Differential Immediate Early Gene Expression in Conditional Hepatitis B Virus pX-transforming Versus Nontransforming Hepatocyte Cell Lines*

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We report construction and characterization of tetracycline-controlled hepatitis B virus pX-expressing hepatocyte (AML12) cell lines. These cell lines were constructed in AML12 clonal isolates (clones 3 and 4), which express constitutively the tetracycline-controlled transactivator. Since pX is implicated in HCC, this immortalized hepatocyte model system was used to investigate the mechanism of pX in transformation. Clonal isolates of 3pX and 4pX lineages display conditional synthesis of pX mRNA and protein and a 2-fold increase in growth saturation density following tetracycline removal, implicating pX in monolayer overgrowth. Interestingly, only 3pX clones display pX-dependent anchorage independence. Clone 3 lineages express hepatocyte nuclear factor-1a and hepatocyte-specific marker genes; clone 4 lineages express hepatocyte nuclear factor-1β and reduced levels of hepatocyte-specific marker genes, suggesting the importance of the differentiated hepatocyte in pX-mediated oncogenic transformation.

Importantly, 3pX and 4pX lineages display differential expression of immediate early genes c-fos and ATF3. The pX-transforming 3pX lineage displays early, pX-dependent induction of ATF3 and prolonged induction of c-fos. The nontransforming 4pX cells display an absence of pX-dependent ATF3 induction and transient induction of c-fos. Our results support the direct link of pX expression to oncogenic transformation in 3pX lineage clones and underscore the advantage of this conditional cellular model system for studying mechanisms of pX-mediated oncogenesis.

Hepatitis B virus (HBV)1 infection causes acute hepatitis in humans, 10% of cases resulting in chronic hepatitis (1), which is linked to development of hepatocellular carcinoma (HCC) (1); integrated HBV DNA is present in virtually all HBV-mediated liver cancers (2, 3). However, the mechanism of HBV-mediated hepatocarcinogenesis remains elusive.

HBV encodes a 16.5-kDa protein termed the X antigen (pX) (4). pX is expressed during viral infection (5–7), is required for the viral life cycle (7), and is highly conserved in all oncogenic mammalian hepadnaviruses (8, 9). In contrast, the avian hepatitis virus, which lacks oncogenic potential, is devoid of an X open reading frame (10). pX promotes liver tumor formation in transgenic mice expressing high levels of pX (11), potentiates c-Myc-induced hepatocarcinogenesis in c-myc/pX bitransgenics (12), and acts as a tumor promoter in hepatocarcinogenesis (13). While the published data clearly implicate pX in HCC, the direct oncogenic effect of pX in hepatocyte transformation has not been demonstrated. Herein, we provide evidence demonstrating the causal link of pX expression to the transformation of immortalized hepatocytes and describe a conditional pX expression system that is amenable to the study of the mechanism of pX-mediated transformation.

pX is a multifunctional protein, with reported activities affecting transcription (14), cell growth (15, 16), and apoptotic cell death (17, 18). Although pX does not directly bind double-stranded DNA, pX acts as a promiscuous transactivator (reviewed in Ref. 14), via interaction with several components of the transcriptional apparatus (19–23). In addition, specific pX-responsive cis-acting elements have been identified, e.g. NF-κB (24–28), AP-1 (29–34), AP-2 (29), and CRE sites (35–37). Transcriptional activation of the AP-1 and NF-κB sites by pX is via cytoplasmic signaling pathways, including the protein kinase C pathway (31, 32) and the Ras, Raf, MAP kinase (33, 38), and c-Jun N-terminal kinase (39) pathways. By contrast, pX activation of CRE-mediated transcription involves direct protein-protein interactions of pX with the bZip factors CREB/ATF2 (35, 36). Importantly, in addition to CREB, pX also interacts directly with other inducible bZip transcription factors, activators or repressors (37), which play important roles in hepatocyte physiology (40–42).

This dual mechanism of pX action, namely the activation of mitogenic signaling cascades in the cytoplasm and the increased transcriptional efficacy of bZip transcription factors in the nucleus, clearly implicate pX in deregulation of hepatocyte gene expression, resulting in oncogenic transformation. However, the role of these activities of pX in cellular transformation cannot be assessed by the available model systems (15, 18, 33). To demonstrate a direct effect of pX in hepatocyte transformation and investigate basic in vivo mechanisms of pX-mediated oncogenicity, we established a conditional pX-expressing cellular model system, composed of the immortalized mouse hepatocyte AML12 cell line (43) and the tetracycline-controlled system of Gossen and Bujard (44).

We present data linking conditional pX expression in AML12 cells to several criteria characteristic of oncogenic transformation. Importantly, we demonstrate that this pX-mediated oncogenic transformation occurs only in differentiated hepatocytes, suggesting the importance of the hepatocyte environ-

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1 The abbreviations used are: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; CRE, cAMP response element; CREB, cAMP response element-binding protein; bZip, basic region leucine zipper; HNF, hepatocyte nuclear factor; TGF-α, transforming growth factor α; MAP, mitogen-activated protein; PCR, polymerase chain reaction; EGF, epidermal growth factor; CAT, chloramphenicol acetyltransferase; TRE, 12-O-tetradecanoylphorbol-13-acetate response element.
ment in pX-mediated transformation. These conditional pX-expressing cell lines provide an ideal cellular model system to investigate signaling mechanisms of pX and its potential links to hepatocyte transformation, especially valuable when considering the multifunctional nature of HBV pX and the clinical significance of HCC.

MATERIALS AND METHODS

Plasmids—Plasmids PUHD15–1, PUHC13–3, and PUHD10–3, required for the tetracycline-repressible system, were kindly provided by Gossen and Bujard (44). The HBV pX gene was cloned into PUHD10–3 plasmid, pHD300, resulting in plasmid PUHD15–1. Tissue Culture—AML12 cells were kindly provided by Dr. N. Fausto. Cells were propagated in Dulbecco’s modified Eagle’s medium/F-12 medium containing 10% fetal calf serum; a mixture of insulin, transferrin, and selenium (ITS, Life Technologies); 1.0 μM dexamethasone; and 50 μg/ml gentamicin (43). Transfections were performed by calcium phosphate co-precipitation, using the Life Technologies, Inc. transfection kit. Briefly, 10–20% sub-confluent cultures were transfected with 5 μg of PUHD15–1 plasmid encoding the tTA activator gene, in the presence of 2 μg of pKNeo plasmid, encoding the neomycin phosphotransferase-selectable marker. Stable transformants were selected with 600 μg/ml G418 (Geneticin; Life Technologies). G418-resistant cells were subcloned and propagated. tTA expression was verified by transient transfection of PUCH13–3 reporter (luciferase) plasmid, in the presence of increasing concentrations of tetracycline.

RESULTS

Tetracycline-regulated cell lines expressing pX were generated from the hepatocyte AML12 cell line, derived from the liver of transgenic mice overexpressing TGF-α (43). These tetracycline-regulated cell lines were constructed by generating two successive cell lines; the first was engineered to express the tetracycline-controlled activator (tTA); the second cell line was constructed in this tTA+ background, to express pX under tTA control (44). Several AML12 clonal isolates expressing tTA were obtained displaying activation ratios ranging from 2- to 56-fold (Table I). The tTA-responsive PUHD10–3-X plasmid-encoding pX was stably introduced in the tTA+ clone 3 and 4 cell lines, resulting in 3pX and 4pX lineages. These lineages are thus composed of clonal isolates (3pX-1, 3pX-2, 4pX-1, 4pX-2, etc.) derived from 3pX and 4pX transfections. The conditional pX clonal isolates were selected using a pX/CREB-dependent functional assay (36), based on the enhanced transcriptional efficacy of endogenous CREB by pX (36). Accordingly, putative 3pX-1 and 4pX-1 cell lines display increased CREB-dependent transcription following tetracycline removal, whereas tetracycline removal did not affect CREB-dependent transcription in parental clones 3 and 4 (data not shown). Furthermore, the addition of tetracycline did not alter the transcriptional efficacy of a Rous sarcoma virus-luciferase reporter in AML12 cells (data not shown).

Northern blot analyses (Fig. 1A) established the tetracycline-regulated expression of pX mRNA in these putative pX-expressing cell lines. Expression of pX mRNA was detected at 1 h after tetracycline removal, reaching maximal levels by 12–48 h. In comparison with the 3pX-1 cell line, the 4pX-1 cell line expressed approximately 3-fold more pX mRNA (Fig. 1B) following tetracycline removal. Northern blot analyses of two additional, independently isolated putative pX-expressing clones from both lineages also demonstrated conditional pX mRNA expression (data not shown).

To conclusively demonstrate the conditional synthesis of pX, we carried out immunoprecipitation reactions of cellular extracts isolated from 3pX-1 and 4pX-1 cells, metabolically labeled with [35S]methionine, following tetracycline removal. We observed pX expression in extracts of cells grown without tetracycline (Fig. 1C). pX was not detected in extracts from either 3pX-1 or 4pX-1 cells grown in the presence of tetracycline or in control clones 3 and 4, demonstrating the tetracycline-regulated synthesis of the X protein.

Overgrowth of the Monolayer by pX in 3pX-1 and 4pX-1 Cell Lines—Saturation density analyses of 3pX-1 and 4pX-1 cell lines (Fig. 2) were used to assess the potential of cells expressing pX to overgrow the monolayer, a criterion indicating oncogenic transformation. The growth saturation density of 3pX-1 and 4pX-1 cell lines in media containing tetracycline is similar to the respective parental cell lines. Removal of tetracycline,
FIG. 1. Tetracycline-regulated pX expression in AML12 cells. A, Northern blot of poly(A)⁺ mRNA from 3pX-1 and 4pX-1 cell lines at the indicated times after tetracycline removal. B, quantitation of pX mRNA expression in 3pX-1 and 4pX-1 cell lines from 30 μg of total RNA isolated at the indicated times after tetracycline removal. Histograms represent the average of three independent experiments of RNA isolated from cells of passages 5-7. -Fold induction is expressed relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal control). C, [³⁵S]methionine-labeled cell extracts from clones 3 and 4 and cell lines 3pX-1 and 4pX-1, immunoprecipitated with pX-specific antibody. Immunoprecipitates were analyzed by 14% SDS-polyacrylamide gel electrophoresis and fluorography. The sizes of molecular weight markers are shown; the arrows denote pX.

FIG. 2. Growth saturation density analyses. A, 3pX-1 cell line; B, 4pX-1 cell line. Aliquots containing 10⁵ cells of each culture, seeded in quadruplicates in 60-mm culture dishes, were fed daily with media with or without 5 μg/ml tetracycline and counted at the days indicated.
thus allowing pX synthesis, resulted in an approximately 2-fold increase in saturation density of both 3pX-1 and 4pX-1 cell lines, in comparison with the parental control clones 3 and 4, also grown without tetracycline. This observation demonstrates that pX promotes overgrowth of the monolayer in 3pX-1 and 4pX-1 cells.

Anchorage-independent Growth of 3pX-1 and 4pX-1 Cell Lines—Anchorage-independent growth is often correlated with tumorigenesis and is an important criterion for cultured cell transformation (46). Therefore, we tested the potential of the 3pX-1 and 4pX-1 cell lines for anchorage-independent growth in soft agar, following pX induction (Fig. 3). 3pX-1 cells grown in the absence of tetracycline, formed large foci in soft agar, whereas the addition of tetracycline suppressed focus formation (Fig. 3A). The transformation efficiency of clone 3 is similar to that observed with 3pX-1 cells grown in the presence of tetracycline; by comparison, removal of tetracycline promoted a 10-fold increase in transformation efficiency of 3pX-1 cells (Fig. 3B). This observation demonstrates the importance of pX induction and activity in the anchorage-independent growth displayed by the 3pX-1 cell line. Importantly, additional independent clonal isolates of the 3pX lineage also demonstrated anchorage-independent growth, similar to the 3pX-1 cell line (data not shown).

Surprisingly, the 4pX-1 cell line failed to demonstrate anchorage independence under conditions of pX induction (Fig. 3). Analysis of two additional, independently isolated clonal cell lines from the 4pX lineage (data not shown) demonstrated that they also lacked the ability to form foci in soft agar.

pX Expression Promotes Morphologic Changes in 3pX-1 Cells—We noted a marked difference in cell morphology between cells of the 3pX and 4pX lineages (Fig. 4A). 3pX-1 cells, even at 48 h after passage, contained clusters of rounded, epithelioid cells that adhered loosely to the surface; this phenotype was even more pronounced when cells were grown in the absence of tetracycline. By comparison, 4pX-1 cells appeared mesenchymal and did not display the rounded form characteristic of nonadhering cells, suggesting that anchorage...
independence is linked to pX expression in a lineage-specific manner.

More than 90% of the nonadherent 3pX-1 cells, observed after passage in tetracycline-free medium (Fig. 4A), were viable, as confirmed by trypan blue staining. Since anchorage independence is an important criterion of cultured cell transformation (46), we reasoned that nonadherent 3pX-1 cells may represent transformed hepatocytes. To test this hypothesis, nonadherent cells from cultures grown in the absence of tetracycline for 48 h were grown as suspension cultures, without tetracycline for 48 h and used in soft agar assays. 3pX-1 + Tet, grown without tetracycline; 3pX-1 + Tet, grown with 5 μg/ml tetracycline in soft agar assay only; 3pX-1 + Tet, grown with 5 μg/ml tetracycline as nonadherent cultures and in soft agar; clone 3, parental clone 3 cells. Relative transformation is expressed as percentage of foci formed and is the ratio of iodonitrotetrazolium-staining foci larger than 80 μm in diameter versus the total number of small iodonitrotetrazolium-stained foci.

Parental Clone 4 and 4pX Cell Lines Are Dedifferentiated Hepatocytes—To address this seeming discrepancy of the transforming potential of pX in the 3pX and 4pX lineages, we investigated their stage of differentiation. Clones 3 and 4 and cell lines 3pX-1 and 4pX-1 were examined for the expression of HNF-1α, a transcription factor characterizing differentiated hepatocytes (50, 51). Nuclear extracts were analyzed by gel retardation assays, employing the PE-56 binding site of HNF-1, recognized by both HNF-1α and HNF-1β (50). Supershift assays employing the HNF-1α antibody demonstrated the presence of high levels of transcription factor HNF-1α in extracts obtained from parental clone 3 and 3pX-1 cells (Fig. 5A), a characteristic of differentiated hepatocytes (50). By contrast, in extracts from clone 4 and 4pX-1 cells, the addition of HNF-1α antibody resulted in only a minor supershift of the DNA-protein complex (Fig. 5A). This suggests that the HNF-1 DNA-protein complex forms instead with transcription factor HNF-1β, which also binds the HNF-1 site and is expressed in dedifferentiated hepatocytes (50, 51). The addition of the un-
related CREB antibody to these reactions did not affect super-shift of HNF-1 DNA-protein complexes (data not shown).

Comparative PCR (53) analysis to monitor the expression of hepatocyte-specific markers (54) in AML12 cells and the AML12-derived clones 3 and 4 is shown in Fig. 5B. Expression of hepatocyte markers in clone 3, closely resembled the expression pattern seen in parental AML12 cells. In contrast, clone 4, which expresses high levels of HNF-1β (Fig. 5A), displays markedly reduced levels of hepatocyte-specific gene expression, further indicating that clone 4 is a dedifferentiated hepatocyte.

pX-dependent Early Induction of ATF3 and c-fos mRNAs in 3pX-1 Cell Lines—To characterize further the 3pX-1 and 4pX-1 cell lines, we analyzed the expression pattern of the immediate early genes c-fos and ATF3 by a time course study after pX synthesis. c-fos is induced by pX, via activation of the Ras-Raf-MAP kinase pathway (39), whereas ATF3 is induced by anisomycin (55), a known activator of the c-Jun N-terminal kinase pathway (56), and via c-Jun N-terminal kinase pathway activa-

**Fig. 5.** Clone 4 lineages are dedifferentiated hepatocytes. A, analysis of HNF-1 binding activities was by gel retardation/supershift assays. Nuclear extracts (10 μg) were isolated from clones 3 and 4, and cell lines 3pX-1 and 4pX-1 were analyzed by gel retardation assays employing the HNF-1 binding site (PE-56 albumin), as the radiolabeled probe. Supershift assays employed 0.5 μl of HNF-1α antibody. DNA-protein binding reactions were performed as described (50). B, PCR analysis. Total RNA isolated from AML12 cells and clones 3 and 4 was digested with DNase I and subjected to RT-PCR analysis, as described (53). One-tenth of the resulting cDNA was used in the presence of 32P-ATP with specific primers for albumin, α-fetoprotein transferrin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The linear range of amplification was empirically determined. Glyceraldehyde-3-phosphate dehydrogenase and transferrin were amplified for 17 cycles; α-fetoprotein and albumin were amplified for 30 cycles. Analysis of PCR products was by agarose gel electrophoresis and autoradiography. Control PCR reaction was with no template added, in the presence of the respective primers.

**Fig. 6.** pX-dependent induction of ATF3 mRNA. Northern blot analysis is shown of ATF3 mRNA expression employing 30 μg of total RNA isolated from 3pX-1 and 4pX-1 cell lines (A) and clone 3 and 4 cell lines (B), grown in 10% serum, at the indicated times after tetracycline (5 μg/ml) removal. C, reverse transcription-PCR (53, 70) analysis of RNA isolated from 3pX-1 and 4pX-1 cell lines at 1 and 2 h after tetracycline (5 μg/ml) removal, employing pX-specific primers (5′-primer, 5′-GGA CGT CCT TTG TTT ACG-3′; 3′-primer, 5′-CCT ACA GCC TCC TAA TAC-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. Amplification for pX is for 40 cycles. Control PCR reaction with no template added, in the presence of the respective primers. D, quantitation of ATF3 mRNA expression in 3pX-1 and 4pX-1 cell lines. Histograms represent the average of four independent experiments of RNA isolated from cells of passages 5–8; -fold induction is expressed relative to the parental clone.
FIG. 7. 

**pX-dependent induction of c-fos mRNA.** Northern blot analysis is shown of c-fos mRNA expression employing 30 µg of total RNA isolated from serum-starved 3pX-1 and 4pX-1 cell lines (A) and clone 3 and clone 4 cell lines (B), at the indicated times after tetracycline (5 µg/ml) removal. Conditions of serum starvation and pX induction are described under “Results.” C, reverse transcription-PCR of RNA isolated from serum-starved 3pX-1 and 4pX-1 cell lines at 1 and 2 h after tetracycline removal (5 µg/ml), employing pX-specific primers. Amplification conditions are as described in the legend of Fig. 6C. D, quantitation of c-fos mRNA expression in 3pX-1 and 4pX-1 cell lines. Histograms represent the average of four independent experiments of RNA isolated from cells of passages 5–8; fold induction is expressed relative to the parental clone.
Conditional HBV pX Expression in AML12 Hepatocytes

FIG. 8. pX-dependent AP-1 activation in 3pX-1 and 4pX-1 cells. TRE-CAT DNA (5 μg) was transfected by the CaPO4 method in serum-starved 3pX-1 and 4pX-1 cells (lanes 1–8) and parental clone 3 cells (lanes 9–12), as a function of pX synthesis, with or without 5 μg/ml tetracycline. Serum starvation conditions are shown as follows. Lanes 1 and 2, 24 h without serum and with 10 μM MEK-1 inhibitor; lanes 3 and 4, 24 h without serum and with 10 μM EGF receptor inhibitor; lanes 5–12, 24 h without serum and with 10 μM MEK-1 inhibitor and pX synthesis (without Tet, lanes 2, 4, 6, and 8) was carried out for 24 h prior to transfection in 2% serum and in the presence of the indicated inhibitors. Following CaPO4 transfection, media contained 2% serum 10 μM EGF receptor inhibitor with or without 5 μg/ml tetracycline. Lanes 7 and 8, cells stimulated with 20% serum. CAT assays (36, 37) were performed 24 h after transfection, quantitated, and expressed per μg of cellular extract.

pX-dependent AP-1 Activation—The pX-dependent induction of c-fos mRNA in 3pX-1 and 4pX-1 cells demonstrates the early induction of ATF3 mRNA exclusively in 3pX-1 cells (Fig. 6A). Maximal ATF3 mRNA expression is induced 1 h after pX synthesis, whereas basal ATF3 mRNA expression is detected in 3pX-1 cells treated with tetracycline. By contrast, tetracycline addition and removal in clone 3 cells did not result in ATF3 induction (Fig. 6B). Reverse transcription-PCR analysis of RNA isolated from the 3pX-1 cell line at 1 and 2 h after tetracycline treatment confirms the early expression of pX mRNA (Fig. 6C). Interestingly, ATF3 induction is not observed in 4pX-1 cells in response to pX synthesis (Fig. 6A), despite the early expression of pX mRNA (Fig. 6C). These comparative studies (Fig. 6D) demonstrate a differential, pX-dependent, 3-fold induction of ATF3 mRNA in the pX-transforming 3pX-1 cell line. Accordingly, this differential induction of ATF3 mRNA in 3pX-1 cells is one major difference in response to pX, between the transforming 3pX-1 and nontransforming 4pX-1 cell lines.

pX-dependent Early c-fos Induction—Comparative Northern blot analysis of c-fos mRNA expression, following pX synthesis in the 3pX-1 and 4pX-1 cell lines (Fig. 7A), was carried out with RNA isolated from serum-starved cells (24 h), also treated for 24 h with the specific EGF receptor inhibitor PD 153035 (58); we use the EGF receptor inhibitor during the serum starvation because AML12 cells were derived from transgenic mouse liver overexpressing TGF-α. TGF-α signals via the EGF receptor and its autocrine effect activate the Ras-Raf-MAP kinase pathway. We included the PD 153035 inhibitor to block the autocrine effect of TGF-α. Following serum starvation, pX synthesis was initiated by tetracycline removal in media containing 2% serum and 10 μM EGF receptor inhibitor. We observed induction of c-fos mRNA 1 h after pX synthesis in both 3pX-1 and 4pX-1 cell lines, whereas tetracycline addition and removal in parental clone 3 and 4 cell lines did not induce c-fos mRNA expression (Fig. 7B). We did not observe expression of pX mRNA, under these growth conditions, as demonstrated by reverse transcription-PCR analysis of RNA isolated from 3pX-1 and 4pX-1 cell lines at 1 and 2 h after tetracycline removal (Fig. 7C). Interestingly, c-fos induction is sustained to 6 h after pX synthesis in the 3pX-1 cell line, whereas in the 4pX-1 cell line, c-fos induction is reduced to basal level by 2 h after pX synthesis (Fig. 7D).

Quantitation of c-fos mRNA induction by pX demonstrates approximately a 2.5-fold induction in 3pX-1 cells, whereas only a transient, 2-fold induction is observed in the nontransforming 4pX-1 cell line (Fig. 7D). Together, these results support the pX-dependent induction of c-fos mRNA (Fig. 7).

pX-dependent Induction of AP-1 Activity—The pX-dependent induction of c-fos and ATF3 mRNAs (Figs. 6 and 7) suggests the pX-dependent activation of the mitogenic Ras-Raf-MAP kinase and c-Jun N-terminal kinase pathways, respectively, as shown to occur in Chang liver cells (33, 38, 39). The combined activation of these pathways results in enhanced AP-1 activity (33, 38, 39). Consequently, we examined, by transfection assays employing the TRE-CAT reporter, whether the conditional synthesis of pX would result in pX-dependent AP-1 activity in the 3pX-1 and 4pX-1 cell lines (Fig. 8). Due to high background expression of the TRE-CAT reporter following 24-h serum starvation (lanes 1–4), we established the conditions for minimal background expression. These conditions are 24-h serum starvation in the presence of 10 μM EGF receptor inhibitor (PD 153035) and 50 μM MEK-1 inhibitor (PD 98059) (59) (compare lanes 1–4 and lanes 5 and 6). pX synthesis in the absence of tetracycline was carried out for 24 h prior to transfection in media containing 2% serum, 10 μM EGF receptor inhibitor and 50 μM MEK-1 inhibitor. Earlier studies reported the reactivation of MAP kinase by serum or growth factor stimulation, following inhibition by the MEK-1 inhibitor (59). Similarly, we demonstrate that the effect of pX synthesis (lanes 5 and 6) or 20% serum stimulation (lane 7) on serum-starved cells is the induction of AP-1 activity. Specifically, we observe that pX synthesis in 3pX-1 cells promotes a 2-fold induction in TRE-CAT expression (lanes 5 and 6), whereas tetracycline treatment of parental clone 3 cells does not affect TRE-CAT expression (lanes 9 and 10). Importantly, under the same serum starvation conditions, 4pX-1 cells display minimal induction in AP-1 activity in response to pX synthesis (5–6). Interestingly, pX synthesis by tetracycline removal in either 3pX-1 or 4pX-1 cell lines, further potentiates the effect of 20% serum stimulation (lanes 7 and 8). By contrast, tetracycline removal from parental clone 3 cells does not further increase AP-1 induction (lanes 11 and 12), indicating that tetracycline does not have a direct effect on the observed enhancement in AP-1 activity. These results are in agreement with the observed transient induction of c-fos mRNA in 4pX-1 cells and the prolonged induction of c-fos mRNA in 3pX-1 cells (Fig. 7).
DISCUSSION

We report construction and characterization of tetracycline-regulated pX-expressing hepatocyte cell lines. The mouse hepatocyte AML12 cell line was employed because AML12 cells are not transformed and display properties of normal, differentiated hepatocytes (43). Accordingly, AML12 cells provide an ideal system to study the growth-regulating and oncogenic potential of pX in an hepatocyte cell culture system. We employed the tetracycline-controlled gene expression system (44) for expressing pX to avoid potential cytotoxic effects on cell growth due to overexpression of pX. During all steps of construction and propagation of the pX-expressing cell lines, 5 μg/ml of tetracycline was included in the media to repress pX expression. Selection of tetracycline-regulated pX-expressing cell lines utilized a functional pX/CREB-dependent assay (36), which enabled us to eliminate putative pX-expressing clones displaying constitutive pX expression.

The evidence that demonstrates the conditional expression of pX in the 3pX-1 and 4pX-1 clonal cell lines includes the tetracycline-regulated expression of pX mRNA and protein (Fig. 1) assessed by Northern blot and immunoprecipitation analyses, respectively. These clonal cell lines display increased CREB-dependent transcriptional activation following tetracycline removal (data not shown), indicating that pX maintains its transcriptional activation potential (36, 37). Accordingly, these conditional pX-expressing cell lines provide an excellent model system to investigate the role of pX in hepatocyte growth and transformation. Importantly, in addition to the mitogenic effects of pX (33, 38), pX is also known to promote apoptosis (16, 17, 60).

We examined the oncogenic properties of these cell lines by growth density saturation analyses (Fig. 2) and soft agar assays (Fig. 3). All clonal isolates of the 3pX lineage following pX expression, demonstrate increased growth saturation density (Fig. 2A) and anchorage-independent growth in soft agar (Fig. 3), indicating the direct role of pX in oncogenic transformation. Furthermore, the rounded epithelioid phenotype of 3pX-1 cells grown without tetracycline (Fig. 4A), agrees with the well-documented cell morphology changes characteristic of transformed epithelia (61, 62).

Clones of the 4pX lineage express pX in a tetracycline-regulated manner (Fig. 1), display increased transcriptional efficacy of endogenous CREB (data not shown), and display increased growth saturation density (Fig. 2B). However, they lack anchorage-independent growth (Fig. 3) and display mesenchymal morphology (Fig. 4A). Both parental clone 4 and 4pX cell lines express high levels of HNF-1α (Fig. 5A), which is associated with repression of hepatocyte-specific genes (51, 52). In agreement with these earlier observations (50, 51, 54), we demonstrate that clone 4 expresses markedly reduced levels of hepatocyte-specific genes, in comparison with AML12 cells. We conclude that clone 4 and 4pX-1 cell lines are differentiated, immortalized hepatocyte cell lines. The lack of oncogenic characteristics in this lineage following pX expression (Fig. 3) suggests the importance of employing differentiated hepatocytes for analysis of pX oncogenicity and corroborates earlier findings that the constitutive expression of pX does not promote focus formation in fibroblasts (15).

Our observations are in agreement with earlier studies (63), describing cell culture transformation of rodent hepatocytes immortalized by the SV40 T-antigen via stable transfection of the HBV genome. Similarly, the immortalized AML12 cell line, derived from TGF-α transgenic mice (43), expresses constitutively TGF-α, a growth factor known to have proliferative effects in hepatocytes (64, 65). Thus, in our AML12 model system, pX may synergize with TGF-α to bring about the oncogenic characteristics we describe.

In contrast to the earlier reports describing the oncogenic potential of pX via stable constitutive pX expression (63, 66), the unique feature of our cellular model system is that it offers the potential of defining the early, direct signaling events activated by pX and to link these events to pX-mediated transformation. Our analyses of c-fos and ATF3 mRNA expression, comparing pX-transforming (3pX) and nontransforming (4pX) lineages, demonstrate a differential pattern of pX-dependent immediate early gene expression. Specifically, within 1 h following pX expression, ATF3 mRNA is induced selectively in the 3pX-1 cell line (Fig. 6), suggesting a differential activation of the c-Jun N-terminal kinase pathway between the 3pX and 4pX lineages. This differential ATF3 induction may be attributed to either the differentiation state of the hepatocytes (Fig. 5) or the different levels of pX expressed in the 3pX and 4pX lineages (Fig. 1). Interestingly, the expression of ATF3 is also induced in adenosine EIA-transformed cells (57), suggesting that one aspect of the mechanism of HBV pX- and adenosine EIA-mediated cellular transformation may be the induction of the immediate early gene ATF3.

Likewise, the pX-dependent induction of c-fos differs between the two cell lines, in terms of both the magnitude of the pX-dependent induction and the duration of its expression (Fig. 7). Earlier studies (39) demonstrated that pX-dependent c-fos induction occurs via activation of the Ras-Raf-MAP kinase pathway. It is well established that sustained Ras-Raf-MAP kinase activation is linked to cellular transformation in other cellular model systems (68, 69). Our studies suggest that the combinatorial activation of the Ras-Raf-MAP kinase and c-Jun N-terminal kinase pathway is involved in pX-mediated oncogenic transformation. Studies are under way to assess the onset and duration of these mitogenic pathways in the 3pX and 4pX cell lines. Accordingly, our pX-conditional cellular model system provides an excellent model system to decipher the molecular mechanism of pX-mediated transformation.

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REFERENCES
1. Beasley, R. P., Hwang, L. Y., Lin, C. C., and Chien, C. S. (1981) Lancer II, 1129–1133
2. Brehédi, C., Pourcel, C., Louise, A., Bain, B., and Tiollais, P. (1980) Nature 286, 533–535
3. Chakraborty, P. R., Ruiz-Opazo, N., Shouval, D., and Schafritz, D. A. (1980) Nature 286, 531–533
4. Tiollais, P., Pourcel, C., and Dejean, A. (1985) Science 227, 429–433
5. Siddiqui, A., Jameel, S. and Mapoles, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2513–2517
6. Chen, H. S., Kaneko, S., Girones, R., Anderson, R. W., Hornbuckle, W. E., Tennant, B. C., Cote, J. P., Gerin, J. L., Purrell, R. H., and Miller, R. H. (1993) J. Virol. 67, 1218–1226
7. Koike, K., Moriya, K., Yotsuyanagi, H., Iino, S., and Kurokawa, K. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 489–495
8. Kay, A., Mandert, E., Trepo, C., and Galibert, F. (1985) Nature 312, 489–495
9. Papper, H., Roth, L., Purrell, R. H., Tennant, B. C., and Gerin, J. L. (1987) Proc. Natl. Acad. Sci. U. S. A. 85, 867–870
10. Duftel, A., Mehrrota, R., Yu, S. Z., Barraud, L., Trepo, C., and Covea, L. (1995) Hepatology 21, 1483–1491
11. Koike, K., Moriya, K., Yotsuyanagi, H., Iino, S., and Kurokawa, K. (1994) Science 227, 429–433
Conditional HBV pX Expression in AML12 Hepatocytes

143–150
Buck, M., Turler, H., and Chojkier, M. (1994) EMBO J. 13, 3413–3420

20. Haviv, I., Vaizel, D., and Shaul, Y. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10578–10583

21. Lin, Y., Nomura, T., Cheong, J., Dorjsuren, D., Iida, K., and Murakami, S. (1997) J. Biol. Chem. 272, 7123–7129

22. Haviv, I., Shamay, M., Doteich, G., and Shaul, Y. (1998) Mol. Cell. Biol. 18, 1562–1569

25. Siddiqui, A., Gaynor, R., Srinivasan, A., Mapoles, J., and Farr, R. W. (1989) Virology 169, 479–484

26. Levrero, M., Balsano, C., Natoli, G., Avantaggiati, M. L., and Elfassi, E. (1990) J. Virol. 64, 3082–3086

27. Twu, J.-S., Wu, J.-Y., and Robinson, W. S. (1990) Virology 177, 406–410

28. Lucito, R., and Schneider, R. J. (1992) J. Virol. 66, 983–991

29. Seto, E., Rapp, J. H., and Yamamoto, K. R. (1994) FASEB J. 8, 252–262

30. Twu, J.-S., Wu, J.-Y., and Robinson, W. S. (1990) J. Virol. 64, 746–749

31. Kekule, A. S., Lauer, U., Weiss, L., Luber, B., and Hofsneider, P. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 8078–8082

32. Benn, J., and Schneider, R. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10350–10354

33. Natoli, G., Avantaggiati, M. L., Chirillo, P., Costanzo, A., Artini, M., Balsano, C., and Levrero, M. (1994) Mol. Cell. Biol. 14, 2, 989–998

34. Maguire, H. F., Hoeffler, J. P., and Siddiqui, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5547–5551

35. Williams, J. S., and Andrisani, O. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1157–1161

36. Barnabas, S., Hai, T., and Andrisani, O. M. (1997) J. Biol. Chem. 272, 20684–20690

37. Doria, M., Klein, N., Lucito, R., and Schneider, R. J. (1995) EMBO J. 14, 4747–4757

38. Benn, J., Su, F., Doria, M., and Schneider, R. J. (1996) EMBO J. 15, 4978–4955

39. Buck, M., Turler, H., and Chojkier, M. (1994) EMBO J. 13, 851–860

40. Chen, E. P., C., Wolfgang, C. D., and Hai, T. (1996) Mol. Cell. Biol. 16, 1157–1168

41. Servillo, G., Penna, L., Foulkes, N. S., Magni, M. V., Fazia, M. A. D., Sassone-Corsi, P. (1997) Oncogene 14, 1601–1606

42. Wu, J. C., Merlino, G., and Fausto, N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 674–678

43. Gossen, M., and Bujard, H. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 5547–5551

44. Andrisani, O. M., Pot, D. A., Zhu, Z., and Dixon, J. E. (1989) Mol. Cell. Biol. 8, 1947–1956

45. Shin, S., Freedman, V. H., Risser, R., and Pollack, R. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4435–4439

46. Cox, A. D., and Der, C. J. (1994) Methods Enzymol. 238, 277–294

47. Fiol, C. J., Williams, J. S., Chou, C.-H, Wang, Q. M., Roach, P. J., and Andrisani, O. M. (1995) J. Biol. Chem. 269, 32187–32193

48. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489

49. Cerchigni, S., Blumenfeld, M., and Yaniv, M. (1988) Genes Dev. 2, 957–974

50. Mendel, K. B., Hansen, L. P., Graves, M. K., Conley, P. B., and Crabtree, G. R. (1991) Genes Dev. 5, 1042–1056

51. De Schutter, M., and Weiss, M. C. (1974) Biochimie (Paris) 56, 1603–1611

52. Fiol, C. J., Williams, J. S., Chou, C.-H, Wang, Q. M., Roach, P. J., and Andrisani, O. M. (1995) J. Biol. Chem. 269, 32187–32193

54. Crabtree, G., Schibler, U., and Scott, M. P. (1992) Transcriptional Regulation (McKnight, S. L., and Yamamoto, K. R., eds) Vol. 2, pp. 1063–1102, CSHL Press, Plainview, NY

55. Liang, G., Wolfgang, C. D., Chen, B. P. C., Chen, T.-H., and Hai, T. (1995) J. Biol. Chem. 270, 1659–1701

56. Kyriakis, J. M., Banerjee, P., Nikolakakis, E., Dai, T., Rubie, E. A., Ahmad, M. F., Avruch, J., and Woodgett, J. R. (1994) Nature 369, 156–160

57. Hagemeyer, B. M., Duyndam, M. C., Angel, P., de Groot, R. P., Verlaan, M., Elfferich, P., van der Ee, H., and Zanzena, A. (1996) Oncogene 12, 1025–1032

58. Fry, D. W., Krakor, A. J., McMichael, A., Ambrosio, L. A., Nelson, J. M., Leopold, W. R., Connors, R. W., and Bridges, A. J. (1994) Science 265, 1093–1095

59. Aliess, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) J. Biol. Chem. 270, 27489–27494

60. Chirillo, P., Pagano, S., Natoli, G., Puri, P. L., Burgio, V. L., Balsano, C., and Levrero, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8162–8167

61. Birchmeier, W., Weidner, K. M., and Behrens, J. (1993) J. Cell. Biol. 157, 159–164

62. Kinch, M. S., and Burridge, K. (1995) Biochem. Soc. Trans. 23, 446–450

63. Hohne, M., Schaefer, S., Seifer, M., Petielsen, M. A., Pulin, D., and Gerlich, W. H. (1990) EMBO J. 9, 1137–1145

64. Michalopoulos, G. K. (1990) FASEB J. 4, 176–187

65. Mead, J. E., and Fausto, N. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 1538–1542

66. Ogaya, D., Dumenco, L. L., Pierce, R. H., and Fausto, N. (1996) Hepatology 24, 1024–1033

67. Bartosch, D., Ghirardi, M., Skehel, P. A., Karl, K. A., Herder, S. P., Chen, M., Bailey, C. H., and Kandel, E. R. (1995) Science 269, 1562–1569

68. Russell, M. E., Utans, U., Wallace, A. F., Liang, P., Arceci, R. J., Karnovsky, M. J., Wyner, L. R., Yamashita, Y., and Tarn, C. (1994) J. Clin. Invest. 93, 722–729
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