Hepatitis B Virus X Protein Differentially Activates RAS-RAF-MAPK and JNK Pathways in X-transforming Versus Non-transforming AML12 Hepatocytes

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The hepatitis B virus (HBV) X protein (pX) is implicated in hepatocarcinogenesis of chronic HBV patients by an unknown mechanism. Activities of pX likely relevant to hepatocyte transformation include activation of the mitogenic RAS-RAF-MAPK and JNK pathways. To assess the importance of mitogenic pathway activation by pX in transformation, we employed a cellular model system composed of two tetracycline-regulated, pX-expressing cell lines, constructed in AML12-immortalized hepatocytes. This system includes the differentiated 3pX-1 and the de-differentiated 4pX-1 hepatocytes. Our studies have demonstrated that conditional pX expression transforms only 3pX-1 cells. Here, comparative in vitro kinase assays and various in vivo analyses demonstrate that pX affects an inverse activation of RAS-RAF-MAPK and JNK pathways in 3pX-1 versus 4pX-1 cells. Sustained pX-dependent RAS-RAF-MAPK pathway activation is observed in pX-transforming 3pX-1 cells, whereas sustained pX-dependent JNK pathway activation is observed in pX non-transforming 4pX-1 cells. This differential, pX-dependent mitogenic pathway activation affects differential activation of cAMP-response element-binding protein and c-Jun and determines the proliferative response of 3pX-1 and 4pX-1 cells. Furthermore, tetracycline-regulated, pX-NLS-expressing cell lines demonstrate that expression of the nuclear pX-NLS variant minimally activates the RAS-RAF-MAPK pathway and results in markedly reduced transformation. These results link sustained, pX-mediated activation of RAS-RAF-MAPK pathway to hepatocyte transformation.

Patients chronically infected with hepatitis B virus develop hepatocellular carcinoma (HCC) in their 4th or 5th decade (1). The 16.5-kDa X protein, encoded by HBV, is implicated in HCC pathogenesis (2). Despite numerous studies describing pX activities, the molecular mechanism by which pX affects hepatocyte transformation is unknown. This study addresses aspects of the pX-mediated mechanism of hepatocyte transformation.

It is well accepted that pX is a multifunctional protein affecting transcription (2, 3), cell growth (4, 5), and apoptosis (6–8). Although not DNA binding, pX is a promiscuous trans-activator of diverse cis-acting elements, including AP-1 (9–12), NF-κB (13–15), AP-2 (11), and CRE (16–18) sites. This transcriptional promiscuity of pX is understood to be due to its dual mechanism of action (19). pX interacts directly with specific components of the basal transcriptional apparatus (20–24) and with bZip (CREB/ATF) transcription factors (16–18), resulting in enhanced CRE/bZip-mediated transcription (16, 18). In addition to affecting direct transcriptional induction, pX activates the RAS-RAF-MAPK (19, 25) and JNK (26) pathways, resulting in enhanced transcription from AP-1 and NF-κB cis-acting elements.

pX-mediated activation of the RAS-RAF-MAPK pathway has been linked to accelerated entry of cells into S phase (4); however, the significance of the pX-dependent activation of this mitogenic pathway in hepatocyte transformation has not been demonstrated directly. Furthermore, the significance of the pX-dependent activation of the JNK pathway also remains unclear, since JNK pathway activation in some cases has been linked to transformation (27–29) and in other cases to apoptosis (30–33).

To understand the significance of pX-induced reprogramming of the mitogenic process in hepatocyte transformation, we employed a new cellular model, composed of immortalized hepatocytes, AML12 cells (34), expressing pX via the tetracycline-regulated expression system (35). We have characterized two hepatocyte lineages derived from AML12 cells as follows: 1) a differentiated hepatocyte (3pX-1) cell line, similar to the parental AML12 cells, and 2) a de-differentiated hepatocyte (4pX-1) cell line. We have demonstrated that conditional expression of pX leads to transformation in the differentiated 3pX-1 hepatocyte cell line; by contrast, expression of pX in the de-differentiated 4pX-1 cell line does not lead to transformation (35). Importantly, our observations agree with clinical data reporting the majority of HCC is derived from differentiated hepatocytes (36) versus the progenitor “oval cells.” Thus, our cellular model system is ideal for investigating physiologically relevant, early signaling events activated by pX during hepatocyte transformation, and for comparing these to signaling events occurring in a cellular environment refractory to pX-mediated cellular transformation.

Herein, we characterize the mitogenic status of the 3pX-1 and 4pX-1 cell lines affected by pX, thus providing for the first time information regarding early mechanistic events linked to pX-mediated transformation. We observe a differential and sustained pX-dependent activation of RAS-RAF-MAPK pathway in the pX-transforming 3pX-1 cells and a sustained pX-de-
DEPENDENT JNK PATHWAY ACTIVATION IN THE pX-NON-TRANSFORMING 4pX-1 CELLS. We demonstrate that this differential pX-mediated mitogenic pathway activation results in differential activation of the downstream effectors, c-Jun and CREB. In addition, we demonstrate that the proliferative response of each cell line is mediated via the mitogenic pathway that displays sustained activation by pX. Furthermore, we report that tetracycline-regulated cell lines expressing a pX-NLS variant, localized exclusively to the nucleus (19), display minimal mitogenic pathway activation and markedly reduced transformation. Our results demonstrate the causal role of the sustained RAS-RAF-MAPK pathway activation in pX-mediated hepatocyte transformation.

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines 3pX-1, 4pX-1, and 3pX-NLS, derived from AML12 cells (34), were propagated as described (35). All experiments employed the tetracycline-regulated cell lines at passages 4–10.

Tetracycline-regulated, pX-NLS-expressing cell lines were constructed in AML12 clones 3 and 4 (G418, tTA), stably transfected with 5 μg of PUH150-3–X-NSL plasmid via selection with hygromycin (35). Putative and conditional pX-NLS-expressing clones were selected by transfecting the CRE3–CHOP reporter in the presence of 10 μM forskolin, ±5 μM tetracycline, and further verified with TRK- and NF-κB-luciferase expression as negative controls.

Serum Starvation Conditions—All experiments were carried out under serum starvation, as described previously (35). Cells were grown in Dulbecco’s modified Eagle’s medium/F-12 medium without fetal bovine serum (0%) and insulin/transferrin/seelenium (ITS) (18–24 h, in the presence of 5 μg/ml tetracycline, 10 μM epidermal growth factor receptor inhibitor PD 153035 (Calbiochem), and 25 μM PD 98059 (Calbiochem). Following serum starvation, cells were washed three times in phosphate-buffered saline and incubated an additional 2 h in medium lacking ITS and containing 2% fetal bovine serum and 10 μM PD 153035. pX synthesis by tetracycline removal was carried out for the indicated time course in medium lacking ITS and containing 2% fetal bovine serum and 10 μM PD 153035.

Transient Transfection Assays—The in vivo signal transduction pathway trans-reporting system (Stratagene) includes the pFR-luciferase reporter and expression vectors pFC-dbd (negative control), pFC-MEK1 (positive control for Elk-1), pFC-MEK1K (positive control for c-Jun), and the transactivators pFA-c-Jun, pFA-Erk1, pFA-CHOP. Each transactivation assay was performed in triplicate 3.5-cm plates, employing Fugene 6 reagent (Roche Molecular Biochemistry). Transfected plasmids included 500 ng of pFR-luciferase reporter, co-transfected in the presence of 50 ng of pFA-Erk1 or 100 ng of pFA-c-Jun. Positive and negative controls also contained 50 ng each of pFC-MEK1, pFC-MEK1K, or pFC-dbd, respectively. CREB transreporter assays employed 100 ng of pFR-luciferase with 100 ng of pFA-CHOP in 3pX-1 cells and 200 ng of pFR-luciferase and 50 ng of pFA-CHOP for 3pX-1 cells. Cyclin A-luciferase assays (37) were performed in serum-starved cells (10-h serum starvation for 3pX-1 cells and 18–24 h for 4pX-1 cells) using 400 ng of cyclin A-luciferase for 3pX-1 cells and 200 ng for 4pX-1 cells, in the presence of 100 ng plasmid encoding dn RasN17, dn ACREB, or dn MEKK1. DNAs were added onto serum-starved cells, which were washed three times with PBS and placed in media containing 2% fetal bovine serum, 10 μM PD 153035, and 5 μg/ml tetracycline. Following 12–24 h incubation, transfected cells were washed three times in PBS and incubated for an additional 4 h in media containing 2% fetal bovine serum, 10 μM PD 153035, ±5 μg/ml tetracycline, and ±25 μM PD 98059, as indicated in figure legends. Luciferase assays were performed as described previously (16) and expressed per μg of total protein extract.

DNA Synthesis Assays—3pX-1 and 4pX-1 cells plated in 96-well dishes were serum-starved 10 h, washed three times in PBS, and incubated in media containing 2% fetal bovine serum, 10 μM PD 153035, ±5 μg/ml tetracycline, and ±25 μM PD 98059. DNA synthesis was measured at 6, 18, and 30 h following tetracycline removal by labeling cells in medium containing 20 μCi/ml [methyll-3H]thymidine, for the last 4, 12, and 18 h, respectively. Each measurement was performed in triplicate, and the data represent averages from three independent experiments.

In Vitro Kinase Assays—MAPK (ERK1,2) enzyme assays, serum-starved cells grown for the indicated interval ± 5μg/ml tetracycline were collected in lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 1 mM orthovanadate, 10 mM sodium pyrophosphate dehydrate, 1 mM phenylmethylsulfonyl fluoride, 5 mM p-nitrophenol phosphate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and normalized to protein content. 500 ng of whole cell extract was used for ERK1,2 activity using the immunoprecipitation-immunocomplex kinase assay (38). ERK1,2 immunoprecipitations were performed in RIPA buffer with 4 μl of polyclonal MAPK antibody (38) in the presence of 30 μl of 50% protein A-Sepharose (Sigma) slurry for 30 min at room temperature. The protein A-Sepharose-antibody complex was washed with 25 μl of Tris-HCl (pH 7.4), 140 mM NaCl, and 3 mM KCl and resuspended in 20 μl of kinase buffer (20 mM Tris (pH 7.5), 10 mM MgCl2, 5.4 mM p-nitrophosphoryl phosphate, 50 μM ATP, 2.5 mM myelin basic protein (MBP, Sigma), and 10 μCi (53)PAP). Kinase reactions were performed for 30 min at 30 °C and terminated by addition of 1× SDS sample buffer. Analysis was by 12% SDS-polyacrylamide gel electrophoresis and autoradiography.

JNK kinase assays employed cellular extracts prepared from serum-starved cells (35). Kinase assays were performed with modifications of the solid phase kinase protocol (39), using 1 μg of GST-c-Jun (1–79) (Stratagene) as substrate. WCE (500 μg) was assayed for JNK activity for 20 min at 30 °C. Analysis was by 12% SDS-polyacrylamide gel electrophoresis and autoradiography.

JNK activation was measured by digital densitometry using OPTIMAS 6.1 software. The value obtained for the phosphorylated substrate (i.e. MBP or GST-c-Jun) was normalized to that of the corresponding total ERK1,2 or JNK1,2 enzyme detected by Western blot analyses. The ratio (−Tet/+Tet) of these values is plotted in the histogram, derived from at least three independent assays.

Western Blot Analyses—For detecting total ERK1,2 or JNK1,2 enzymes, 10 μg of WCE was analyzed by Western blot assays using the ECL detection reagent (Amersham Pharmacia Biotech) and anti-MAPK (Promega) or anti-JNK (Santa Cruz Biotechnology), respectively. For detecting phosphorylated CREB, cells were grown for the indicated time course ±5 μg/ml tetracycline, ±10 μM SB 203580, or ±25 μM PD 98059. Incubation with SB 203580 (10 μM) was initiated during the time when serum starvation was extended, and the JNK synthesis following tetracycline removal. Prior to harvesting, cells were washed in PBS containing 5 mM NaF, and nuclear extracts were prepared in buffer (Nonidet P-40 (0.2% for 3pX-1 cells and 0.5% for 4pX-1 cells), 5 mM NaF, 5 mM orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 5 mM p-nitrophenol phosphate, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). For detecting phosphorylated c-Jun, cells were lysed directly in 1× SDS sample buffer (62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol, and 0.15 M β-mercaptoethanol). Western blot analyses were performed with 15 μg of extract per lane, employing phospho-CREB and phospho-c-Jun antibodies (New England Biolabs), respectively. OPTIMAS 6.1 software was used for quantification of Western blot analyses. Values obtained by digital densitometry for phosphorylated CREB or c-Jun were normalized to those of total CREB or c-Jun, respectively.

The ratio (−Tet/−Tet) of these values, plotted in the histogram, is derived from five independent experiments.

Statistical Analyses—Prism software was employed for statistical analyses.

RESULTS

DIFFERENTIAL, pX-DEPENDENT ACTIVATION OF ERK1,2 AND JNK IN pX-TRANSFORMING (3pX-1) VERSUS pX-NON-TRANSFORMING (4pX-1) CELL LINES—We demonstrated previously (35) that pX expression, following tetracycline removal, affects a differential induction of the immediate-early genes (IEG) c-fos and ATF3 in the 3pX-1 and 4pX-1 cell lines. Induction of IEGs follows activation of the mitogenic cascades (40–42). Therefore, the distinct, pX-dependent expression pattern of IEGs in 3pX-1 and 4pX-1 cells suggests that pX differentially activates mitogenic RAS-RAS-MAPK and JNK pathways and that this differential activation may be linked to different cell growth outcomes (35). In support of our hypothesis, recent studies (43) have demonstrated that the repertoire of IEG expression is determined by the duration of activation of mitogenic cascades. Sustained mitogenic pathway activation was shown to elicit a different IEG expression profile, when compared with transient activation (43).

To study the kinetics of pX activation of the RAS-RAS-MAPK pathway, a variety of reagents were used to inhibit ERK1,2 and JNK. In vitro kinase assays were performed with cellular extracts prepared from serum-starved cells (35). Kinase assays were performed with modifications of the solid phase kinase protocol (39), using 1 μg of GST-c-Jun (1–79) (Stratagene) as substrate. WCE (500 μg) was assayed for JNK activity for 20 min at 30 °C. Analysis was by 12% SDS-polyacrylamide gel electrophoresis and autoradiography.
and JNK pathways, we measured activation of ERK1,2 (Fig. 1) and JNK (Fig. 2) enzymes by in vitro assays employing cellular extracts prepared at different intervals after pX synthesis. Importantly, we have demonstrated previously by reverse transcriptase-polymerase chain reaction that expression of X mRNA is detected as early as 1 h after tetracycline removal in serum-starved 3pX-1 and 4pX-1 cells (35). In the present study we employ these same serum starvation conditions (35), including treatment with epidermal growth factor receptor inhibitor PD 153035 (44), in order to block the autocrine effect of TGF-α expressed by the AML12 cells (34). Following serum starvation, expression of pX mRNA is detected as early as 1 h after tetracycline removal in 3pX-1 and 4pX-1 cells, derived from at least three independent assays. Quantitation was performed by digital densitometry using OPTIMAS 6.1 software. pX-dependent MAPK activation in 3pX-1 cells is statistically significant (p < 0.05).

Activation of ERK1,2 by pX was examined using ERK1,2 kinase assay and GST-c-Jun as the substrate (39). Representative ERK1,2 assays using cellular extracts from 3pX-1 and 4pX-1 cells are shown in Fig. 1, A and B, respectively. Quantitation of at least three independent assays is shown in Fig. 2. In the pX-transforming 3pX-1 cells (Fig. 1, A and C), we observe a modest 1.5-fold activation of ERK1,2 1 h after pX synthesis is initiated that progressively increases to nearly 3-fold activation by 1 h, returning to basal level activation after 6 and 15 h. This moderate activation of ERK1,2 by pX (Fig. 1A) is in agreement with observations by others (45, 46). By contrast, activation of ERK1,2 in 4pX-1 cells (Fig. 1, B and C) is more pronounced at 1–2 h after pX synthesis is initiated, reaching nearly 3-fold activation by 1 h, but returning to basal activation after only 2 and before 6 h. Western blot analyses of the same cellular extract with antibody detecting total MAPK demonstrated equal amounts of total ERK1,2.

Likewise, in vitro JNK assays were performed with cellular extracts from 3pX-1 and 4pX-1 cells, using the solid phase kinase assay and GST-c-Jun as the substrate (39). Representative JNK assays are shown in Fig. 2, A and B; quantitation of at least three independent assays is shown in Fig. 2C. In 3pX-1 cells, activation of JNK by pX is not detectable in the intervals analyzed (Fig. 2, A and C). By contrast, in 4pX-1 cells, activation of JNK is observed as early as 30 min after pX synthesis is initiated. A 3.5-fold activation of JNK is observed within 1 h after pX synthesis in 4pX-1 cells and remains detectable even at 15 h after tetracycline removal (Fig. 2B). Equivalent amounts of total JNK were analyzed in the respective tetracycline treated and untreated samples, verified by Western blot analyses (Fig. 2), using antibody detecting total JNK.
Differential, pX-dependent Activation of Elk-1, CREB, and c-Jun in 3pX-1 and 4pX-1 Cells—To assess the in vivo consequence of pX-dependent mitogenic pathway activation observed in our in vitro kinase assays (Figs. 1 and 2), we examined activation of their downstream effectors, namely activation of Elk-1 (47), CREB (48), and c-Jun (49). These transcription factors are activated by phosphorylation at serine residues within their transactivation domains (47, 49). Elk-1 and c-Jun phosphorylation is mediated directly by activated ERK1,2 and JNK enzymes, respectively, whereas CREB is a distal target of ERK1,2 (48), being phosphorylated by p90RSK, the downstream target of the RAS-RAF-MAPK pathway (48, 50, 51). In addition, CREB is phosphorylated in response to activation of the p38 MAPK pathway (51, 52).

To monitor this activation of Elk-1, CREB, and c-Jun, we performed transient transfections using the in vivo signal transduction pathway transreporting system (Stratagene). In this assay system the Gal4-luciferase reporter, pFR-luciferase, is co-transfected with expression vectors encoding a fusion protein composed of the transactivation domain of Elk-1 (pFA-Elk-1), CREB (pFA-CREB), and c-Jun (pFA-c-Jun) in frame with the DNA binding domain of Gal4(-1–147). Since the expression of pX activates the ERK1,2 (Fig. 1) and JNK (Fig. 2) enzymes, we expected that pX expression will lead to activation by phosphorylation of their nuclear effectors, including the chimeric Elk-1-Gal4, CREB-Gal4, and c-Jun-Gal4.

Initially, we employed the Elk-1 transreporter assay, as it is the established assay to monitor in vivo activation of ERK1,2 (47). When Elk-1-Gal4 vector is co-transfected with the luciferase reporter in 3pX-1 cells (Fig. 3A), the ratio of luciferase activity (−Tet/+Tet) is ~7-fold higher than in 4pX-1 cells. Addition of MEK-1 inhibitor PD 98059 or co-transfection of dn RasN17 mutant reduced to nearly basal levels the pX-dependent induction (Fig. 3A), confirming that Elk-1-mediated activation is dependent on activation of RAS-RAF-MAPK pathway by pX. Likewise, to test for pX-dependent activation of the JNK pathway, we co-transfected the Gal4-luciferase reporter with the c-Jun-Gal4 expression vector. The ratio of luciferase activity (−Tet/+Tet) is ~5-fold higher in 4pX-1 than 3pX-1 cells (Fig. 3A), demonstrating a nearly 5-fold increased activity of the JNK pathway in 4pX-1 cells. Co-transfection of dn MEKK-1 mutant blocked this pX-dependent expression of the luciferase reporter (Fig. 3A), confirming that pX activates the JNK pathway in 4pX-1 cells. Importantly, both 3pX-1 and 4pX-1 cells respond to the positive effectors of the RAS-RAF-MAPK and JNK pathways (data not shown).

To confirm our results further we examined the pX-dependent activation of CREB in the two cell lines, using the CREB transreporter assay. Interestingly, although both 3pX-1 and 4pX-1 cells exhibit pX-dependent CREB activation, only in 3pX-1 cells is activation of CREB sensitive to RAS-RAF-MAPK pathway inhibitor PD 98059 (Fig. 3B); in 4pX-1 cells, pX-dependent CREB activation is insensitive to PD 98059 (Fig. 3B). We conclude that both the Elk-1 and CREB transreporter assays (Fig. 3, A and B) confirm that in 3pX-1 cells, pX expression mediates the transcriptional activation of Elk-1 and CREB via activation of the RAS-RAF-MAPK pathway. By contrast, the pX-dependent RAS-RAF-MAPK activation (Fig. 1) only minimally activates these transcription factors (Fig. 3).

Differential pX-dependent Phosphorylation, in Vivo, of CREB and c-Jun in 3pX-1 and 4pX-1 Cells—To demonstrate the in vivo consequence of mitogenic pathway activation by pX on endogenous downstream effectors, we examined the pX-dependent phosphorylation status of CREB and c-Jun. We focused our analysis on CREB as the downstream effector of the RAS-RAF-MAPK pathway because pX interacts directly with CREB/ATF proteins and increases their DNA binding affinity (1, 2, 16, 18). To detect in vivo phosphorylated transcription factors, cellular extracts were isolated at specific intervals after pX synthesis and used in Western blot analyses with phospho-specific antibodies.

In 3pX-1 cells (Fig. 4A), pX synthesis results in the appearance of phosphorylated CREB starting at 1 h and lasting at least 6 h (Fig. 4, A and E) after tetracycline removal. By contrast, in 4pX-1 cells, pX-dependent CREB phosphorylation is detectable only for 2 h after initiation of pX synthesis (Fig. 4, C and E). Since CREB phosphorylation occurs via activation of either RAS-RAF-MAPK or p38 MAPK pathways, we examined whether the p38 MAPK-specific inhibitor SB 203580 affects the
level of pX-mediated CREB phosphorylation (Fig. 4, B and D). In 3pX-1 cells (Fig. 4B), addition of 10 μM SB 203580 does not affect CREB phosphorylation mediated by pX synthesis, whereas addition of 25 μM PD 98059 does inhibit pX-dependent CREB phosphorylation. The opposite occurs in 4pX-1 cells; pX-dependent CREB phosphorylation is sensitive to SB 203580 but not to PD 98059 treatment (Fig. 4D). Importantly, both cell lines respond to the inhibitors PD 98059 and SB 203580, demonstrated by using the respective RAS-RAF-MAPK- and p38 MAPK-responsive transporter assays (Fig. 4F). In summary, these results (Fig. 4) agree with those derived from the CREB transreporter assays (Fig. 3B) and indicate that in 3pX-1 cells but not in 4pX-1 cells pX mediates CREB phosphorylation by activating the RAS-RAF-MAPK pathway.

Western blot analyses of pX-dependent c-Jun phosphorylation (Fig. 5, A and B) demonstrate only minimal c-Jun phosphorylation in 3pX-1 cells (Fig. 5, A and C). By contrast, c-Jun phosphorylation was sustained for at least 6 h in 4pX-1 cells, reaching basal phosphorylation after 6 and before 15 h following pX expression (Fig. 5, B and C). This prolonged phosphorylation of c-Jun in 4pX-1 cells agrees with the sustained activation of the JNK enzyme observed in the in vitro kinase assays (Fig. 2).

**Differential Dependence of Proliferation on RAS-RAF-MAPK Pathway in 3pX-1 and 4pX-1 Cells**—Since pX expression in 3pX-1 and 4pX-1 cells elicits a differential activation of RAS-RAF-MAPK and JNK pathways (Figs. 1–3), we explored the contribution of each mitogenic pathway in the proliferative response of these cells. Accordingly, we measured pX-dependent DNA synthesis as a function of treatment with 25 μM PD 98059, i.e. the ratio of [3H]thymidine incorporation in cells with pX expression (−Tet) versus that observed in cells without pX expression (+Tet), at 6, 18, and 30 h following tetrandrine removal. In agreement with similar observations by others (4), pX expression promotes DNA synthesis in both 3pX-1 (Fig. 6A) and 4pX-1 (Fig. 6B) cell lines. Addition of 25 μM PD 98059 inhibited pX-dependent DNA synthesis only in 3pX-1 cells (Fig. 6A). Surprisingly, addition of 25 μM PD 98059 to 4pX-1 cells promoted a small increase in pX-dependent cell proliferation (Fig. 6B), suggesting that additional signaling pathways may be involved.

The differential effect of PD 98059 in 3pX-1 and 4pX-1 cells is also shown (Fig. 6C) as the ratio of the pX-dependent proliferation obtained in the presence versus absence of PD 98059. A ratio smaller than 1.0 is indicative of sensitivity to treatment with PD 98059. Accordingly, our data (Fig. 6C) clearly show that the pX-dependent proliferative response in 3pX-1 cells is mediated via the RAS-RAF-MAPK pathway, whereas the proliferative response in 4pX-1 cells is independent of the RAS-RAF-MAPK pathway.

**Differential Regulation of Cyclin A Transcription in 3pX-1 and 4pX-1 Cells**—It is well established that onset of replication in mammalian cells requires the cyclin A protein (53). Transcription of the cell cycle-regulated cyclin A gene (54, 55) is mediated via CRE/ATF (56, 57) and Erk-1 (58) cis-acting elements. Based on the differential pX-dependent activation of CREB (Fig. 4) and c-Jun (Fig. 5) in 3pX-1 and 4pX-1 cells, and the selective dependence of 3pX-1 cells on the RAS-RAF-MAPK pathway for pX-mediated proliferation (Fig. 6), we explored regulation of cyclin A gene transcription as a function of pX synthesis in these two cell lines.

We employed the cyclin A-luciferase reporter (37) in transient transfection assays in 3pX-1 and 4pX-1 cells, and we demonstrated that pX expression induces transcription from the cyclin A promoter in both cell lines (Fig. 7). In 3pX-1 cells addition of 25 μM PD 98059 or use of dn RasN17 reduces cyclin A promoter transcription significantly, by 50 and 70%, respectively (Fig. 7A). However, in 4pX-1 cells addition of 25 μM PD 98059 does not have an inhibitory effect on cyclin A promoter transcription (Fig. 7B), and expression of dn RasN17 exerts only a minimal effect. By contrast, expression of dn MEKK1, which interferes with the JNK pathway, inhibits by nearly 70% transcription from the cyclin A promoter in 4pX-1 cells (Fig. 7B), without a substantial effect in 3pX-1 cells (Fig. 7A). Moreover, expression of dn ACREB (59) reduced cyclin A promoter transcription in 3pX-1 by ~70% but results in only a minimal reduction in 4pX-1 cells (Fig. 7, A and B).

We conclude that in 3pX-1 cells sustained, pX-dependent RAS-RAF-MAPK pathway activation (Fig. 1) affects CREB activation (Figs. 3 and 4) which, in turn, mediates transcription of the cyclin A gene (Fig. 7A). This dependence of cyclin A gene

**Fig. 4. Differential pX-dependent in vivo CREB phosphorylation in 3pX-1 and 4pX-1 cells.** Western blot analyses of nuclear extract were isolated from serum-starved cells, at indicated times after pX synthesis, employing CREB- and phospho-CREB antibodies. Nuclear extracts were isolated in parallel from 3pX-1 cells, ±10 μM SB 203580 or ±25 μM PD 98059 where indicated (A and B), and 4pX-1 cells, ±10 μM SB 203580 or ±25 μM PD 98059 where indicated (C and D). Forkin treatment (20 μM) was for 15 min. E, fold induction of pX-mediated CREB phosphorylation in 3pX-1 ((Db) and 4pX-1 (Db) cells derived from five independent assays, using OPTIMAS 6.1 software. pX-mediated CREB phosphorylation in 3pX-1 cells is statistically significant (p < 0.05). F, control assays demonstrate PD 98059 and SB 203580 effect on endogenous RAS-RAF-MAPK and p38 MAPK pathways, respectively, in 3pX-1 and 4pX-1 cells. Transient transfections of pFR-luciferase plasmid cotransfected with pFA-Elk1 or pPA-CHOP in 3pX-1 (Db) and 4pX-1 (Db) cells were stimulated with 20% FCS or 500 mM sorbitol ±25 μM PD 98059 or ±10 μM SB 203580, respectively.
transcription on the RAS-RAF-MAPK pathway, mediated by CREB activation (Fig. 7A), explains the sensitivity of the proliferative response of 3pX-1 cells to PD 98059 (Fig. 6). In 4pX-1 cells, nearly 70% of the cyclin A gene transcription is regulated via the pX-dependent, sustained activation of the JNK pathway (Fig. 7B), thus explaining the insensitivity of the mitogenic response of 4pX-1 cells to PD 98059 (Fig. 6).

pX-NLS Variant Displays Markedly Reduced Transformation—To demonstrate and assess the significance of the mitogenic pathway activation affected by pX in cellular transformation, we also constructed tetracycline-regulated cell lines expressing the pX-NLS variant. This variant is localized exclusively in the nucleus and does not activate the cytoplasmic mitogenic pathways (19). pX-NLS was cloned in the tetracycline-responsive PUHD10–3 vector (60) and stably introduced in clones 3 and 4 (35) derived from the AML12 cells (34).

Northern blot analysis (Fig. 8A) demonstrates tetracycline-regulated expression of pX-NLS mRNA in 4pX-NLS1 and 3pX-NLS1 clonal cell lines. Additional functional evidence supporting conditional expression of pX-NLS is based on its exclusive nuclear localization (Fig. 8B). pX-NLS does not activate cytoplasmic signaling cascades, as evidenced by its inability to induce expression of TRE- and NF-κB-driven reporters (19). Accordingly, upon tetracycline removal, our pX-NLS cell lines do not appreciably induce TRE- or NF-κB-driven luciferase expression. Importantly, as we reported previously (35), tetracycline removal induces CRE-luciferase expression in the presence of forskolin induction, based on the direct interaction of pX-NLS and CREB (18). Together, these data (Fig. 8, A and B) demonstrate that the constructed cell lines express the pX-NLS variant, in a tetracycline-regulated manner.

To examine the significance of mitogenic pathway activation by pX in transformation, we focused our analyses on clonal isolates from the 3pX-NLS lineage. In vitro ERK1,2 and JNK assays directly assessed activation of these mitogenic pathways by pX-NLS (Fig. 9A). In contrast to the sustained activation of ERK1,2 affected by pX in 3pX-1 cells (Fig. 1), the 3pX-NLS1 cells (Fig. 9A) display only transient pX-NLS-dependent activation of ERK1,2 and no activation of JNK (data not shown). We attribute this transient activation of ERK1,2 by pX-NLS to expression. Importantly, as we reported previously (35), tetracycline removal induces CRE-luciferase expression in the presence of forskolin induction, based on the direct interaction of pX-NLS and CREB (18). Together, these data (Fig. 8, A and B) demonstrate that the constructed cell lines express the pX-NLS variant, in a tetracycline-regulated manner.
the transient presence of pX-NLS in the cytoplasm during its synthesis, prior to its transport to the nucleus. Likewise, in vitro kinase assays employing the 4pX-NLS1 cell line demonstrate a transient activation of JNK (data not shown), in contrast to the sustained activation of JNK observed with 4pX-1 cells.

To confirm these in vitro kinase results by an in vivo assay, we carried out in vivo signal transduction transreporting assays, using 3pX-NLS1 cells (Fig. 9B). Whereas pX activates the RAS-RAF-MAPK pathway in 3pX-1 cells, monitored by the Elk-1-dependent expression of the reporter plasmid (Fig. 3), the expression of pX-NLS by tetracycline removal does not induce the Gal4-luciferase reporter, in the presence of either co-transfected Elk-1-Gal4 or c-Jun-Gal4 encoding vectors. These results agree with the published properties of pX-NLS (19) and indicate that pX-NLS does not activate cytoplasmic signaling in our tetracycline-regulated pX-NLS cell lines.

To address the significance of the cytoplasmic function of pX in transformation, we employed soft agar assays following pX synthesis in several 3pX-NLS clonal isolates having characteristics similar to the 3pX-NLS1 clone (data not shown). Soft agar assays assess anchorage-independent growth and are a criterion of cellular transformation (61). By this assay we have demonstrated previously (35) that pX expression results in cellular transformation.

Most interestingly, soft agar assays employing clonal isolates 3pX-NLS1, 3pX-NLS2, and 3pX-NLS3, in the absence of tetracycline, display markedly reduced transformation. Compared with 3pX-1 cells, pX-NLS clones display a nearly 70% reduction in transformation (Fig. 10). Since pX-NLS elicits minimal activation of the mitogenic pathways (Fig. 9) and significantly reduced transformation, these results (Fig. 10) directly demonstrate the crucial importance of cytoplasmic pX in activating...
the mitogenic RAS-RAF-MAPK pathway, consequently affecting hepatocyte transformation.

DISCUSSION

Activation of RAS-RAF-MAPK and JNK pathways is a well established function of pX (4, 10, 25, 26, 45). However, the significance of pX-mediated mitogenic pathway activation in transformation remains unresolved, due to the cellular model systems employed previously. These cellular systems employed either already transformed hepatocyte cell lines (26, 62), hepatocytes constitutively expressing pX (62, 63), or immortalized cell lines of questionable relevance to hepatocyte transformation (5, 64). Studies of NIH 3T3 cells have conclusively demonstrated that constitutive (65) or aberrant (66) RAS-RAF-MAPK activation is causally linked to transformation. However, regarding the mechanism of pX-mediated transformation, pX has been shown to activate not only the RAS-RAF-MAPK pathway (4, 25) but also the JNK pathway (26). The role of the JNK pathway remains enigmatic since, in some cases, it is associated with transformation (28) and in other cases with apoptosis (30–32). Therefore, determination of the molecular mechanisms of pX-mediated transformation requires the definition of the contribution of each mitogenic pathway to cell growth and transformation in the cellular environment of the hepatocyte.

In this paper, we examined the mechanism of pX-mediated hepatocyte transformation using a cellular model system, which models the normal, differentiated hepatocyte and, importantly, allows the determination of the molecular mechanisms of transformation. The system employs two immortalized hepatocyte cell lines (3pX-1 and 4pX-1), expressing pX conditionally. The 3pX-1 cells are a differentiated hepatocyte cell line in which pX expression leads to transformation and induction of IEGs, ATF3, and c-fos (35). By contrast, the 4pX-1 cells are a de-differentiated hepatocyte cell line, in which pX expression results in no transformation and only a modest induction of c-Fos (35). This differential induction by pX of c-Fos and ATF3 in 3pX-1 and 4pX-1 cells underscores the merit of this cellular system for delineating the early signaling events activated by pX. Those early events that we describe here are direct effects of pX action, in contrast to data derived from constitutive pX expression systems. Importantly, since pX expression is directly linked to transformation in 3pX-1 cells, the pX-mediated, reprogrammed mitogenic status of these cells allows the identification of molecular pathways leading to transformation.

In this study we provide evidence that pX initiates a differential activation of RAS-RAF-MAPK and JNK pathways in the pX-transforming (3pX-1) versus non-transforming (4pX-1) cell lines. This evidence is derived both from in vitro (Figs. 1 and 2) and in vivo assays (Figs. 3–7).

First, we used kinetic biochemical assays and assessed the duration of activation of mitogenic pathways by pX, from the beginning of pX synthesis. These analyses include in vitro kinase assays, starting at 1 h and until 15 h after pX synthesis. We have followed the work of others (30–32, 67), reviewed by Marshall (68), who proposed that mitogenic pathway activation lasting more than 3 h is sustained, whereas activation lasting less than 2 h is transient. Accordingly, we demonstrate that within the first 15 h of pX expression, pX affects a differential mitogenic pathway activation in the 3pX-1 versus 4pX-1 cells (Figs. 1 and 2). In the pX-transformable 3pX-1 cells (35), pX mediates sustained activation of ERK1,2 (Fig. 1A). By contrast, in the non-transformable 4pX-1 cells, pX leads to transient activation of ERK1,2 (Fig. 1B) and sustained activation of JNK (Fig. 2B).

Second, we confirmed these in vitro observations by in vivo analyses, using both the signal transduction pathway transreporting assays (Fig. 3) and the kinetic analyses of CREB (Fig. 4) and c-Jun phosphorylation (Fig. 5). The transreporting analyses (Fig. 3) demonstrate that only in 3pX-1 cells does pX induce Elk-1 and CREB activation via pX-mediated activation of the RAS-RAF-MAPK pathway. In 4pX-1 cells, pX induces selective activation of the JNK pathway (Fig. 3A). These results are further supported by the differential, pX-dependent phosphorylation of CREB (Fig. 4) and c-Jun (Fig. 5) in the two cell lines. In 3pX-1 cells pX synthesis initiates sustained CREB phosphorylation (Fig. 4A) which is insensitive to treatment with SB 203580 but was inhibited by PD 98059 (Fig. 4B). By contrast, in 4pX-1 cells pX-dependent CREB phosphorylation is sensitive to SB 203580 and insensitive to PD 98059 (Fig. 4D). Collectively, we interpret these results (Figs. 3–5) to mean that CREB phosphorylation in 3pX-1 cells is mediated by pX-dependent activation of the RAS-RAF-MAPK pathway (Fig. 1), whereas in 4pX-1 cells it is mediated by pX-dependent activation of the p38 MAPK pathway. Likewise, the in vivo phosphorylation of c-Jun demonstrates differential, pX-dependent phosphorylation only in 4pX-1 cells, in agreement with the sustained activation of JNK we detected in vitro (Fig. 2). Conclusions by others (68) indicate that sustained activation of mitogenic pathways results in different cellular responses than transient activation. Our results demonstrate that pX-dependent activation of the RAS-RAF-MAPK in the two cell lines (Figs. 1 and 3) elicits different cellular responses (Figs. 4, 6, and 7), depending on the duration of pX activation. The mechanism by which sustained versus transient activation of the RAS-RAF-MAPK pathway in 3pX-1 and 4pX-1 cells affects the differential activation of CREB remains to be determined.

Third, further supporting differential activation of the mitogenic pathways mediated by pX in these two cell lines, we demonstrate that the proliferative responses of 3pX-1 and 4pX-1 cells are causally linked to pX-mediated activation of the mitogenic pathways. Our results demonstrate that in 3pX-1 cells, pX-dependent DNA synthesis is sensitive to treatment with PD 98059 (Fig. 6), supporting that the RAS-RAF-MAPK pathway accounts for the observed, pX-dependent proliferative response. In contrast, PD 98059 treatment of 4pX-1 cells does not inhibit pX-dependent DNA synthesis.

Finally, we demonstrate a mechanistic link of (a) the pX-mediated mitogenic pathway activation (Figs. 1 and 2), (b) the resultant pX-dependent transcription factor activation (Figs.

\[ \text{C. Tarn and O. M. Andrisani, unpublished observations.} \]
Differential Mitogenic Pathway Activation by HBV X Protein

In 3pX-1 cells pX promotes sustained activation of the RAS-RAF-MAPK pathway leading to activation by phosphorylation of the pX-interacting transcription factor CREB (16, 18, 73). pX-CREB interactions lead to enhanced CREB/CRE binding and increased CRE-driven cyclin A gene expression via concomitant pX interactions with specific components (RPB5, TFIIB, and TFIIH) of the basal transcriptional apparatus (20–23). The link between the pX-mediated activation of the RAS-RAF-MAPK pathway and CREB phosphorylation is revealed by the inhibitory effect of dn RasN17 and PD 98059. The central role of CREB in pX-mediated hepatocyte growth is revealed by the effect of dn ACREB on cyclin A gene transcription.

We demonstrate that pX-induced transcription from the cyclin A promoter in 3pX-1 cells is dependent primarily on activation of the RAS-RAF-MAPK pathway and is inhibited by treatment with PD 98059 and expression of dn RasN17 and dn ACREB (Fig. 7A). The observed transcriptional efficacy of CREB in cyclin A transcription in 3pX-1 cells (Fig. 7A) may also reflect the nuclear effect of pX in further enhancing the CRE-binding potential of CREB (18). Furthermore, the interfering effect of dn ACREB in cyclin A transcription in 3pX-1 cells supports our mechanistic model (Fig. 11), namely that CREB is a central molecule in pX-mediated hepatocyte cell growth. Studies are in progress to assess the role of CREB in pX-mediated transformation.

By contrast, in 4pX-1 cells pX-mediated transcriptional induction from the cyclin A promoter is insensitive to inhibitors of the RAS-RAF-MAPK pathway. Moreover, dn MEKK1 expression in 4pX-1 cells reduces cyclin A transcription by nearly 70%, indicating that cyclin A transcription is dependent on the JNK pathway (Fig. 7B). Importantly, the JNK pathway is known to activate by phosphorylation, c-Jun (49) and ATF-2 (69), which act on the CRE/ATF cis-acting element of the cyclin A promoter, either as ATF-2 homodimers or c-Jun/ATF-2 heterodimers (57).

In summary, we have previously shown (35) that pX expression in 3pX-1 and 4pX-1 cells results in different cell growth outcomes. In this study, we extend our observations and demonstrate that pX differentially activates the RAS-RAF-MAPK and JNK pathways in the two cell lines (Figs. 1–5). Our results demonstrate for the first time that pX mediates two distinct phenomena in cells of related cellular backgrounds.

Since pX has been implicated both in mechanisms of cell growth (4, 5) and apoptosis (6–8), our results reveal key aspects of the molecular mechanism by which pX affects these cell growth outcomes. Specifically, our findings reported herein identify the importance of sustained activation of the RAS-RAF-MAPK pathway by pX in hepatocyte transformation. Our results agree with the accepted role of sustained RAS-RAF-MAPK pathway activation in transformation (65, 66, 70) and differentiation (71) and agree with studies reporting that both the expression and activity of ERK1,2 are increased in primary HCC (72).

Furthermore, this mechanism of cytoplasmic pX in hepatocyte transformation is demonstrated by the conditional 3pX-NLS cell lines (Fig. 8). pX-NLS expression affects only minimal RAS-RAF-MAPK pathway activation (Fig. 9) and minimal hepatocyte transformation (Fig. 10). pX-NLS transforms hepatocytes 70% less efficiently than pX (Fig. 10). Although minimal, the observed transforming potential of pX-NLS may be a result of the combined effects of transient MAPK activation observed in 3pX-NLS cells (Fig. 9A) and the activity of pX in enhancing the DNA binding potential of CREB (2, 16, 18, 73). Importantly, since CREB is the downstream target of the RAS-RAF-MAPK pathway (Fig. 4), the combination of these activities may be sufficient to elicit a low level transformation exhibited by pX-NLS (Fig. 10). Since pX-NLS elicits only minimal activation of the mitogenic pathways and significantly reduced transformation, we conclude that our results (Fig. 10) directly demonstrate the crucial importance of pX acting to affect sustained activation of the RAS-RAF-MAPK pathway, thus causing aberrant growth and hepatocyte transformation.

The role of the sustained, pX-mediated activation of the JNK pathway, observed in our 4pX-1 cell line, remains unresolved. In some cases (27–29) sustained JNK activation is associated with transformation and in others (31–33) with apoptosis. Since pX is known to transform hepatocytes (35, 74) and also to sensitize cells to apoptosis (7, 8), we propose that absence of pX-dependent transformation in 4pX-1 cells may be due to the apoptotic function of pX, mediated by the sustained activation of the JNK pathway. Studies are in progress to determine whether pX sensitizes 4pX-1 cells to apoptosis.

Earlier studies employing hepatoma cell lines (4, 7, 8) report data making it difficult to dissect the growth-promoting and -transforming functions of pX versus its function in apoptosis. The strength and novelty of the 3pX-1 and 4pX-1 cell lines are the separate manifestations of these opposite pX-driven growth outcomes, making them amenable to molecular analyses. Our analyses establish a molecular mechanism by which pX mediates these opposing cell growth outcomes. We demonstrate that cells transformed by pX exhibit sustained activation of the RAS-RAF-MAPK pathway, in contrast to cells that do not. We propose that cells, which exhibit sustained activation of JNK pathway by pX, will be sensitized to apoptosis. It is well established that overexpression of cellular and viral oncoproteins results in both transformation and apoptosis (75). It has been proposed that these opposing growth outcomes reflect a linkage of the signaling pathways mediating transformation and apoptosis (76). Our results support this proposed mechanism and suggest that these pathways are inversely linked.

In conclusion, our results are the first report of the hepatocyte mitogenic status affected by pX and the resultant growth outcomes, i.e., transformation versus lack of transformation. We demonstrate that pX expression affects a differential activation of the RAS-RAF-MAPK and JNK pathways. We propose that this
inverse mitogenic pathway activation, affected by pX, is causally linked to the opposing cell growth outcomes.

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