relative fold increase
Quiescent cells	1.0
HBx (20 h)	7.5
HBx/RAS Asn-17 (20 h)	1.8
HBx$_0$/serum stimulated (36 h)	6.0
HBx$_0$/serum/RAS Asn-17 (36 h)	1.5

Chang cells were serum starved, transfected with a plasmid expressing the Asn-17 RAS dominant-negative inhibitor, and infected 5 h later with Ad-CMV-X or Ad-CMV-X$_0$ vectors as described (12). Kinase activity of CDC2 immunocomplexes was determined by phosphorylation of histone H1 as described in the legend to Fig. 3.

icant that when HBx was expressed from transfected plasmids in quiescent cells it still activated cyclin-dependent kinases but at lower levels and at a later time point than in cells infected with Ad-X vectors. HBx is therefore also capable of stimulating a measurable deregulation of checkpoint controls when expressed from plasmids in quiescent cells. It remains to be determined whether HBx expressed from an HBV genome causes deregulation of checkpoint controls and whether this involves its putative interaction with p53 protein (25, 26) or its reported stimulation of *Myc* (37). It is also important to point out that carcinogenesis has not been observed in transgenic mice that contain (and presumably express) the entire HBV genome (38, 39). Tumors only arise when HBx is expressed independently at high levels (7, 8). This suggests that a slight deregulation of cell cycle checkpoint controls mediated by HBx, possibly when expressed at low levels during HBV infection, might be expected to potentiate but not directly mediate the process of cellular transformation that takes place over the many years of chronic viral infection, perhaps by enhancing genetic instability also promoted by factors contributed by the host immune response.

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