HBx Protein of Hepatitis B Virus Promotes Reinitiation of DNA Replication by Regulating Expression and Intracellular Stability of Replication Licensing Factor CDC6*1

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Background: CDC6 is a replication licensing factor, which is tightly regulated to prevent reinitiation of replication.

Results: Hepatitis B virus HBx protein up-regulates CDC6 both transcriptionally as well as post-transcriptionally and promotes its recruitment to β-globin origin of replication.

Conclusion: Accumulation of CDC6 protein under HBx microenvironment facilitates origin licensing.

Significance: Deregulation of replication factors could be a mechanism associated with viral oncogenesis.

Prevention of re-replication via negative regulation of replication initiation proteins, such as CDC6, is key to maintenance of genomic integrity, whereas their up-regulation is generally associated with perturbation in cell cycle, genomic instability, and potentially, tumorigenesis. The HBx oncoprotein of hepatitis B virus is well known to deregulate cell cycle and has been intricately linked to development of hepatocellular carcinoma. Despite a clear understanding of the proliferative effects of HBx on cell cycle, a mechanistic link between HBx-mediated hepatocarcinogenesis and host cell DNA replication remains poorly perused. Here we show that HBx overexpression in both the cellular as well as the transgenic environment resulted in the accumulation of CDC6 through transcriptional and post-translational up-regulation. The HBx-mediated increase in CDK2 activity altered the E2F1-Rb (retinoblastoma) balance, which favored CDC6 gene expression by E2F1. Besides, HBx impaired the APCCdh1-dependent protein degradation pathway and conferred intracellular stability to CDC6 protein. Increase in CDC6 levels correlated with increase in CDC6 occupancy on the β-globin origin of replication, suggesting increment in origin licensing and re-replication. In conclusion, our findings strongly suggest a novel role for CDC6 in abetting the oncogenic sabotage carried out by HBx and support the paradigm that pre-replicative complex proteins have a role in oncogenic transformation.

CDC62 is an indispensable player in regulation of DNA replication in eukaryotic cells. CDC6 gene is transcriptionally regulated in a cell cycle- and E2F1-dependent manner in mammalian cells (1–3), Drosophila (4), and plants (5). In early G1 phase of the cell cycle, CDC6, along with Cdt1 (chromatin licensing and DNA replication factor 1), forms a pre-replication complex with a six-subunit origin recognition complex on the origins of replication followed by recruitment of the minichromosome maintenance complex 2–7 helicases. These molecular events lead to licensing of origins, from which DNA synthesis begins at the onset of S-phase (6, 7).

CDC6 protein must be rendered incompetent for its function after origin firing to ensure prevention of replication reinitiation. In accordance, the cellular levels of CDC6 are under tight control of post-translational modifications, e.g. phosphorylation by cyclin-dependent kinases at serine residues (Ser-54, Ser-74, and Ser-106) localized within the N-terminal domain (NTD) of CDC6 (8–11). During quiescence and G1 phase, CDC6 is a target for proteolysis by the anaphase-promoting complex APCCdh1, which recognizes destruction boxes (D-box and KEN-box) located at the CDC6-NTD (1, 9, 10, 12, 13). The cyclin E/CDK2-mediated phosphorylation of CDC6-NTD masks these destruction boxes and prevents its APCCdh1-mediated proteolysis (14). In support of these studies, it has been reported that p53 activation indirectly triggers degradation of CDC6 by APCCdh1, via down-regulation of CDK2 activity and therefore CDC6 phosphorylation at Ser-54 (15).

It is expected that deregulation of CDC6 has a negative impact on genomic integrity. Indeed, there is mounting evidence strongly suggesting that CDC6 protein possesses oncogenic features, underlying its critical role in malignant cancers (16–19). In fact, elevated levels of CDC6 protein have been reported in brain tumors (20), non-small cell lung carcinomas (21), and a subset of mantle cell lymphomas (22).

Viral oncoproteins have also been known to deregulate replication and cell cycle control (23). HBx, a pleiotropic modular protein encoded by the hepatitis B virus genome, is widely regarded as the etiological factor in development of hepatocellular carcinoma. HBx brings about its pro-proliferative effects through activation of growth-promoting signal transduction cascades and transactivation of genes involved in promoting
proliferation, cell growth, and survival (24, 25). One of the major perturbations caused by HBx in host cells includes dysregulation of cell cycle showing accelerated progression through G1/S phase into S-phase of cell cycle (26–28). Despite ever increasing data proving that HBx adversely affects cell cycle regulatory proteins and causes DNA replication defects, there is no direct evidence linking viral HBx with DNA replication. Further, the regulation of DNA replication proteins such as CDC6 and Cdt1 by HBx is also poorly understood (29).

In the present study, we sought to determine the molecular mechanisms by which HBx could modulate CDC6 functions. HBx appears to exercise a multipronged control over CDC6 activity through transcriptional activation of CDC6 gene via E2F1-Rb axis and stabilization of CDC6 protein via post-translational modifications and impairment of APC/Cdh1 proteolytic machinery. Further, we show that increased CDC6 levels correlated with its specific enrichment on the β-globin origin of replication that seems to favor origin licensing, re-replication, and unscheduled entry of cells into S-phase.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and DNA Transfections**—Maintenance of human hepatoma HuH7, HepG2, HEK293 (ATCC CRL-1573), and HepG2.2.15 cells was described elsewhere (30). Immortalized human hepatocyte (IH) cell line and HepG2.2.15 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO2. Transfection was carried out in a 60-mm culture dish (0.6 × 106 cells) with 2.0 μg of the indicated plasmids by Lipofectamine (Invitrogen) according to the manufacturer’s instructions. EGFP-N1 (0.5 μg) was used in each experiment as a transfection control. For reporter assays, 0.2 μg of Luciferase reporter plasmids was co-transfected with 0.5–1 μg of indicated expression plasmids.

**Recombinants, Reagents, and Antibodies**—Construction of the eukaryotic expression vectors for wild-type HBx (X0) and its deletion mutant (X9) was as described previously (31). pEGFP-N1 was procured from Clontech. Luciferase reporter constructs CDC6-WT, CDC6-SM1, pGL3-E2F1-WT, and pGL3-E2F1-mut were kindly provided by Kristian Helin (1, 32). The expression vectors for wild-type E2F1 (E2F1-WT) and its deletion mutant (X9) was as described previously (31). Antibodies were obtained from the following sources: CDC6, phospho-CDC6 Ser-54, E2F1, Rb, phospho-Rb Ser-807, p27Kip1, phospho-p27Kip1, and GAPDH were from Santa Cruz Biotechnology, and Cdh1 was from Abcam. The production of monoclonal antibody against HBx has been reported earlier (34). The polyclonal antiserum against HBx was raised in rabbit using recombinant HBx produced in *Escherichia coli* (34).

**Luciferase Assay**—Luciferase assay was performed according to the manufacturer’s instructions (Promega). The relative luciferase activities were measured after normalizing each sample with protein amount, and transfection efficiency.

**Double Thymidine Block**—48 h after transfection, cells were incubated in complete medium containing 2 mM thymidine for 10–12 h following which cells were washed twice with 1× PBS and incubated in complete medium for 12 h. Subsequently, for second block, cells were again incubated in complete medium containing 2 mM thymidine for 10–12 h. Later, cells were washed twice with 1× PBS and induced to enter S-phase by incubation in complete medium. Harvesting of cells was carried out at the indicated time points.

**Flow Cytometry (FACS, Fluorescence-Activated Cell Sorting)**—HuH7 and IHH cells 24 h after transfection were starved for 72 h and then stimulated with serum for the indicated time periods. Flow cytometry of cells was done as described earlier (35).

**RNA Isolation and Quantitative RT-PCR Assays**—Total RNA was isolated from cells using TRIzol reagent as per the supplier’s instructions (Invitrogen). Reverse transcription-PCR (RT-PCR) was performed with M-MuLV reverse transcriptase (Fermentas) according to the manufacturer’s guidelines. The real-time quantitative PCR (qPCR) was done using specific primers (supplemental Table 1) as described previously (30).

**Western Blotting**—Western blotting of protein samples was done as described previously (30).

**EMSA (Electrophoretic Mobility Shift Assay)**—In vitro binding of E2F1 to E2F1 response elements was performed by EMSA as described earlier (30). The end labeling of oligonucleotides (supplemental Table 2) was done using [γ-32P]ATP and T4 polynucleotide kinase (Fermentas) as per the supplier’s protocol.

**ChIP-qPCR Assay**—Chromatin immunoprecipitation (ChIP) assay was carried out as described earlier (30). Chromatin obtained was purified using the QIAquick PCR purification kit (Qiagen). The eluted genomic DNA was subjected to either semiquantitative PCR or SYBR Green real-time qPCR with the indicated primer sets (supplemental Table 3). The results were expressed as -fold enrichment over mock.

**X15-myc Transgenic Mouse Model**—Development of the X15-myc transgenic mouse model has been reported earlier (36). The liver samples were processed for RNA isolation and Western blotting according to Janbandhu et al. (30).

**Immunoprecipitation**—Cell lysates were prepared in cell lysis buffer. Protein estimation was carried out for cleared lysates using Bradford’s reagent. Equal amounts of protein diluted in cell lysis buffer were incubated with 1 μg of antibody overnight at 4 °C. Protein A-Sepharose beads were added, and further incubation was carried out at 4 °C. Subsequently, the beads bound to protein complexes were washed with lysis buffer and then resuspended in 2× SDS dye and boiled, and eluates were resolved on 10% SDS-PAGE.

**Statistical Analysis**—Data are expressed as mean ± S.E. Means were compared by one-factor analysis of variance followed by Fisher’s protected least significant difference to assess specific group differences. Differences were considered significant at *p* < 0.05.
RESULTS

HBx Regulates Stability of Origin Licensing Factor CDC6—As deregulation of CDC6 is frequently seen in many human cancers (19–22), its involvement in hepatocellular carcinoma was investigated under the HBx microenvironment. The human hepatoma Huh-7 cells were transfected with control vector or expression vectors for wild-type HBx (X0) or its mutant X9 lacking the mitogenic signaling domain (35). After synchronization at G1/S transit by double thymidine block, the cells were stimulated by the addition of serum to growth medium to enter S-phase and harvested at the indicated time points. The distribution of cells in various phases of cell cycle is shown in supplemental Fig. 1C. There was a progressive increase in CDC6 protein levels in the presence of HBx, but it remained unaffected in the case of control and mutant X9 (Fig. 1A). The cells were also subjected to serum starvation followed by release as above. It has been reported earlier that CDC6 levels remain undetectable or very low in quiescent and early G1 cells (13, 14). This indeed was the case with vector and X9-transfected cells in our experiments. However, HBx overexpression led to CDC6 accumulation in G1 cells, whereas the control or X9-expressing cells failed to do so (Fig. 1B). Thus, viral HBx appeared to confer stability to CDC6 protein or induce its expression in cells.

HBx Promotes CDC6 Stability by Increasing Its Phosphorylation at Ser-54—CDC6 phosphorylation at Ser-54 by CDK2 is well known to interfere with its association with the anaphase-promoting complex/cyclosome, prevent its proteolytic degradation, and promote origin licensing (9, 10, 14). Because viral HBx is known to potentiate the kinase activity of CDK2 (35), we examined the status of CDC6 phosphorylation in the HBx microenvironment. As shown in Fig. 1C, only X0 overexpression promoted CDC6 phosphorylation at Ser-54 as cells re-entered S-phase. Thus, HBx seemed to stabilize CDC6 protein by increasing its specific phosphorylation.

HBx Helps in Accumulation of CDC6 in Transgenic Environment—As our studies on synchronized cell population alluded to increase in CDC6 levels in the presence of HBx, we further investigated the regulation of CDC6 under more physiological conditions. The levels of total and phosphorylated CDC6 were measured in HepG2.2.15 cells that carry chromosomally integrated sequences of HBV genome and express HBx gene (37). In agreement with transfection studies, the levels of total and phosphorylated CDC6 were observed to be higher in HepG2.2.15 cells as compared with HepG2 cells (Fig. 2A). Besides, the level of APCCdh1 was found to be down-regulated. To ascertain the role of CDK2 in CDC6 phosphorylation, HepG2.2.15 cells were treated with CDK2 inhibitor II (Calbiochem), and the levels of CDC6 and other physiological targets of CDK2 were measured. As shown in Fig. 2B, there was a conspicuous decrease in the levels of both total and phosphorylated CDC6 Ser-54. Further, as expected, a decline in the levels of phosphorylated retinoblastoma (pRb) and p27Kip1-Thr-187 was also observed.
Next we investigated the status of CDC6 in the X15-myc transgenic mouse model of hepatocellular carcinoma that shows hepatotropic expression of HBx gene (36). Expectantly, a 2-fold increase in total CDC6 levels and a 4.8-fold rise in phosphorylated CDC6 Ser-54 levels were observed in the transgenic mouse liver as compared with control. Further, concurrent with the observation made in HepG2.2.15 cells, a notable decrease in Cdh1 level of mouse liver was also observed. Taken together, these results suggested that HBx promotes CDC6 stability by promoting its phosphorylation at Ser-54 and destabilizing APCCdh1.

HBx Cooperates with E2F1 for Transcriptional Activation of CDC6 Gene

HBx has been implicated in the transcriptional up-regulation of DNMT1 gene via a regulatory circuit involving the p16INK4a-cyclin D1-CDK4/6-pRb-E2F1 pathway (38), whereas E2F1 is known to regulate the cell cycle-dependent expression of the human CDC6 gene (1, 2). In view of these studies, we probed the possibility of CDC6 gene activation by HBx. The real-time quantitative RT-PCR (RT-qPCR) analysis of RNA isolated from cells overexpressing HBx showed a significant increase (p < 0.05) in the levels of CDC6 transcripts (Fig. 3A). Interestingly, a 2.5-fold increase (p < 0.01) in CDC6 expression was also seen in HepG2.2.15 cells (Fig. 3B). HBx-induced DNA re-replication by CDC6 protein.

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Next we examined the modulatory action of E2F1 on CDC6 promoter under the HBx microenvironment. Huh7 and HEK293 cells were transfected with wild-type HBx along with pGL3 basic (vector). CDC6-WT, or CDC6-SM1 luciferase reporter constructs (containing wild-type and mutated E2F1-binding site, respectively) (supplemental Fig. 2A). A 2-fold increase in luciferase activity was observed in Huh7 cells in the presence of HBx (Fig. 4A), whereas an 8-fold increase was observed in HEK293 cells with CDC6-WT under similar conditions (supplemental Fig. 2B). Interestingly, HBx could not stimulate CDC6-SM1 reporter. These results suggested that E2F1 and HBx cooperate to stimulate CDC6 gene. The molecular cooperation between HBx and E2F1 was further investigated in our co-expression studies using the above reporter recombinants. As shown in Fig. 4B, a massive 14-fold increase in the reporter activity was observed upon co-expression of HBx and E2F1 as compared with HBx alone, suggesting the existence of a concert between HBx and E2F1 in modulating CDC6 promoter activity. The synergy between HBx and E2F1 was specific because co-expression of HBx with mutant E2F1-(1–374) failed to stimulate the reporter gene. To substantiate the synergy between HBx and E2F1, the relative levels of CDC6 transcript were also examined using specific primers.
We observed a 3-fold increase in the *CDC6* transcripts in the presence of HBx and E2F1, which was abrogated in the presence of mutant E2F1 (1:1 ratio) (Fig. 4C). Thus, HBx and E2F1 seem to work cooperatively to stimulate *CDC6* gene.

**HBx Augments E2F1 Occupancy on CDC6 Promoter**—In view of the synergy shown by HBx and E2F1 in the transactivation of *CDC6* gene, we next analyzed the binding characteristics of E2F1 to its response element in the presence of HBx. Nuclear extracts of Huh-7 cells expressing control vector or HBx were incubated with consensus E2F1-binding element or that derived from *CDC6* gene (supplemental Table 2), and the protein-DNA complexes were resolved by electrophoretic mobility shift assay (EMSA). As depicted in Fig. 5, the *CDC6*-derived E2F1-binding element showed a specific band (lanes 4 and 5) akin to the oligonucleotides harboring consensus E2F1-binding motifs (lanes 1 and 2). It is noteworthy that nuclear extracts derived from the HBx-expressing cells produced a more prominent protein-DNA complex than the control extract. The specificity of the E2F1-DNA complex was evident from its competitive displacement by 50-fold molar excess of unlabeled wild-type response element (lanes 6 and 7), but not by mutant response element (lanes 8 and 9), and its characteristic supershift in the presence of anti-E2F1 antibody (lanes 10 and 11).

Thus, these data provide compelling evidence in favor of the role of HBx in *CDC6* gene transcription, which involves a surge in E2F1 recruitment on *CDC6* promoter.

As HBx promoted the recruitment of E2F1 on *CDC6* promoter for modulating its transcription, we next studied the regulation of E2F1 promoter that is also known to carry two proximal E2F1-binding sites (supplemental Fig. 3). The E2F1 luciferase reporter constructs harboring either wild-type (pGL3-E2F1-WT) or mutant E2F1-binding site (pGL3-E2F1-mut) were transfected into asynchronously growing Huh7 cells along with HBx or control expression vectors. As shown in Fig. 6A, HBx expression led to a significant 2-fold increase (p < 0.05) in the pGL3-E2F1-WT reporter gene activity as compared with control. As expected, the pGL3-E2F1-mut reporter gene was essentially unresponsive to HBx, supporting our earlier observations on the molecular cooperation between HBx and E2F1 in activation of its target genes. The involvement of other host factors in the stimulation of E2F1 gene is not ruled out because of partial stimulation of the mutant reporter in the presence of HBx.
HBx Induced DNA Re-replication by CDC6 Protein

The tumor suppressor Rb is well documented to sequester E2F1, which is essential for the activation of S-phase genes. Further, hyperphosphorylation of Rb by CDKs functions as a molecular switch for the progression of cell cycle, differentiation, and apoptosis (39). Not surprisingly, there is deregulation of the critical balance between Rb-E2F1 in most human cancers. In light of the molecular cooperation shown by E2F1 and HBx in the up-regulation of CDC6 and E2F1 genes, we monitored the status of pRb-E2F1 axis in the transgenic environment. The Western blot analysis of the liver tissue lysate of transgenic mice showed a marked (3-fold) increase in the level of hyperphosphorylated Rb as compared with control mice (Fig. 6B). Besides, a conspicuous decrease in the Rb-E2F1 interaction was also observed in these animals despite an overall increase in E2F1 protein levels (Fig. 6C). These results were corroborated by our findings in HepG2.2.15 cells where, similar to transgenic mice, elevated levels of E2F1 and phosphorylated-Rb were observed. Collectively, these data suggest that HBx overexpression in both cellular and transgenic environment disturbs the delicate balance between Rb and E2F1, resulting in more free E2F1 available for stimulation of target genes, including CDC6.

HBx Promotes CDC6 Occupancy on Human β-Globin Origin of Replication—CDC6 and Cdt1 are two well established origin licensing factors that nucleate the assembly of pre-replication complex that controls DNA synthesis during S-phase (6). In view of the profound effect of HBx on the expression and intracellular stability of CDC6, we checked the occupancy of CDC6 on the human β-globin origin of replication in the presence of HBx. The chromatin extracts of HuH7 cells having enforced expression of control, HBx (X0), or X9 expression vectors were immunoprecipitated with control serum or antibodies against CDC6 or CDC6 Ser-54 and analyzed by RT-qPCR (Fig. 7) or semiquantitative PCR (supplemental Fig. 4) using primers specific to the human β-globin locus and non-origin γ-globulin locus (supplemental Table 3). A significant 3-fold increase (p < 0.01) in CDC6 occupancy was observed on human β-globin origin in the presence of HBx as compared with control (Fig. 7A). Intriguingly, a similar increase in the association of CDC6 Ser-54 was observed on the origin sequence (Fig. 7B). Under these conditions, no enrichment of CDC6 was seen at the non-origin human γ-globulin locus (supplemental Fig. 4B). In concurrence with the aforementioned observations, the occupancy of human β-globin origin of replication by CDC6 protein was also found to be significantly enriched (p < 0.01) in HepG2.2.15 cells as compared with HepG2 cells (Fig. 7C). Thus, the surplus CDC6 available in the cellular milieu as a consequence of HBx overexpression is actively and specifically recruited to the origins of replication, which may promote pre-replication complex formation and origin licensing.

HBx Facilitates Entry of Cells into S-phase—As HBx up-regulated CDC6 levels and promoted its occupancy on the human β-globin origin of replication, we hypothesized that HBx may facilitate inappropriate entry of cells into cell cycle, specifically S-phase. To test this hypothesis, HepG2 and HepG2.2.15 cells were labeled with 5-bromo-2′-deoxy-uridine (BrDU) for 1 h prior to fixation and stained for BrDU incorporation. Confocal microscopy of HepG2.2.15 cells showed a significantly higher incorporation of BrDU label as compared with HepG2 cells (Fig. 8A). Besides, the number of BrDU-positive cells was significantly higher (~2.5-fold, p < 0.05) in HepG2.2.15 as compared with HepG2 cells (Fig. 8A, right panel). These observations were further validated in IHH cells after transiently transfecting control or HBx expression vectors (X0 and X9). As expected, the HBx-expressing cells exhibited a significant 4-fold higher BrDU incorporation (p < 0.01) when compared with either control or X9-expressing cells (Fig. 8B). These observations strongly suggested that HBx-dependent dysregulation of CDC6 could lead to unscheduled entry of cells into S-phase.

DISCUSSION

The replication licensing factor CDC6 is essential for the maintenance of genomic integrity, and its deregulation is generally associated with perturbation in cell cycle, genomic instability, and tumorigenesis. The HBx oncoprotein, on the other hand, is well known to deregulate cell cycle and has been implicated in the development of hepatocellular carcinoma. Although the proliferative effects of HBx on cells have been extensively studied, there is no direct evidence linking viral HBx with DNA replication. Further, the regulation of DNA replication proteins like CDC6 and Cdt1 by HBx is also poorly understood. In the present study, we have probed into the molecular mechanisms by which HBx could regulate CDC6 functions.

Although CDC6 has been known to be tightly regulated in cell cycle, among other factors, by APC\(^{Cdh1}\) master controller of origin licensing, the important link between HBx oncoprotein,
HBx Induced DNA Re-replication by CDC6 Protein

![Diagram A](image1.png)  ![Diagram B](image2.png)  ![Diagram C](image3.png)

**FIGURE 7.** CDC6 occupancy on human β-globin origin of replication in presence of HBx. IHH cells were transfected with control or expression vectors for wild-type (X0) or mutant HBx (X9). After synchronization by serum starvation for 72 h, cells were incubated in complete medium and harvested after 12 h for ChIP assay using anti-CDC6 (A) or anti-phospho-CDC6 antibodies (B). C. asynchronously growing HepG2 and HepG2.2.15 cells were subjected to ChIP using anti-CDC6 antibody. Normal rabbit serum was used as negative control in ChIP assays. -Fold DNA enrichment over mock due to CDC6 occupancy on human β-globin origin of replication and non-origin γ-globulin locus was measured by ChIP-qPCR using primers specific for each locus (supplemental Table 3). The data are shown as mean ± S.D. of three independent experiments. * and #, statistically significant difference at $p < 0.05$ and $p < 0.01$, respectively.

CDC6, and APC\textsuperscript{Cdh1} has not previously been appreciated. Our clear demonstration that Cdh1 is down-regulated in the presence of HBx leading to elevation in the levels of CDC6 emphasizes both the significance of regulation of this well known ubiquitin/proteasome system in mammalian cells and the establishment of this system in HBx-induced oncogenic phenotype. The targeted degradation of CDC6 by anaphase-promoting complex/cyclosome may be part of a mechanism designed to restrict cell proliferation. Indeed, CDC6 has been found to be up-regulated in cervical, lung, and brain cancer cells (19, 21, 40, 41). Complementing these observations, it has been reported that APC\textsuperscript{Cdh1} substrates are overexpressed in many tumors and are associated with chromosomal instability and poor prognosis (43), adding relevance to our conclusions. More significantly, down-regulation of Cdh1 has been reported in many cancers, including those of prostate, ovary, liver, and brain, and during the malignant progression of a B-lymphoma cell line (43, 44). CDC6 stabilization in quiescence and in early to late G\textsubscript{1} and S-phase by HBx-mediated Cdh1 down-regulation makes this replication factor available in a window of time where under normal circumstances it is kept under check. This surplus CDC6 can participate in licensing of origins in quiescence and early G\textsubscript{1} phase, which may induce untimely initiation of DNA replication. In agreement with this observation, it has been reported that HBx-expressing murine AML12 hepatocytes overexpress CDC6 and Cdt1 origin licensing factors and exhibit polyploidy (29).

The phosphorylation of CDC6 at Ser-54 by cyclin E-CDK2 activity is well known to confer protection against proteolytic degradation in cell (14). The present study provides first evidence of CDC6 accumulation in the HBx microenvironment (both in cell culture as well as in vivo) via CDK2-mediated enhanced phosphorylation at Ser-54. Earlier we showed the potentiated CDK2 activity in the presence of HBx (35). However, this study did not include the influence on CDC6, which is a well known target of CDK2. Significantly, the CDK2 inhibitor assay in HepG2.2.15 cells provided irrefutable evidence substantiating that CDK2-mediated phosphorylation at Ser-54 was indeed instrumental in stabilizing CDC6 protein in the HBx microenvironment. Considering that APC\textsuperscript{Cdh1} and cyclin E-Cdk2 have opposing effects on CDC6 stabilization, it is interesting to note that HBx, setting yet another example of its pleiotropic behavior, manipulates both the pathways to bring about accumulation of CDC6, and thus, help the nucleation of pre-replication complex assembly.

We also report here that HBx regulates CDC6 expression during early G\textsubscript{1}-phase of cell cycle where E2F1 and HBx seem to work in concert to transcriptionally stimulate CDC6. Further, the observations made in transgenic mice liver tissue convincingly exhibit perturbation of the Rb-E2F1 axis in the presence of HBx by transcriptional up-regulation of E2F1 gene itself and hyperphosphorylation of tumor suppressor Rb. Consequently, more E2F1 is available in free form to transcriptionally activate its target genes, most of which are involved in progression through G\textsubscript{1} and into S-phase of cell cycle. Our observations are in concert with the finding that in most human neoplasia, genetic or epigenetic alterations occur that ultimately result in the deregulation of E2F1-dependent transcription (45).

It is of utmost importance to this study to perseus the physiological fate and activity of increased levels of CDC6 in the HBx microenvironment. Previously, several studies have dwelt upon the consequences of deregulation of CDC6 and bestowed upon it certain oncogenic features, i.e. accelerated G\textsubscript{1} to S transition observed in G\textsubscript{1} nuclei incubated with S-phase extracts supplemented with CDC6 (16), the cooperation of CDC6 with cyclin E to induce DNA replication in quiescent cells (17), and the DNA re-replication observed in tumor cells upon ectopic expression of CDC6 and Cdt1 (18). An important addition to these studies is the recent finding that pre-replication complex proteins, including CDC6, are present more abundantly on origins of replication in transformed cells as compared with normal cells (46). In light of these studies, a comparative analysis of the in vivo association of CDC6 to human β-globin origin of replication has revealed that surplus CDC6 is actively and specifically recruited to origins of replication in the presence of HBx, facilitating increased origin licensing. The differential binding of
CDC6 to human β-globin origin of replication in the absence and presence of HBx is concurrent with the suggestion that there is a pre-replication complex checkpoint lacking in cancer cells (47). As a consequence, more numbers of origins of replication will be competent for firing in the S-phase, eventually leading to early S-phase entry and progression. This hypothesis was proven by applying the BrdU incorporation assay, which showed a greater number of BrdU-positive cells in the HBx-overexpressing cell population as well as in HepG2.2.15 cells (Fig. 8, A and B). Not only has it been reported that overexpression of CDC6 leads to S-phase entry (1), but also that HBx hastens the S-phase progression (35). Hence, our observations provide compelling evidence that HBx overexpression causes unscheduled entry of cells into S-phase. Collectively, it may be inferred that HBx-mediated oncogenesis can, in part, be owed to CDC6 being increasingly recruited to origins of replication, heralding higher than normal origin licensing, ultimately leading to faster and unscheduled entry and progression through S-phase.

Our results indicate that deregulated increase in levels of CDC6 protein in the presence of HBx may be an early event in the stepwise progression to cancer. It is conceivable that the
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