Hepatitis B Virus X Protein Differentially Regulates Cell Cycle Progression in X-transforming Versus Nontransforming Hepatocyte (AML12) Cell Lines*

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Hepatitis B virus (HBV) X protein (pX) is implicated in hepatocarcinogenesis of chronically infected HBV patients. To understand mechanism(s) of pX-mediated cellular transformation, we employed two tetracycline-regulated, pX-expressing cell lines, constructed in AML12 immortalized hepatocytes: one a differentiated (3pX-1) and the other a de-differentiated (4pX-1) hepatocyte cell line. Only 3pX-1 cells undergo pX-mediated transformation, via sustained Ras-Raf-mitogen-activated protein kinase pathway activation. pX-nontransforming 4pX-1 cells display sustained, pX-dependent JNK pathway activation. To understand how pX mediates different growth characteristics in 3pX-1 and 4pX-1 cells, we report, herein, comparative cell cycle analyses, pX-transforming 3pX-1 cells display pX-dependent G1, S, and G2/M progression evidenced by cyclin D1, A, and B1 induction, and Cdc2 kinase activation. pX-nontransforming 4pX-1 cells display pX-dependent G1 and S phase entry, followed by S phase pause and absence of Cdc2 kinase activation. Interestingly, 4pX-1 cells exhibit selective pX-induced expression of cyclin-dependent kinase inhibitor p21Cip1, tumor suppressor p19ARF, and proapoptotic genes bax and IGFBP-3. Despite the pX-mediated induction of growth arrest and apoptotic genes and the absence of pX-dependent Cdc2 activation, 4pX-1 cells do not undergo pX-dependent G2/M arrest or apoptosis. Nocodazole-treated, G2/M-arrested 4pX-1 cells exhibit pX-dependent formation of multinucleated cells, similar to human T-cell lymphotropic virus type I Tax-expressing cells. We propose that in 4pX-1 cells, pX deregulates the G2/M checkpoint, thus rescuing cells from pX-mediated apoptosis.

Epidemiological evidence (1) links chronic hepatitis B virus (HBV) infection in humans to development of hepatocellular carcinoma (HCC). Evidence derived from comparative studies of mammalian and avian hepadnaviruses, transgenic animal models of mammalian and avian hepadnaviruses, transgenic animal models of hepatocarcinogenesis (4–7), woodchuck hepatitis virus-mediated HCC (8), and human liver pathologies (9, 10) point to the undifferentiated (oval cell) or less-differentiated (transitional) hepatocyte as the precancerous precursor in HCC development.

Activities ascribed to pX probably linked to HBV-mediated pathology include activation of the Ras-Raf-MAPK (11–14), JNK (15), and STAT3 pathways (16, 17); direct interactions with specific components of the basal transcriptional apparatus (18–21) and with the CREB/ATF family of transcription factors (22–25); interaction with DNA repair proteins (26); and activation of the proteasome complex (27). Importantly, many studies have demonstrated that pX expression in different cell types (Chang cells, NIH3T3 cells, immortalized differentiated AML12 hepatocytes) results in distinct and opposing cellular responses, including cell cycle progression (28), G1/S phase arrest (29, 30), transformation (31, 32), and apoptosis (32–35). However, despite evidence supporting the growth-promoting (36) versus the antiproliferative or apoptotic function of pX (37), the molecular mechanisms by which pX effects these processes, for the most part, remain to be deciphered. Likewise, the significance of these pX-mediated processes in HCC development is poorly understood.

In our studies, we employ a cellular model system linked to pX-mediated hepatocyte transformation (32) and suitable to molecular analyses. It is composed of two tetracycline-regulated, pX-expressing cell lines, a differentiated hepatocyte 3pX-1 cell line, and a dedifferentiated hepatocyte 4pX-1 cell line. Conditional pX expression selectively transforms the 3pX-1 cell line. We recently demonstrated (36) that an early pX-mediated event in 3pX-1 cells is sustained activation of the Ras-Raf-MAPK pathway, an activation that is causally linked to pX-mediated transformation. In the pX-nontransforming 4pX-1 cell line, pX expression results in sustained activation of the JNK pathway and only transient activation of the Ras-Raf-MAPK pathway. Since pX expression mediates distinct growth characteristics between the 3pX-1 and 4pX-1 cell lines (i.e. transformation in the differentiated 3pX-1 cells versus absence of transformation in the less differentiated 4pX-1 cells and differential activation of the Ras-Raf-MAPK and JNK pathways), we examined how pX expression affects progression of these two cell lines into the cell cycle. The rationale for comparative studies between the differentiated (3pX-1) and less-differentiated (4pX-1) cell line is that we will gain further
insights regarding mechanism(s) of pX-mediated cellular transformation. Since the hepatic progenitor (oval cell) is the likely precursor leading to HCC (4–10), our comparative studies will define the effect of pX on the less-differentiated 4pX-1 hepatocyte cell line.

In this study, we examined the expression pattern of endogenous cell cycle regulators following pX expression in the 3pX-1 and 4pX-1 cell lines. We report 1) a differential pX-dependent cell cycle entry between the two cell lines; 2) a mechanistic link of cell cycle progression to the pX-reprogrammed mitogenic status of each cell line; and 3) the novel observation that pX expression effects a pronounced S phase pause or growth retardation in the dedifferentiated 4pX-1 cell line. Despite initiating this S phase pause, pX expression in the interval analyzed does not sensitize 4pX-1 cells to apoptosis, suggesting that pX deregulates the G1/M checkpoint.

MATERIALS AND METHODS

Cell Culture and Serum Starvation Conditions—Cell lines 3pX-1 and 4pX-1, derived from AML12 cells (38), were propagated as described in Ref. 32. All experiments employed the tetracycline-regulated cell lines at passages 4–10.

Indicated experiments in this study were carried out under the serum starvation conditions as described in Refs. 32 and 36. Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium without fetal bovine serum (0%) and insulin-transferrin-selenium for 18–24 h in the presence of 5 μg/ml tetracycline, 10 μg/ml epidermal growth factor receptor inhibitor PD 153035 (CalBiochem), and 25 μg/ml PD 98059 (Calbiochem). Following serum starvation, cells were washed three times in phosphate-buffered saline. pX synthesis by tetracycline removal was carried out for the indicated time course in medium lacking insulin-transferrin-selenium and containing 2% fetal bovine serum and 10 μg/ml epidermal growth factor receptor inhibitor.

Transient Transfection Assays—cdc2-CAT reporter transient transfections were carried out in cells grown in 10% FCS with or without 5 μg/ml tetracycline and employing the calcium phosphate method (22). CAT assays were performed as previously described (22). p21CIP1, bax-luciferase, and JGFBR-3-luciferase assays were performed by the Fugene protocol, as previously described (36).

Real Time Quantitative PCR—Total RNA was isolated by the Trizol method (Invitrogen) from 3pX-1 and 4pX-1 cultures, grown under serum starvation conditions (32, 36) with or without 5 μg/ml tetracycline for the indicated time course. cDNA (20 μl) was synthesized following DNase I treatment from 10 μg of total RNA; cDNA (2 μl) was used in quantitative real time PCR, using fluorescent SYBR-Green dye (Perkin-Elmer) and the Perkin-Elmer 5700 GeneAmp Sequence Detection System. The results are derived from three independent RNA preparations employing identical triplicates in each analysis and quantitated using the GAPDH standard curve (Fig. 1A) corresponding to the cycle of threshold value (Ct) (i.e. fluorescence intensity for a given mRNA species), and X represents the log copy number (C0) from which the relative content of mRNA is quantitated.

In Vitro Kinase Assays—For Cdk4 (39) and Cdk2 (40) enzyme assays, cells grown for the indicated interval in 10% FCS with or without 5 μg/ml tetracycline were collected in radioimmuno precipitation buffer containing 20 mm phosphate buffer, pH 7.4, 150 mm NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 2.5 mm EDTA, 10% glycerol, 0.1 mm Na3VO4, and 1 mm NaF. Cellular extract (500 μg) was immunoprecipitated with Cdk4 antibody (H-22; Santa Cruz Biotechnol., Inc., Santa Cruz, CA), Cdk2 antibody (H-297; Santa Cruz Biotechnology), or Cdk2 and cyclin B1 (H-433; Santa Cruz Biotechnology) antibodies for 2 h at 4 °C, followed by the addition of 25 μl of a 50% slurry of protein A-agarose (Santa Cruz Biotechnology) for 1 h at 4 °C. The protein-agarose complex was washed three times with 500 μl of radioimmuno precipitation buffer lacking detergents and resuspended in 20 μl of kinase buffer containing 40 mM Hepes, pH 7.0, 10 mm EDTA, 20 mm MgCl2, 20 μM ATP, 10 μM of [γ-32P]ATP, and 1 μg of GST-Rb (Santa Cruz Biotechnology) substrate for Cdk4 or Cdk2 kinase assays or 5 μg of histone H1 (Sigma) substrate for Cdk2 kinase assays. The kinase reaction was carried out for 30 min at 30 °C and terminated by the addition of 2× SDS sample buffer. Analysis was by 12% SDS-PAGE and autoradiography; quantitation was via the Scion Image software.

Western Blot Assays—p21CIP1 and p19ARF Western blot assays employed 50 μg of whole cell extract (WCE) isolated from 3pX-1 and 4pX-1 cells under no tetracycline or serum deprivation for p19ARF, with or without 5 μg/ml tetracycline for the indicated time course, using p21CIP1 antibody (C19; Santa Cruz Biotechnology) or p19ARF antibody (Novus Biologicals), respectively. Cdc2 phosphorylation at Tyr15 was examined by Western blots with TyrP) antibody (PY99; Santa Cruz Biotechnology). Whole cell extracts (500 μg), isolated from cells grown in 10% FCS with or without 5 μg/ml tetracycline for the indicated time course, were immunoprecipitated with Cdc2 antibody. Immunoprecipitates were analyzed by 12% SDS-PAGE, immunoblotted with TyrP antibody, and visualized using the ECL reagent (Amersham Biotechnology, Inc.).

Flow Cytometric Analysis—3pX-1 and 4pX-1 cells, 70–90% confluent, were grown in 10% FCS with or without 5 μg/ml tetracycline for the indicated time course. Cells were resuspended in PBS at a density of 2 × 106 cells/ml. An equal volume of Vindelev’s propidium iodide solution, containing 10 nm Tris, pH 8.0, 10 nm NaCl, 10 μg/ml RNase A, 50 μg/ml propidium iodide, and 0.001% Nonidet P-40, was added to the cell suspension, and cells were incubated on ice for 30 min. An XL-MCL flow cytometer was used to analyze DNA content; emitted light was measured at 675 nm.

RESULTS

Differential, pX-dependent G1 Entry of 3pX-1 and 4pX-1 Cells—Our studies to date, employing the two conditional pX-expressing 3pX-1 and 4pX-1 cell lines, have identified distinct and different pX-dependent properties. First, pX expression causes cellular transformation only in the differentiated hepatocyte 3pX-1 cell line and not in the dedifferentiated hepatocyte 4pX-1 cell line (32). Second, pX activates in a sustained manner the Ras-Raf-MAPK pathway in 3pX-1 cells and the JNK pathway in 4pX-1 cells (36). Given these differences, we examined the pX-dependent cell cycle progression of these two cell lines and the implications of this cell cycle progression for their distinctive cell growth characteristics.

Entry of cells into the G1 phase of the cell cycle is characterized by transcriptional induction and synthesis of cyclin D1 protein (41–43), which complexes and activates G1-specific Cdk4/6 kinases, required for progression through the early G1 phase (44, 45). Accordingly, we performed kinetic analyses of the expression pattern of cyclin D1 mRNA in the two cell lines, as a function of pX synthesis by tetracycline removal. Total RNA was isolated at 6, 12, 18, and 24 h after pX expression, from 3pX-1 and 4pX-1 cells grown in 2% FBS after serum starvation (32, 36). The pX-dependent expression of cyclin D1 mRNA was quantified by real time PCR, using the PerkinElmer 5700 GeneAmp sequence detection system and GAPDH as the internal control (Fig. 1A). The GAPDH standard curve employed for quantitation of our real time PCR assays is shown in Fig. 1A and is described under “Materials and Methods.”

We observe (Fig. 1) that expression of cyclin D1 mRNA displays a differential rate of pX-dependent (−Tet/+Tet) transcriptional induction in the two cell lines. In 4pX-1 cells (Fig. 1B), within 6 h of pX synthesis expression of cyclin D1 mRNA increased by nearly 2-fold, in contrast to the +Tet control. Interestingly, this increased pX-dependent expression of cyclin D1 mRNA is maintained at nearly the same level by 12 h of pX synthesis and is slightly decreased to ~1.5-fold by 18 and 24 h following pX synthesis.

To confirm these observations by another assay, we measured in vitro the activity of cyclin D1-activated Cdk4 kinase (39), employing cellular extracts isolated from 4pX-1 cells in a time course (6–24 h), following pX synthesis by tetracycline removal (Fig. 1B). In vitro Cdk4 kinase assays (Fig. 1B) demonstrate that in 4pX-1 cells, the Cdk4 kinase is activated...
within 6 h of pX expression, with maximal activation occurring within 12 h of pX expression. This pattern of Cdk4 kinase activation in 4pX-1 cells (Fig. 1B) agrees with the pX-dependent expression profile of cyclin D1 (Fig. 1B).

In 3pX-1 cells (Fig. 1C), pX-dependent transcriptional induction of cyclin D1 displays a progressive increase from 1.5-fold to nearly 2-fold, starting at 6 h and reaching maximal expression by 12 h after pX synthesis. In contrast to 4pX-1 cells (Fig. 1B) in 3pX-1 cells, pX-dependent cyclin D1 expression decreases to 10% by 18 h and ceases by 24 h of pX synthesis. The in vitro Cdk4 assays employing cellular extracts from 3pX-1 cells demonstrate a delayed pX-dependent activation, occurring on or before the 12-h time point of pX synthesis. Maximal Cdk4 kinase activation is observed at 18 h of pX expression (i.e. after the maximal pX-dependent cyclin D1 induction, which occurs at 12 h of pX expression) (Fig. 1C). We conclude that in 3pX-1 cells, the maximal pX-dependent cyclin D1 expression agrees with the delayed Cdk4 kinase activation (Fig. 1C). In summary, both 3pX-1 and 4pX-1 cells display pX-dependent cyclin D1 expression and Cdk4 kinase activation, indicating that both cell lines undergo pX-dependent cell cycle entry, in agreement with similar observations by others (28). These comparative analyses demonstrate that the pX-nontransforming 4pX-1 cells display an earlier cell cycle entry, in contrast to the delayed cell cycle progression of the pX-transforming 3pX-1 cells.

Differential, pX-dependent S Phase Progression between 3pX-1 and 4pX-1 Cells—Expression of the cyclin A gene occurs during late G1 and S phase (46). Studies have demonstrated
that the presence of cyclin A protein coincides with incorporation of bromodeoxyuridine into DNA (47). Thus, the cyclin A protein serves as a marker for progression of cells into S phase. Additional S phase-specific genes include dihydrofolate reductase and DNA polymerase \( \alpha \), among others (48). Accordingly, we have employed the real time quantitative PCR to assess expression of cyclin A and DHFR mRNAs as indicators for pX-dependent entry of 3pX-1 and 4pX-1 cells into S phase (Fig. 2).

4pX-1 cells display a 1.6-fold pX-dependent increase in expression of cyclin A mRNA within 6 h after pX synthesis, reaching a maximal 2.6-fold increase by 12 h after pX synthesis (Fig. 2A). Importantly, the pX-dependent expression pattern of cyclin A mRNA parallels the pX-dependent induction of DHFR mRNA (Fig. 2A). Considering that maximal cyclin D1 (Fig. 1B) and cyclin A and DHFR (Fig. 2A) expression in 4pX-1 cells occur at 6 and 12 h, respectively, following pX expression, we conclude that these cells display a short, pX-dependent progression through G1 phase.

By contrast, pX-dependent cyclin A and DHFR mRNA expression in 3pX-1 cells progressively increase from 1.3-fold, starting at 6 h, reaching a maximum 2.2-fold induction 18 h after pX synthesis (Fig. 2B). Thus, in 3pX-1 cells, maximal pX-dependent transcripational induction of cyclin D1 (Fig. 1C) and cyclin A (Fig. 2B) occur at 12 and 18 h, respectively, after pX synthesis, indicating that, in comparison with 4pX-1 cells, 3pX-1 cells display delayed, pX-dependent progression to S phase.

**Progression of 3pX-1 and 4pX-1 Cells into G2/M Phase—** Activation of mitotic Cdk-cyclin complexes promotes progression through the G2/M transition (49, 50). The mitotic Cdc2 kinase is a cell cycle-regulated gene (51) whose expression is undetectable in quiescent cells but increases starting at the G1/S transition, reaching maximal levels at G2 phase (52). Activation of Cdc2 kinase required for G2/M progression is mediated by association with cyclin B1 (53).

We employed real time quantitative PCR to assess pX-dependent induction of cdc2 and cyclin B1 mRNAs in 3pX-1 and 4pX-1 cells (Fig. 3, A and B). We observe a progressive pX-dependent expression of cdc2 mRNA in 3pX-1 cells, starting at 6 h and reaching a pronounced maximal 5-fold induction at 18 h after initiation of pX synthesis (Fig. 3A). Maximal 2.2-fold pX-dependent increase in cyclin B1 mRNA is observed at 12 h following pX synthesis (Fig. 3A). Surprisingly, pX-dependent cdc2 mRNA induction in 4pX-1 cells is small, ranging from 1.4-fold at 6 h to a maximal 2.5-fold induction by 12 h of pX synthesis. pX-dependent induction of cdc2 mRNA is not observed at 18 and 24 h after pX synthesis in 4pX-1 cells (Fig. 3B). Likewise, in 4pX-1 cells, the maximal pX-dependent cyclin B1 induction is only 1.5-fold, occurring by 12 h of pX synthesis.
MEKK1 and dominant negative RasN17 mutants (Fig. 4C). In agreement with the differential pX-mediated activation of the Ras-Raf-MAPK and JNK pathways in 3pX-1 and 4pX-1 cells (36), respectively, we observe a major inhibitory effect, ~90% inhibition, by the dominant negative RasN17 mutant on cdc2 expression in 3pX-1, whereas the dominant negative MEKK1 does not display this inhibitory effect. By contrast, dominant negative MEKK1 blocks ~60% of pX-dependent cdc2 expression in 4pX-1 cells. The effect of dominant negative RasN17 on cdc2 expression in 4pX-1, ~40% inhibition, suggests either that JNK pathway activation is Ras-dependent or that the transient, pX-dependent Ras-Raf-MAPK activation observed in 4pX-1 cells (36) also effects cdc2 transcription.

Selective pX-dependent Cdc2 Kinase Activation in 3pX-1 Cells—Entry into mitosis is controlled by the Cdc2 kinase. The activity of Cdc2 kinase is regulated by multiple mechanisms, including 1) association with the Cdc2 activator protein cyclin B1 (49); 2) phosphorylation of Cdc2 at threonine 161, which positively regulates Cdc2 kinase activity (59), whereas phosphorylation at threonine 14 (60, 61) and tyrosine 15 inhibits Cdc2 kinase activity (62–64); dephosphorylation of tyrosine 15 and threonine 14 by Cdc25 phosphatase at the onset of mitosis results in activation of Cdc2 kinase; and 3) in specific cell types, the cyclin-dependent kinase inhibitor p21<sup>Cip1</sup> negatively regulates Cdc2 kinase activity (65).

Accordingly, we have employed several approaches to measure pX-mediated Cdc2 kinase activity. Cdc2 immunokinase complex assays were performed with cellular extracts prepared in a time course after pX synthesis from 3pX-1 (Fig. 5A) and 4pX-1 (Fig. 5B) cells. The immunokinase complex was used to measure in vitro Cdc2 kinase activity employing GST-Rb (66) or histone H1 (40) as substrates. In parallel assays from the same immunoprecipitated Cdc2-cyclin B1 complex, we both measured in vitro phosphorylation of histone H1 substrate and assessed the presence of the inhibitory tyrosine phosphorylation (pTyr15) of cdc2 by Western blot analysis (Fig. 5). Collectively, the results demonstrate that 3pX-1 cells (Fig. 5A) exhibit pX-dependent Cdc2 kinase activation. Maximal pX-dependent Cdc2 kinase activation in 3pX-1 cells, by all three assays (Fig. 5A), occurs at 24 h after pX expression. By contrast, in 4pX-1 cells, our assays do not detect significant pX-dependent Cdc2 kinase activation (Fig. 5B).

To further investigate the mechanism that is probably contributing to the absence of pX-dependent Cdc2 activation in 4pX-1 cells, we also examined expression of the cdk inhibitor p21<sup>Cip1</sup>. We assessed by real time quantitative PCR the transcriptional induction of p21<sup>Cip1</sup> mRNA (Fig. 6A) and confirmed these results both by transient transfection assays of p21-luciferase reporter (Fig. 6B) and Western blot assays of p21<sup>Cip1</sup> protein (Fig. 6C). All three assays demonstrate that only the pX-nontransforming 4pX-1 cells exhibit a selective, pX-dependent p21<sup>Cip1</sup> induction, suggesting its involvement in Cdc2 kinase inhibition in 4pX-1 cells.

Selective pX-dependent Induction of Proapoptotic Genes in 4pX-1 Cells—When one considers the accelerated pX-dependent entry of 4pX-1 cells into G2 and S phases, the absence of detectable pX-mediated Cdc2 kinase activation (Fig. 5) implies the accumulation of cells at the G2/M transition. G2/M phase arrest is rather unusual for vertebrate cells; it is a transient phase during which the cells either repair and overcome the block and proceed to mitosis or undergo apoptosis.

To investigate further these possibilities, we examined whether pX induces the proapoptotic genes bax (67) and IGFBP-3 (68). We performed transient transfection assays employing IGFBP-3 and bax-luciferase reporters (69). We observe selective pX-dependent induction of both proapoptotic genes in 3pX-1 cells (Fig. 6B) and repression in 4pX-1 cells (Fig. 6C). The mitogenic pathways involved in this pX-dependent cdc2 induction were determined by using the dominant negative cdc2 mRNA induction in the two cell lines, we carried out in 3pX-1 and 4pX-1 cells transient transfections employing the cdc2-CAT reporter (Fig. 4A). In both cell lines, pX expression by tetracycline removal mediates transcriptional induction from the cdc2-CAT reporter (Fig. 4A). However, cdc2 transcription is known to be negatively regulated by p53 (54). Accordingly, we examined in transient transfection assays the effect of co-transfected WT p53 on cdc2-CAT reporter activity (Fig. 4B). We observe that co-transfected WT p53 selectively represses expression from the cdc2 promoter in 4pX-1 cells (Fig. 4B). Interestingly, the pX-dependent induction of the cdc2-CAT reporter in 3pX-1 cells is 12.5-fold, versus a 4-fold induction observed in 4pX-1 cells (Fig. 4C). Based on these results (Fig. 4), we suggest that the small pX-dependent Cdc2 mRNA induction in 4pX-1 cells (Fig. 3B) may be due to the differential transcriptional efficacy of WT p53 between the two cell lines. Importantly, the expression of both cyclin B1 (55, 56) and Cdc25 (57), the dual specificity phosphatase required for Cdc2 activation (58), are known to be negatively regulated at the transcriptional level by WT p53. Although we have not examined the promoter activity of cyclin B1 in our cell lines, the observed pX-dependent expression pattern of cyclin B1 (Fig. 3) suggests its negative regulation by WT p53 in 4pX-1 cells.
4pX-1 cells (Fig. 7A). Since both bax and IGFBP-3 genes are known to be regulated transcriptionally by the p53 protein (69), we tested by transient transfections the effect of co-transfected WT p53 (Fig. 7B) in 4pX-1 cells. Interestingly, co-transfection of these reporters with WT p53 expression vector does not further increase transcription from bax and IGFBP-3 promoters (Fig. 7B). These results indicate that the endogenous p53 is functional (i.e. WT) and mediates the observed pX-dependent expression from both of these p53-responsive promoters in 4pX-1 cells (Fig. 7, A and B).

Selective pX-dependent Expression of p19ARF in 4pX-1 Cells—We were intrigued by the selective, pX-dependent transcriptional induction in 4pX-1 cells of the p53-regulated genes p21Cip1 (Fig. 6), bax, and IGFBP-3 promoters (Fig. 7B). These results indicate that the endogenous p53 is functional (i.e. WT) and mediates the observed pX-dependent expression from both of these p53-responsive promoters in 4pX-1 cells (Fig. 7, A and B).

Selective pX-dependent Expression of p19ARF in 4pX-1 Cells—We were intrigued by the selective, pX-dependent transcriptional induction in 4pX-1 cells of the p53-regulated genes p21Cip1 (Fig. 6), bax, and IGFBP-3 (Fig. 7). In untransfected cells, p53 is latent and maintained at low levels by targeted degradation, mediated by its negative regulator MDM2 (70). Upon cellular stress, p53 is stabilized in the nucleus, where it becomes transcriptionally active, thus performing its antiproliferative function by expression of p53-responsive genes that mediate cell cycle arrest or apoptosis (71). One of the mechanisms leading to p53 protein stabilization involves the p19ARF protein (72). p19ARF activates p53, thus inhibiting cell growth, by binding MDM2, thereby blocking MDM2-mediated degradation of p53 (73–76).

Accordingly, we assessed the expression level of p19ARF in the two cell lines, as a function of pX expression (Fig. 8). Comparative real time PCR analyses demonstrate the pronounced pX-dependent expression of p19ARF in 4pX-1 cells (Fig. 8A). Within 6 h of pX expression in 4pX-1 cells, p19ARF is transcriptionally induced by 7-fold, in comparison with the +Tet control. The p19ARF mRNA level progressively declines to ~3-fold, in comparison with the +Tet control, by 24 h of pX expression (Fig. 8A). By contrast, in 3pX-1 cells, only a minimal 1.5-fold induction in p19ARF mRNA is observed at 12–18 h following pX expression, declining to basal level expression by 24 h (Fig. 8A). These results (Fig. 8A) are further confirmed by Western blot analyses of cellular extracts obtained from 4pX-1 and 3pX-1 cells at the indicated times after tetracycline removal, employing p21Cip1- and anti-α-actin as the internal control.

Selective pX-mediated cdc2 kinase activation in 3pX-1 cells. WCE was isolated from 3pX-1 (A) and 4pX-1 cells (B) at the indicated times after pX synthesis and analyzed in parallel for Cdc2 kinase activity by Cdc2 immuno-complex kinase assays using histone H1 as the substrate and by Western blots with Tyr(P) antibody. Total Cdc2 protein was monitored by Western blot using Cdc2 antibody. In vitro Cdc2 immunocomplex kinase assays were performed with WCE isolated at 24 h with or without tetracycline treatment of 3pX-1 (A) and 4pX-1 cells (B), using GST-Rb as the substrate.

Selective, pX-mediated cdc2 kinase activity in 3pX-1 cells. WCE was isolated from 3pX-1 (A) and 4pX-1 cells (B) at the indicated times after pX synthesis and analyzed in parallel for Cdc2 kinase activity by Cdc2 immunocomplex kinase assays using histone H1 as the substrate and by Western blots with Tyr(P) antibody. Total Cdc2 protein was monitored by Western blot using Cdc2 antibody. In vitro Cdc2 immunocomplex kinase assays were performed with WCE isolated at 24 h with or without tetracycline treatment of 3pX-1 (A) and 4pX-1 cells (B), using GST-Rb as the substrate.
Differential Cell Cycle Progression by HBV X Protein

In this study, we have investigated in comparative analyses the pX-dependent cell cycle progression of the pX-transforming (3pX-1) and pX-nontransforming (4pX-1) cells. Fig. 11 summarizes the pX-dependent cell cycle progression of these two cell lines, based on quantification of the mRNA expression profile of key cell cycle regulators (Figs. 1–3). We employed real time quantitative PCR to assess the pX-dependent effects on mRNA expression of cell cycle regulators, as opposed to measuring protein levels, because it is well documented that pX expressed in physiologically relevant concentrations (32, 35, 77) is only a moderate activator of signal transduction pathways (11, 13, 14, 36) and transcription (23, 32, 36).

In 3pX-1 cells (Fig. 11A), the expression profile of the G1-specific cyclin D1 (Fig. 1C) and the onset of activation of the G2-specific Cdc4 kinase (Fig. 1C) indicate that the pX-dependent G1 phase occurs before 12 h and ends by 18 h of pX expression. Since cyclin A and DHFR expression begin in middle to late G1, and peak transcriptional induction occurs at 18 h of pX synthesis (Fig. 2B), the pX-dependent S phase in 3pX-1 cells occurs before 18–24 h of pX synthesis. By 24 h of pX synthesis, the pX-dependent expression of cyclin A and DHFR reaches the same level as the +Tet control. cdc2 pX-dependent expression progressively increases to a maximal level at 18 h of pX synthesis (Fig. 2A), whereas maximal cyclin B1 expression occurs at 12 h of pX expression (Fig. 2A). Although cyclin B1 and cdc2 mRNAs are expressed in the 12–18-h interval of pX synthesis, it is known that the cdc2-cyclin B1 complex changes its subcellular localization during the G2/M transition, transplanting from the cytoplasm to the nucleus (78). Since our in vitro Cdc2 kinase assays (Fig. 5A) demonstrate maximal pX-dependent Cdc2 kinase activity at 24 h of pX synthesis, we conclude that in 3pX-1 cells G2/M begins on or before 24 h of pX synthesis.

Expression of cyclin A in 3pX-1 cells, as demonstrated in our recent studies (36), is promoted by pX-dependent activation of the Ras-Raf-MAPK pathway. Likewise, transcriptional induction of cdc2 in 3pX-1 cells is mediated via pX-dependent activation of the Ras-Raf-MAPK pathway (Fig. 4C). In 3pX-1 cells, pX effects a small, sustained, 20-fold activation of the Ras-Raf-MAPK pathway (36), consistent with the slow, pX-dependent cell cycle progression of these cells.

**DISCUSSION**

The data presented in this study support the hypothesis that pX rescues cells from the nocodazole-induced block, by deregulating the G2/M checkpoint of the cell cycle. The selective pX-dependent expression of proapoptotic genes bax and IGFBP-3 in 4pX-1 cells, A, transient transfection assays of bax-luciferase and IGFBP-3-luciferase in 3pX-1 and 4pX-1 cells as a function of pX expression. B, co-transfection of bax-luciferase and IGFBP-3-luciferase reporters with WT p53 expression vector in 4pX-1 cells as a function of pX expression. Results shown are from three independent assays performed in triplicates.

**Fig. 7.** Selective, pX-dependent expression of proapoptotic genes bax and IGFBP-3 in 4pX-1 cells. A, transient transfection assays of bax-luciferase and IGFBP-3-luciferase in 3pX-1 and 4pX-1 cells as a function of pX expression. B, co-transfection of bax-luciferase and IGFBP-3-luciferase reporters with WT p53 expression vector in 4pX-1 cells as a function of pX expression. Results shown are from three independent assays performed in triplicates.

...tion of the antiproliferative genes p21<sup>Cip1</sup>, bax, and IGFBP-3 in these cells.

**Flow Cytometric Analysis of 4pX-1 Cells**—The selective pX-dependent induction in 4pX-1 cells of p21<sup>Cip1</sup> (Fig. 6), p19<sup>WAF1</sup> (Fig. 8), bax, and IGFBP-3 (Fig. 7) suggests that these cells undergo pX-dependent growth arrest or apoptosis. To directly assess the effect of pX expression on their cell cycle progression, we monitored their growth profiles by flow cytometry. Subconfluent cultures (70–90%) were grown in parallel in 10% FCS for 12, 18, and 24 h with or without tetracycline treatment. Table I shows the percentage of 4pX-1 cells in G1, S, and G2/M phases of the cell cycle, 12–24 h following pX expression. A representative flow cytometric profile at 18 h with or without pX expression is shown in Fig. 9. 4pX-1 cells display a nearly 45% increase in pX-dependent S phase entry, observable at 12 h and maintained to the same level at 24 h following pX expression (Table I). Similar flow cytometric analyses with 3pX-1 cells demonstrate a 26% pX-dependent increase in S phase entry (data not shown). However, in contrast to 3pX-1 cells, 4pX-1 cells do not demonstrate pX-dependent activation of the mitotic Cdc2 kinase within the 12–24-h interval after pX synthesis (Fig. 5). Furthermore, the flow cytometric data of 4pX-1 cells do not support the idea that these cells arrest in the G2/M transition or that they undergo pX-dependent apoptosis (Fig. 9 and Table I). Annexin V staining of 4pX-1 cells grown under the same conditions as described in Fig. 9, and for the same time interval of pX expression (i.e. 12, 18, and 24 h), also failed to detect evidence of pX-dependent apoptosis. Accordingly, we interpret the results of Table I and Fig. 9 to indicate that 4pX-1 cells undergo a growth retardation or pause in S phase.

To investigate how pX-expressing 4pX-1 cells overcome the pronounced S phase pause, we simulated G2/M arrest in 4pX-1 cells by treatment with nocodazole, a known mitotic spindle inhibitor. Cells were synchronized by serum starvation followed by treatment for 6 h with 10% FCS with or without tetracycline removal before the addition of nocodazole. Incubation with nocodazole as a function of pX synthesis was an additional 36 h; cells were stained with 4′,6-diamidino-2-phenylindole and examined by fluorescence microscopy (Fig. 10). We observe that 4pX-1 cultures grown in the presence of both nocodazole and pX (Fig. 10A) have nuclei that appear less pyknotic in comparison with those grown only in the presence of nocodazole (Fig. 10B) and display numerous cells that have multiple, smaller nuclei (Fig. 10A). Electron microscopy revealed that in these multinucleated cells, the nuclei have an internal structural organization typical of interphase and are enveloped by intact nuclear membranes (Fig. 10C). The pX-dependent appearance of multinucleated cells in 4pX-1 cells displays a 3-fold increase when cells are treated with nocodazole and pX for 24 h (Fig. 10D). Furthermore, similar analyses employing additional clonal isolates of the 4pX lineage (i.e. clones 4pX-2 and 4pX-3) also demonstrate the pX-dependent appearance of multinucleated cells in nocodazole-treated cultures (Fig. 10D). The analyses of the additional 4pX-2 and 4pX-3 clonal isolates exclude the possibility that the observed phenomenon (Fig. 10) is due to clonal variation of the 4pX-1 cell line. In conclusion, we interpret these observations (Fig. 10) to suggest that pX rescues cells from the nocodazole-induced block, by deregulating the G2/M checkpoint of the cell cycle.
tetracycline removal, employing p19ARF−4pX−1 cells at the indicated times after real-time quantitative PCR of total RNA isolated from serum-starved 3pX-1 and 4pX-1 cells at the indicated times after tetracycline removal, employing p19ARF−4pX−1 cells at the indicated times after real-time quantitative PCR of total RNA isolated from serum-starved 3pX-1 and 4pX-1 cells, as a function of pX synthesis, using p19ARF and α-actin antibodies.

Tetracycline removal for 18 h. We and basal level expression by 18 h of pX synthesis (Fig. 2B). We propose that in 4pX-1 cells, due to this pX-dependent expression of p19ARF (Fig. 8) and the sustained activation of the JNK pathway (36). We interpret these observations to mean that in 4pX-1 cells, progression into S phase occurs at or before the 6-h time point and terminates at or before 18 h of pX synthesis (Fig. 11B). However, the absence of pX-dependent Cdc2 kinase activation (Fig. 5B) suggests that 4pX-1 cells do not proceed to the G2/M phase.

4pX-1 cells exhibit a small pX-dependent cdc2 mRNA induction (Fig. 3B). Our co-transfection assays of WT p53 with the cdc2-CAT reporter (Fig. 4B) demonstrate selective transcriptional cdc2-CAT repression in 4pX-1 cells. Although the mechanism of transcriptional repression by p53 is not yet understood, these results (Fig. 4B) suggest that the small, 2.5-fold pX-dependent cdc2 mRNA induction in 4pX-1 cells (Fig. 3B), versus the 5-fold induction observed in 3pX-1 cells (Fig. 3A), may be due to the differential transcriptional efficacy of the endogenous p53 between the two cell lines. This proposal is also supported by our observations that 4pX-1 cells display selective transcriptional induction of the classic p53-responsive genes p21Cip1 (Fig. 6) (65), bax, and IGFBP-3 (Fig. 7) (65). It has been shown that WT p53 exhibits varying cellular responses, depending on the endogenous level of p53, which depends on its stability (80). One of the mechanisms that regulates p53 stability is mediated by the tumor suppressor p19ARF (72). The p19ARF protein, by interacting and interfering with the p53 negative regulator MDM2 (70), increases the stability and thus the transcriptional efficacy of p53 (73–76). Our results (Fig. 8) demonstrate that pX induces the selective expression of the tumor suppressor p19ARF only in 4pX-1 cells and support the differential stability of p53 between the two cell lines. Furthermore, p53 was shown to be phosphorylated by the JNK enzyme (81). The JNK-phosphorylated form of p53 exhibits increased half-life and reduced degradation by the 26 S proteasome complex (82). Importantly, 4pX-1 cells are characterized by sustained pX-dependent activation of the JNK pathway (36). We propose that in 4pX-1 cells, due to this pX-dependent expression of p19ARF (Fig. 8) and the sustained activation of the JNK pathway by pX (36), the endogenous p53 is stabilized from degradation.

pX-dependent Cdc2 kinase activation is not detected in 4pX-1 cells during the intervals from 6 to 24 h of pX expression (Fig. 5). Since Cdc2 kinase activation requires dephosphorylation of tyrosine 15, we suggest that a likely mechanism contributing to absence of Cdc2 kinase activation is lack of Cdc25 phosphatase activation or expression. In addition, the selective induction of p21Cip1 may also contribute to Cdc2 inhibition, although we did not detect co-immunoprecipitation of p21Cip1 with the cyclin B1-Cdc2 complex (data not shown).

By contrast, in 4pX-1 cells (Fig. 11B), pX-dependent cell cycle entry is rapid, occurring at or before 6 h after pX synthesis. This conclusion is based on the robust cyclin D1 expression seen at 6 h of pX synthesis (Fig. 1B), coupled with the Cdk4 kinase activation occurring at or before the 6-h time point (Fig. 1B). The prolonged (24-h) pX-dependent Cdk4 activation of 4pX-1 cells (Fig. 1B) agrees with the prolonged expression of cyclin D1 observed at 12–24 h of pX synthesis (Fig. 1B). The consequence of this prolonged cyclin D1 expression and Cdk4 kinase activation for the 4pX-1 cells is unknown. Interestingly, prolonged, aberrant expression of G1 cyclins (cyclins D1, D2, and D3) throughout the cell cycle is linked to polyploidy in megakaryocytes (79).

In the 4pX-1 cell line, cyclin A and DHFR display a significant pX-dependent transcriptional induction starting at 6 h of pX synthesis, reaching peak expression at the 12-h time point and basal level expression by 18 h of pX synthesis (Fig. 2A). We

TABLE I

| 4pX-1 cells | Percentages of cells in respective cell cycle phases |
|-------------|---------------------------------------------------|
|             | G1 phase | S phase | G2/M phase |
| 12 h +Tet   | 61.6 ± 2 | 26.0 ± 3 | 11.8 ± 1 |
| 12 h −Tet   | 46.1 ± 2 | 44.6 ± 3 | 9.3 ± 1  |
| 18 h +Tet   | 62.4 ± 3 | 27.6 ± 4 | 10.0 ± 1 |
| 18 h −Tet   | 53.5 ± 1 | 39.5 ± 1 | 7.0 ± 1  |
| 24 h +Tet   | 61.1 ± 2 | 30.5 ± 2 | 8.4 ± 2  |
| 24 h −Tet   | 52.2 ± 2 | 39.6 ± 2 | 8.2 ± 2  |

FIG. 8. Selective pX-dependent expression of p19ARF in 4pX-1 cells. A, real-time quantitative PCR of total RNA isolated from serum-starved 3pX-1 and 4pX-1 cells at the indicated times after tetracycline removal, employing p19ARF-specific primers and GAPDH as the internal control. pX-dependent (−Tet/+Tet) induction was quantitated versus the GAPDH standard. Results shown are from three independent RNA preparations using identical triplicates for each PCR. B, p19ARF Western blot assays of WCE isolated from serum-starved 3pX-1 and 4pX-1 cells, as a function of pX synthesis, using p19ARF and α-actin antibodies.

FIG. 9. Flow cytometric analyses of pX-dependent cell cycle progression of 4pX-1 cells grown in 10% FCS with or without tetracycline removal for 18 h.

FIG. 10. Differential cell cycle progression by HBV X protein. 8737, 3pX-1, and 4pX-1 cells G1 phase S phase G2/M phase

Percentages of cells in respective cell cycle phases

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The consequence of absence of pX-dependent Cdc2 kinase activation in 4pX-1 cells is that they do not proceed to G2/M phase. In mammalian cells, a G2/M phase block will either be repaired and cells will continue their progression through the cell cycle, or they will undergo apoptosis. Although 4pX-1 cells express in a pX-dependent manner the proapoptotic genes \textit{bax} and \textit{IGFBP-3} (Fig. 7), our flow cytometric data do not support the occurrence of pX-dependent apoptosis at 12–24 h of pX expression. It is well established that the effective cellular concentration of opposing activities of \textit{bcl2} family members counteract the proapoptotic function of \textit{bax} (83, 84). Moreover, the apoptotic response can be masked by survival factors produced by cultured cells. We have determined by transient transfection assays and real time quantitative PCR that pX expression increases IGF-II mRNA in both cell lines (data not shown). Based on the observation that IGF-II rescues tumor necrosis factor-\textit{a}-induced apoptosis of pX-expressing cells (35), we propose that the pX-mediated \textit{IFG-II} induction probably rescues 4pX-1 cells from apoptosis.

Despite the absence of detectable pX-dependent Cdc2 kinase activation in 4pX-1 cells (Fig. 5B), our flow cytometric analyses do not display a classic G2/M block but rather a pause or a
growth retardation in S phase (Table I). There are several possibilities to explain such a pause in S phase (Fig. 9). Recently, an S phase-specific checkpoint regulator termed HIRA protein has been identified in yeast and mammalian cells (85). A pX-dependent deregulation of cellular HIRA activity may explain our data. Second, it has been demonstrated that the p38 MAPK pathway activation blocks mitotic entry by inhibiting the anaphase-promoting complex (86, 87). We have evidence to support pX-dependent p38 MAPK activation in 4pX-1 cells.2 We are currently examining the kinetics of this pX-dependent p38 MAPK activation and its effect on this observed S phase pause. Mammalian cells, in addition to Cdc2 kinase, contain Cdc2-related kinases such as Cdc2 PCTAIRE (88), which could compensate for the absence of Cdc2 kinase activation in 4pX-1 cells (Fig. 5). Alternatively, considering the absence of pX-dependent apoptosis in 4pX-1 cells, we suggest that pX deregulates the G2/M checkpoint. We base this proposal on the results shown in Fig. 10, in which we simulated G2/M arrest by treatment of cells with the mitotic spindle inhibitor, nocodazole. Expression of pX in nocodazole-treated cultures results in the increased appearance of multinucleated cells (Fig. 10D), which lack apoptotic features such as chromatin condensation and nuclear envelope breakdown, as supported by our electron microscopic analysis (Fig. 10C). Interestingly, the appearance of the multinucleated cells upon expression of pX in the nocodazole-treated cultures is reminiscent of the effects of another viral regulatory protein, the human T-cell lymphotropic virus type I Tax protein (89). It has been demonstrated that Tax interacts with Mad1, a protein that negatively regulates the anaphase-promoting complex (89). Expression of Tax or a dominant negative Mad1 mutant in nocodazole-treated cultures results in the appearance of multinucleated cells (89). Furthermore, our hypothesis that pX deregulates the G2/M checkpoint is supported by recent studies that identified the expression profiles of genes that become deregulated in HBV-mediated HCC in humans (90). A category of up-regulated genes include proteins playing regulatory roles in the mitotic checkpoint, including BUB1, PLK, Cdc23, Cdc28, PCTAIRE, and 26 S proteasome subunit p31 (90). Loss of checkpoint function is known to induce genomic instability and to alter the ploidy of dividing cells (91). We are currently exploring the role of pX in deregulation of the G2/M checkpoint and its phenotypic consequences for the dedifferentiated 4pX-1 cells.

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Hepatitis B Virus X Protein Differentially Regulates Cell Cycle Progression in X-transforming Versus Nontransforming Hepatocyte (AML12) Cell Lines
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