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The Mitogenic Function of Hepatitis B Virus X Protein Resides within Amino Acids 51 to 140 and Is Modulated by N- and C-Terminal Regulatory Regions[▽]

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The hepatitis B virus (HBV) X protein (pX) is implicated in hepatocarcinogenesis by an unknown mechanism. pX variants encoded by HBV genomes found integrated in genomic DNA from liver tumors of patients with hepatocellular carcinoma (HCC) generally lack amino acids 134 to 154. Since deregulation of mitogenic pathways is linked to oncogenic transformation, herein we define the pX region required for mitogenic pathway activation. A series of pX deletions was used to construct tetracycline-regulated pX-expressing cell lines. The activation of the mitogenic pathways by these pX deletions expressed in the constructed cell lines was measured by transient transreporter assays, effects on endogenous cyclin A expression, and apoptosis. Conditional expression of pX_{51-140} in AML12 clone 4 cell line activates the mitogenic pathways, induces endogenous cyclin A expression, and sensitizes cells to apoptosis, similar to wild-type (WT) pX. By contrast, pX_{1-115} is inactive, supporting the idea that amino acids 116 to 140 are required for mitogenic pathway activation. Moreover, this pX deletion analysis demonstrates that WT pX function is modulated by two regions spanning amino acids 1 to 78 and 141 to 154. The N-terminal X_{1-78}, expressed via a retroviral vector in WT pX-expressing 4pX-1 cells, coimmunoprecipitates with WT pX, indicating this pX region participates in protein-protein interactions leading to pX oligomerization. Interestingly, pX_{1-78} interferes with WT pX in mediating mitogenic pathway activation, endogenous gene expression, and apoptosis. The C-terminal pX region spanning amino acids 141 to 154 decreases pX stability, determined by pulse-chase studies of WT pX and pX_{1-140}, suggesting that increased stability of naturally occurring pX variants lacking amino acids 134 to 154 may play a role in HCC development.

Epidemiologic evidence (6) links chronic hepatitis B virus (HBV) infection in humans to development of hepatocellular carcinoma (HCC). Comparative studies of mammalian and avian hepadnaviruses, transgenic animal studies, and cell culture transformation studies (reviewed in references 2 and 16) implicate the 16.5-kDa X protein (pX) of HBV in HCC development. Although the mechanism by which pX mediates hepatocellular carcinogenesis is not yet understood, it is known that pX is a multifunctional regulatory protein required for viral infection (17, 48). Well-established functions of pX include activation of cellular Src by calcium signaling (12–15, 25), resulting in pX-dependent activation of the mitogenic Ras–Raf–mitogen-activated protein kinase (MAPK), Jun N-terminal protein kinase (JNK), and p38 MAPK pathways (7–9, 41, 42).

Another well-established function of pX is its effect on transcription (2, 14). pX increases transcription of select viral (20) and diverse cellular genes mediated by *cis*-acting elements, including AP1 (9, 22, 32), AP2 (38), *cis*-acting replication element (CRE) (4, 5, 45), and NF-κB (30, 39) sites. Since pX is not a sequence-specific DNA binding protein, the broad effects of pX on transcription are linked to its ability to activate the mitogenic pathways. Specifically, the consequence of the pX-dependent activation of the mitogenic pathways (7, 9, 25, 41, 42) is the activation of their downstream effectors, leading to enhanced pX-dependent transcription. pX-dependent activation of the mitogenic pathways has been linked to increased cell cycle progression (12, 28), apoptosis (44), and cellular transformation of differentiated hepatocytes (40) by deregulation of cellular gene expression. Genes transcriptionally induced by pX and linked to deregulated cell growth include the cyclins (28), tumor necrosis factor alpha/tumor necrosis factor receptor I, Fas/FasL, and p53-responsive genes (44; W.-H. Wang and O. M. Andrisani, unpublished results).

In addition to mitogenic pathway activation, pX activates transcription directly via protein-protein interactions with specific components of the basal transcriptional apparatus (18, 24, 29, 34) and via direct interactions with the bZIP family of CREB/ATF proteins (4, 5, 45). Interestingly, the CREB/ATF family of transcription factors is the downstream effector of the mitogenic pathways, activated by phosphorylation in response to pX action (1). In addition, CREB/ATF proteins are direct interaction targets of pX (4, 5, 45). Thus, the mitogenic and transcription functions of pX integrate at the level of CREB/ATF protein function. The pX-dependent activation of mitogenic pathways and the direct transcriptional effects of pX likely contribute to pX-mediated hepatocyte transformation.

Various studies have mapped the regions of pX required for its transactivation function. Amino acids (aa) 50 to 140 of pX are required for X-protein-dependent transactivation from the simian virus 40 early promoter (3, 35), the Rous sarcoma virus

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long terminal repeat (27), CRE-driven CREB/ATF transactivation (4), and interaction with the RPB5 subunit of RNA polymerase II (18). X_{50-140} also contains the interaction region for CREB/ATF proteins located between amino acids 50 to 115 (4), transcription factor IIB (TFIIB) (amino acids 102 to 136) (29), and TFIIH (amino acids 110 to 143) (34). In contrast to the activating region of pX (aa 51 to 140), the pX region spanning amino acids 1 to 50 was shown to have a repressive effect on X-protein-mediated transactivation and was reported to be involved in protein-protein interactions leading to pX oligomerization (31).

Regarding the pX region(s) required for mitogenic pathway activation, it was reported that amino acids 58 to 119 of pX are sufficient to activate the MAPK pathways, assayed in mouse liver in vivo (33). Since pX is implicated in HBV-mediated HCC (2, 16) and deregulation of mitogenic pathways is causally linked to oncogenic transformation, we were intrigued by the naturally occurring pX deletions found in liver tumors of patients with HCC (36, 43, 46). These pX variants encoded by integrated HBV genomes contain C-terminal deletions generally lacking aa 134 to 154 (36, 43, 46), suggesting that these deletions might alter the potential of pX in activating the mitogenic pathways. Accordingly, we undertook a systematic deletion analysis of pX to define the pX region required for mitogenic pathway activation. We employ the same pX deletion mutants previously analyzed for mediating pX-dependent CREB/ATF transactivation (4) and constructed tetracycline (Tet)-regulated pX-expressing cell lines in the less-differentiated AML12 clone 4 (40). Our earlier studies have demonstrated that tetracycline-regulated expression of wild-type (WT) pX in AML12 clone 4, i.e., the 4pX-1 cell line, induces sustained activation of the JNK and p38 MAPK pathways and transient activation of the Ras-Raf-MAPK pathway (42). Importantly, the consequence of the pX-dependent sustained activation of the cellular stress pathways is the sensitization of 4pX-1 cells to apoptosis (44). Herein, we demonstrate that pX_{51-140}, conditionally expressed in AML12 clone 4 cell line, is essential for activation of all the mitogenic pathways, and importantly, pX_{51-140} sensitizes cells to apoptosis, similar to WT pX. By contrast, pX_{1-115} is inactive in both mitogenic pathway activation and apoptosis. Moreover, we demonstrate that the function of WT pX is modulated by two regions spanning amino acids 1 to 78 and 141 to 154. The C-terminal region determines X-protein stability. The N-terminal region is the site of direct interaction of pX molecules. Interestingly, expression of pX_{1-78} exerts a dominant-negative effect on WT pX function in mediating mitogenic pathway activation, endogenous gene expression, and apoptosis in 4pX-1 cells.

MATERIALS AND METHODS

Plasmid construction. WT X_{1-154} and X-protein deletion mutants X_{51-154}, X_{79-154}, X_{51-140}, X_{51-115}, X_{51-90}, X_{1-140}, and X_{1-115} were amplified by PCR using pT_7-X as a template (45) and cloned into the PvuII and NheI sites of pBI-EGFP vector (BD Biosciences Clontech). pBI-EGFP vector contains a bidirectional tetracycline-responsive promoter. X (adw) cloning primers include a Kozak sequence (26) and a six-histidine tag sequence inserted in frame at the 3′end of X. X_{1-78} was PCR amplified from pT_{7-X} plasmid in frame with the Myc epitope at the 3′ end and cloned in the BglII and EcoRI sites of the retroviral plasmid vector pMSCV (BD Biosciences Clontech), containing the puromycin resistance gene. All plasmid constructs were confirmed by sequencing.

Cell culture. 4pX cell lines derived from mouse AML12 cell line (47) were propagated in Dulbecco's modified Eagle medium/F12 (Life Technologies) containing 10% fetal calf serum (HyClone); a mixture of insulin, transferrin, and selenium (Life Technologies); 1.0 μM dexamethasone; and 50 μg/ml gentamicin with or without 5 μg/ml tetracycline as described previously (40). The PT67 retrovirus packaging cell line (BD Biosciences Clontech) was propagated in α modification medium supplemented with 100 units/ml penicillin G sodium, 100 μg/ml streptomycin, 4 mM L-glutamine, 100 mM sodium pyruvate, 2.4 mg/ml sodium bicarbonate, and 10% fetal bovine serum.

Establishment of stable 4pX cell lines expressing X-protein deletions. Subconfluent cultures of AML12 clone 4 (40) expressing the tetracycline-controlled transactivator tTA (23) were cotransfected with 10 μg of pBI-EGFP-X deletion plasmids containing inserts X_{51-154}, X_{79-154}, X_{51-140}, X_{51-115}, X_{51-90}, X_{1-140}, and X_{1-115}, and 2 μg of pCGN plasmid containing the hygromycin resistance gene by the calcium phosphate coprecipitation method. Stable transformants were selected with 200 units/ml hygromycin in the presence of 5 μg/ml tetracycline. Well-separated colonies were each transferred to a plate and propagated in medium containing 200 units/ml hygromycin. Positive clones were screened by real-time PCR monitoring tetracycline-regulated expression of X, performed as described previously (28).

Transient transfections. Reporter plasmid pFR-luc, CMV-β-gal, and transactivator plasmids pFA-Elk-1/pFA-c-Jun/pFA-chop were cotransfected into 4pX cells using the FuGENE 6 method. WT or X-protein deletion mutants were expressed 24 h after transfection by tetracycline removal. Luciferase activity was determined 24 h following pX expression using the luciferase assay system (Promega), normalized per microgram of protein extract or β-galactosidase activity.

Pulse-chase studies. Whole-cell extracts were isolated in radioimmunoprecipitation assay buffer from 4pX-1 and $4pX_{1-140}$ cultures expressing pX and pX_{1-140}, respectively, for 24 h, labeled with [^{35}S]methionine as described previously (40), and chased for 0 to 4 h as indicated. Immunoprecipitations with X antibody, kindly provided by B. Slagle, were performed as described previously (40) and analyzed by 14% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography.

Establishment of retrovirus-producing cell lines. Plasmids pMSCV, pMSCV-EGFP, and pMSCV-X_{1-78} were transfected into the PT67 cell line. Stable transformants were selected with 2.5 μg/ml puromycin. Single colonies were each transferred to a plate and propagated in medium containing 2.5 μg/ml puromycin. Positive clones were screened by Western blot analyses and immunostaining with Myc antibody recognizing the Myc epitope fused to pX_{1-78}.

Retrovirus production and viral titer determination. Retrovirus-producing PT67 stable cell lines, PT67-Vector, PT67-EGFP, and PT67-X_{1-78}, were grown without puromycin to 60 to 80% confluence. Viral supernatants were harvested in 12-h intervals until the cells were no longer viable. Supernatants were filtered through 0.45-μm cellulose acetate filter and stored at −80°C. Viral titer was determined using NIH 3T3 cells as described by the manufacturer (BD Biosciences Clontech).

Retrovirus infection. A total of 5×10^4 to 10×10^4 AML12 clone 4 or 4pX cells were infected with 1 ml (2×10^5 CFU/ml) of viral supernatant for 12 to 24 h in the presence of 8 μg/ml Polybrene. Confluent cells were passaged to 6-cm dishes, followed by expression of pX for 12 to 24 h as indicated.

In vitro JNK assays. 4pX cells were serum starved as described previously (40) for 12 h; pX synthesis was initiated by tetracycline removal for 1 to 6 h in medium containing 10% fetal calf serum. Cells were harvested in chilled hypotonic lysis buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 0.1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 100 μM Na_3VO_4, 20 μg/ml leupeptin, 50 mM NaF, 1 mM benzamidine) and sonicated on ice for 10 seconds. Lysates were clarified by centrifugation, and protein concentration was determined by the Bio-Rad protein assay. One milligram of cellular extract was incubated with 1 μg of glutathione *S*-transferase (GST)–c-Jun (1-79) beads (Stratagene) for 1 h at 4°C. The beads were collected by centrifugation for 2 min at $500 \times g$, washed three times with cold phosphate-buffered saline containing Tween 20, and kinase reaction was performed by adding to the beads 25 μl of kinase buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 50 μM ATP), 1 μl [γ-^{32}P]ATP (6,000 Ci/mmol; NEN); incubation was for 20 min at 30°C. Analysis was carried out by 12% SDS-polyacrylamide gel electrophoresis, and autoradiography. JNK activity was quantified using the SCION IMAGE software.

RESULTS

To define pX regions required for mitogenic pathway activation, we constructed tetracycline-regulated cell lines express-

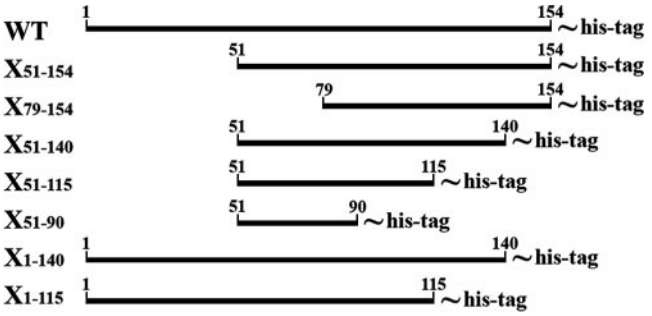


FIG. 1. Diagram of pX deletions cloned in the tetracycline-regulated expression vector pBI-EGFP used for the construction of tetracycline-regulated cell lines.

ing various pX deletions in the AML12 clone 4 cell line that expresses the tetracycline-controlled transactivator tTA (40). Importantly, the AML12 clone 4 cell line supports low-level, tetracycline-regulated pX expression (40, 42, 44), similar to pX expression levels observed during viral infection (21), rendering this cellular model physiologically relevant. Furthermore, our earlier studies have linked pX-dependent mitogenic pathway activation to specific cell growth outcomes, i.e., cell cycle progression (28) and apoptosis (44).

Our rationale for studying these pX deletions (Fig. 1) is based on the results of our earlier studies (4), demonstrating that $pX_{51\text{-}140}$ is required for CRE/CREB-mediated transactivation, whereas $pX_{51\text{-}115}$ is transcriptionally inactive but sufficient for enhanced CREB/ATF CRE binding and CRE-mediated transrepression. Positive clonal cell lines displaying tetracycline-regulated expression of the X transcript were selected by real-time PCR, employing RNA isolated from putative clones grown in the presence and absence of tetracycline for 24 h. The real-time PCR assay is based on the fluorescence emitted by the SYBR green dye upon intercalation to the synthesized double-stranded cDNA. The fluorescence intensity corresponding to the cycle threshold value is the C_T value (28). C_T values obtained for each clonal cell line expressing the indicated X deletions are shown in Table 1. Although the C_T values are not identical for all the generated clonal cell lines, the expression of X is within a similar range and, importantly, displays tetracycline-regulated expression.

Transient transreporter assays in 4pX clonal cell lines. The transreporter assay is comprised of two plasmids transiently cotransfected in a cell line of interest to assess activation of a specific signaling pathway. These include the pFR-luc reporter containing a synthetic promoter with five tandem repeats of the yeast GAL4 binding site regulating luciferase gene expression and the pFA-Elk-1, pFA-c-Jun, and pFA-Chop transactivators, expressing the activation domain of Elk1, c-Jun, or CHOP-10, respectively, in fusion with the yeast GAL4 DNA binding domain. Activation of the endogenous Ras-Raf-MAPK, JNK, and p38 MAPK signaling pathways results in the phosphorylation and activation of the transcriptional activation domain of ELK-1, c-Jun, and CHOP-10, respectively, resulting in luciferase expression.

To assess the activation of the Ras-Raf-MAPK pathway, the pFR-luc reporter and pFA-Elk-1 transactivator were transiently cotransfected in 4pX-1 cells expressing WT pX and in each of the 4pX clonal cell lines expressing the various pX deletions (Fig. 2A). The pX-dependent induction in luciferase expression in each cell line is the ratio of luciferase expression in the presence of pX (in the absence of Tet), WT or pX deletion mutants, versus in the absence of pX expression (in the presence of Tet). In comparison to WT pX, deletion of the 50 or 78 N-terminal amino acids of pX increased luciferase induction (in the absence of Tet/in the presence of Tet) in cell line $4pX_{51\text{-}154}$ or $4pX_{79\text{-}154}$, respectively. Likewise, deletion of the C-terminal 14 amino acids, as in pX deletions expressed in $4pX_{51\text{-}140}$ and $4pX_{1\text{-}140}$ cell lines, results in increased pX-dependent luciferase induction (in the absence of Tet/in the presence of Tet). Importantly, the double deletion mutant $pX_{51\text{-}140}$ exhibits at least 50% increased luciferase induction relative to WT pX. By contrast, a larger deletion of the C-terminal region spanning amino acids 116 to 154, as in $pX_{1\text{-}115}$, results in substantial reduction in mitogenic pathway activation, similar to $pX_{51\text{-}90}$. Intriguingly, in comparison to the inactive $pX_{1\text{-}115}$, expression of $pX_{51\text{-}115}$ activates the transient transreporter to WT pX levels. Qualitatively, similar results were obtained with transreporter assays monitoring activation of the p38 MAPK (Fig. 2B) and JNK pathways (Fig. 2C).

To confirm that the induction (in the absence of Tet/in the presence of Tet) of luciferase activity observed in the 4pX cell lines (Fig. 2A to C) is mediated by the X-mediated activation of the mitogenic Ras-Raf-MAPK, JNK, and p38 MAPK pathways, the respective transreporter assays were performed in the presence of specific inhibitors for these pathways, namely, the MEK-1 inhibitor PD 98059 and the JNK- and p38 MAPK-specific inhibitors SP 600125 and SB 202190, respectively (Fig. 2D). Since $pX_{51\text{-}140}$ displays robust activation of all the mitogenic pathways (Fig. 2A to C), we examined the effects of these inhibitors employing the $4pX_{51\text{-}140}$ cell line. Increased concentration of these inhibitors resulted in nearly 70% inhibition in $pX_{51\text{-}140}$-mediated luciferase induction (Fig. 2D). These results demonstrate that the region of pX spanning amino acids 51-140 mediates the activation of the mitogenic pathways.

Induction of endogenous cyclin A expression in 4pX clonal cell lines. Our earlier studies (28) have demonstrated that in the 4pX-1 cell line, WT pX induces expression of the endogenous cyclin A gene, reaching maximal (2.5-fold) induction at 12 h after pX expression. The consequence of this transcriptional induction of cyclin A, along with the pX-dependent induction of the other cyclins, is progression of 4pX-1 cells in

TABLE 1. Screening of pX-expressing 4pX cell lines

Cell line	C_I value[a]	
	With Tet	Without Tet
$4pX_{1\text{-}154}$	26.4	21.2
$4pX_{51\text{-}154}$	26.0	20.2
$4pX_{79\text{-}154}$	25.5	21.2
$4pX_{51\text{-}140}$	23.3	19.8
$4pX_{51\text{-}115}$	26.4	21.7
$4pX_{51\text{-}90}$	20.7	16.4
$4pX_{1\text{-}140}$	24.3	21.9
$4pX_{1\text{-}115}$	25.0	23.4

[a] C_T values of real-time PCR analyses monitoring the expression of pX deletion mutants in the clone 4 cell lines, grown for 24 hours, in the presence or absence of 5 μg/ml tetracycline.

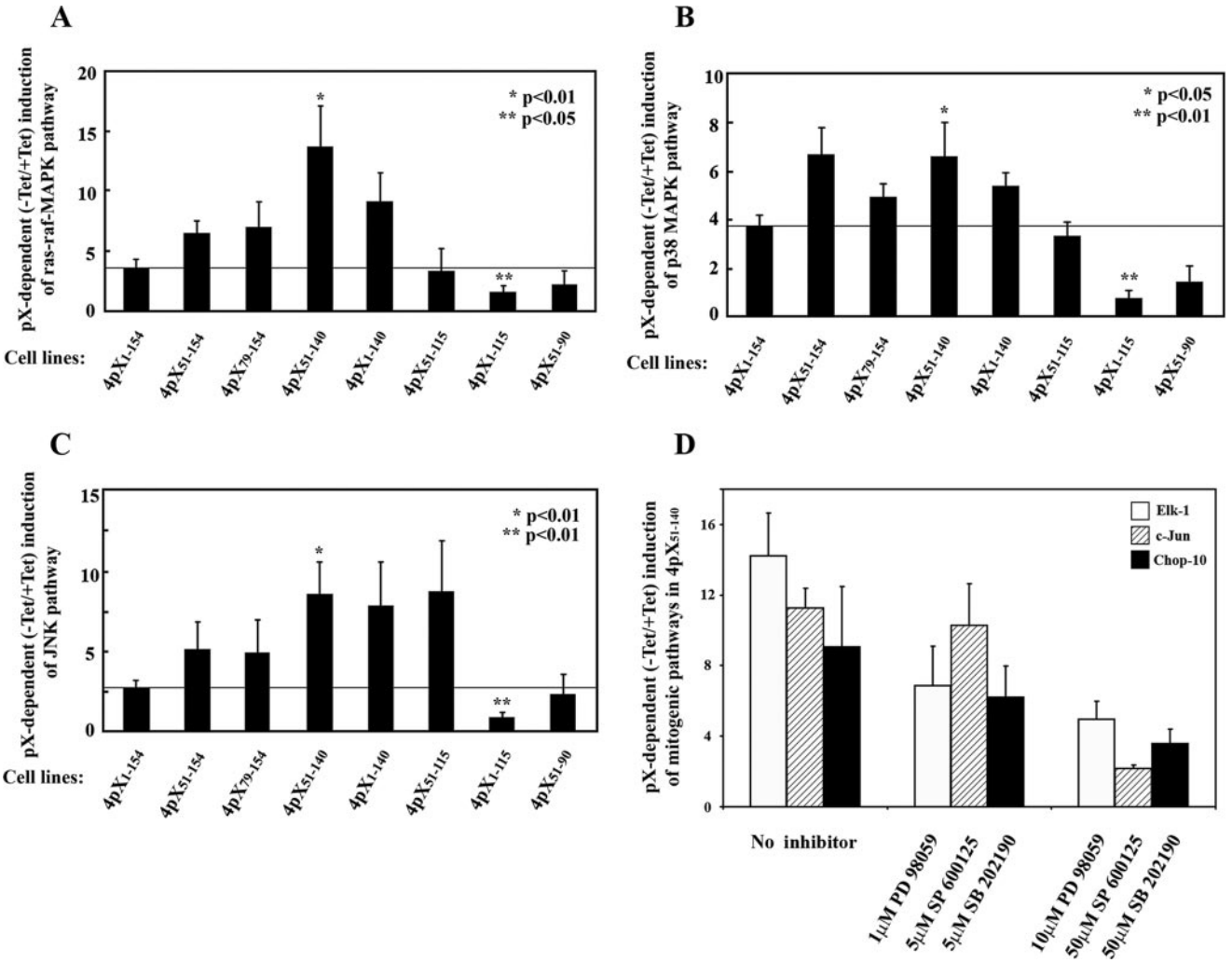


FIG. 2. Transient transreporter assays performed in the tetracycline-regulated 4pX cell lines expressing WT pX (X_{1-154}) and X deletion mutants X_{51-154}, X_{79-154}, X_{51-140}, X_{1-140}, X_{51-115}, X_{1-115}, and X_{51-90}. Reporter plasmid pFR-luc (100 ng), CMV-β-gal (100 ng) and transactivator plasmids pFA-Elk-1 (100 ng) (A), pFA-Chop-10 (25 ng) (B), and pFA-c-Jun (50 ng) (C) were cotransfected into 4pX cells using the FuGENE 6 method. (D) Transient transreporter assays in $4pX_{51-140}$ cell line employing pFR-luc (100 ng) reporter plasmid and transactivator plasmids pFA-Elk-1 (100 ng), pFA-c-Jun (50 ng), and pFA-Chop-10 (25 ng), indicated as Elk-1, c-Jun, and Chop-10, respectively, as a function of the indicated concentration of PD 98059, SP 600125, and SB 2021190. WT or X deletion mutants were expressed 24 h after transfection by tetracycline removal. Luciferase activity was determined 24 h following pX expression using the luciferase assay system (Promega), normalized per microgram of protein extract or β-galactosidase activity. Results are from at least three independent assays, each performed in identical triplicate experiments. Values for the pX deletion that were statistically significantly different from the value for pX_{1-154} are indicated by an asterisk(s). pX-dependent induction of the Ras-Raf-MAPK pathway in the absence of Tet/in the presence of Tet (−Tet/+Tet) is shown.

the cell cycle (28). Moreover, the pX-dependent cyclin A transcription in 4pX-1 cells is dependent on the sustained, pX-dependent activation of the JNK pathway (42). On the basis of these observations, we employed the 4pX cell lines expressing the indicated pX deletions to assess their potential in inducing endogenous cyclin A gene transcription.

Employing real-time PCR, we quantified the pX-dependent cyclin A mRNA levels (in the absence of Tet/in the presence of Tet) in total RNA isolated from 4pX cell lines at 12 h after expression of the indicated pX deletions (Fig. 3A). In comparison to WT pX, only pX_{1-140} and pX_{51-140} demonstrate endogenous cyclin A induction. Interestingly, deletions pX_{51-154}, pX_{79-154} and pX_{51-115}, despite demonstrating activation of all the mitogenic pathways (Fig. 2), lack the ability to induce transcription of endogenous cyclin A gene. On the other hand, deletions pX_{1-115} and pX_{51-90} activate neither the mitogenic pathways (Fig. 2) nor induce transcription of the endogenous cyclin A gene (Fig. 3A).

Since sustained, pX-mediated activation of the JNK pathway is required for cyclin A transcription in the 4pX-1 cell line (42), we investigated the duration of pX-dependent activation of the JNK enzyme in each of the 4pX cell lines expressing the indicated deletions. In vitro JNK enzyme assays were performed, employing cellular extracts isolated in a time course experiment, 1, 2, and 6 h after expression of pX deletions, with GST–c-Jun as the substrate (Fig. 3B).

Deletions pX_{1-140} and pX_{51-140}, which induce expression of the endogenous cyclin A gene (Fig. 3A), exhibit sustained JNK

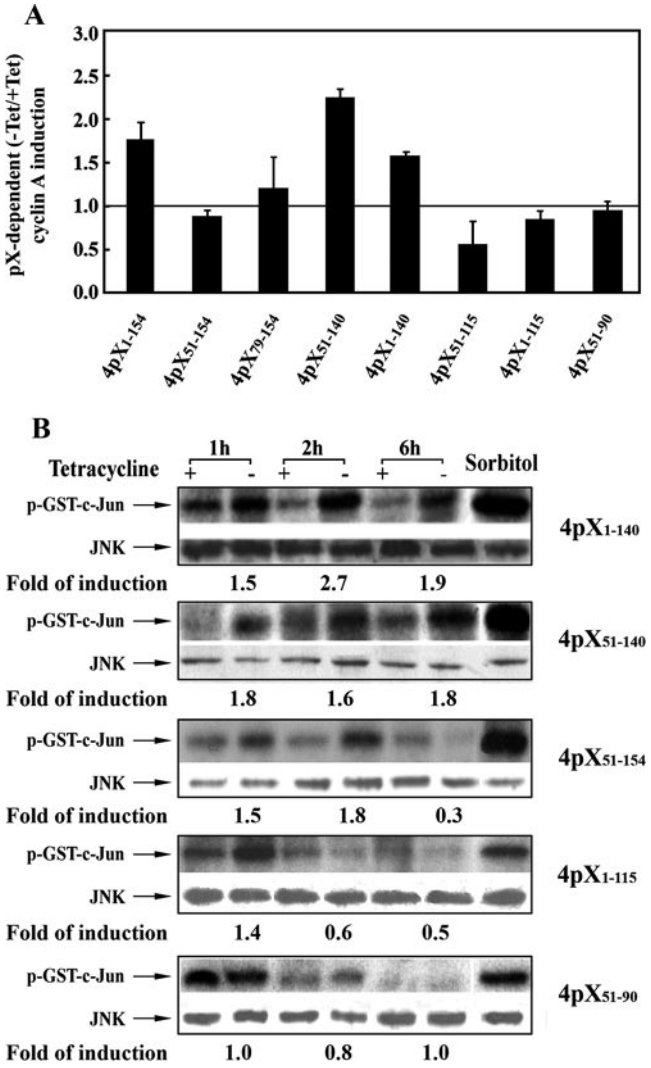


FIG. 3. (A) pX-dependent transcriptional induction (in the absence of Tet/in the presence of Tet [−Tet/+Tet]) of endogenous cyclin A in the indicated 4pX-expressing cell lines. Total RNA was isolated 12 h following expression of WT pX (X_{1-154}) and X deletion mutants X_{51-154}, X_{79-154}, X_{51-140}, X_{1-140}, X_{51-115}, X_{1-115}, and X_{51-90} by tetracycline removal from serum-starved (18-h) 4pX cell lines. Quantification of cyclin A mRNA was by real-time PCR performed in identical triplicate experiments, as described previously (28), using endogenous glyceraldehyde-3-phosphate dehydrogenase as an internal control. Data are from three independent RNA preparations. *P* values of the −Tet/+Tet cyclin A induction are as follows: $P < 0.05$ for pX_{1-154}, $P < 0.01$ for X_{51-140}, and $P < 0.01$ for X_{1-140}. (B) In vitro JNK kinase assays employing cellular extracts isolated from the indicated 4pX cell lines in a time course experiment 1, 2, and 6 h after tetracycline removal using GST–c-Jun as the substrate. Sorbitol treatment (15 min, 500 mM) is used as a positive control. Total JNK enzyme in each sample was determined by Western blot assay using the JNK antibody. A representative assay from the independent experiments is shown. Quantification was performed by the SCION IMAGE software.

enzyme activation, lasting at least 6 h (Fig. 3B). By contrast, the duration of JNK activation by pX_{51-154} and pX_{1-115} is not sustained, lasting 2 h or less; furthermore, pX_{51-90} is incapable of activating the JNK enzyme (Fig. 3B). Importantly, pX_{51-154} and pX_{1-115}, which mediate only transient JNK activation (Fig. 3B), lack the ability to induce endogenous cyclin A transcription (Fig. 3A), in agreement with our earlier observations (42) and those of others (11). Therefore, only deletions pX_{1-140} and pX_{51-140}, which mediate sustained JNK pathway activation (Fig. 3B), induce endogenous cyclin A transcription (Fig. 3A).

Effects of pX deletions in mediating apoptosis. Expression of WT pX sensitizes cells to apoptosis (19, 39, 44). In the 4pX-1 cell line, pX sensitizes cells to apoptosis by activating in a sustained manner the p38 MAPK and JNK pathways (44). Accordingly, we examined the potential of each of the pX deletions to mediate apoptosis in the respective 4pX cell line, employing the same conditions as those used for pX-dependent apoptosis in the 4pX-1 cell line (44). Specifically, 4pX cultures were grown to confluence, followed by expression of pX deletions for 24 h; apoptosis was initiated by growth factor withdrawal, by incubating 4pX cultures in 2% fetal calf serum. 4pX-1 cells grown under these apoptotic conditions undergo apoptosis at 24 h via the combined pX-dependent activation of the p38 MAPK and JNK pathways (44). Accordingly, pX-dependent apoptosis in 4pX cell lines expressing the indicated deletions was monitored at 24 h, employing immunofluorescence microscopy detecting active, cleaved caspase 3 (Fig. 4A).

Quantification of these apoptosis assays (Fig. 4B) demonstrates that only deletions pX_{51-140} and pX_{1-140}, which exhibit sustained JNK activation (Fig. 3B) and induction of endogenous transcription (Fig. 3A), sensitize cells to apoptosis as the WT pX does. Importantly, inhibition of the p38 MAPK and JNK pathways by their specific inhibitors rescues pX_{51-140}-mediated apoptosis (Fig. 4B), directly demonstrating that this apoptosis is a consequence of the activation of the stress pathways by pX_{51-140}, in agreement with our earlier studies (44). By contrast, the level of apoptosis observed with deletions pX_{51-154} and pX_{1-115}, which only transiently activate JNK (Fig. 3B), is similar to that obtained following inhibition of the p38 MAPK or JNK pathway (Fig. 4B).

Thus, our analyses of the activity of the pX deletion mutants (Fig. 1), employing transient transreporter assays (Fig. 2), endogenous cyclin A gene expression (Fig. 3A), and apoptosis (Fig. 4), identify pX_{51-140} as essential in activating the cellular mitogenic pathways as well as in mediating the known mitogenic effects on cellular gene expression and the ensuing consequences on cell growth. Likewise, our analyses demonstrate that pX_{1-115} only transiently activates the mitogenic pathways (Fig. 3C), exerting no effect on endogenous gene expression (Fig. 3A) or apoptosis (Fig. 4).

WT pX contains C- and N-terminal modulatory regions. In comparison to WT pX, deletion mutant X_{51-140} consistently demonstrates increased activity linked to mitogenic pathway activation (Fig. 2 to 4), suggesting that both the N-terminal and C-terminal regions of WT pX modulate its potential to activate the mitogenic pathways.

To understand the role of the C-terminal region spanning aa 141 to 154, we compared the stability of WT pX and pX_{1-140}, employing pulse-chase studies by in vivo labeling with [^{35}S]methionine (Fig. 5). WT pX is not detectable at 30 min following labeling with [^{35}S]methionine (Fig. 5), in agreement with similar observations by others (10). In comparison to WT pX, pX_{1-140} is readily detected at 30 min following labeling with [^{35}S]methionine, reaching basal levels by 2 h (Fig. 5). On the basis of these results, we conclude that the C-terminal region

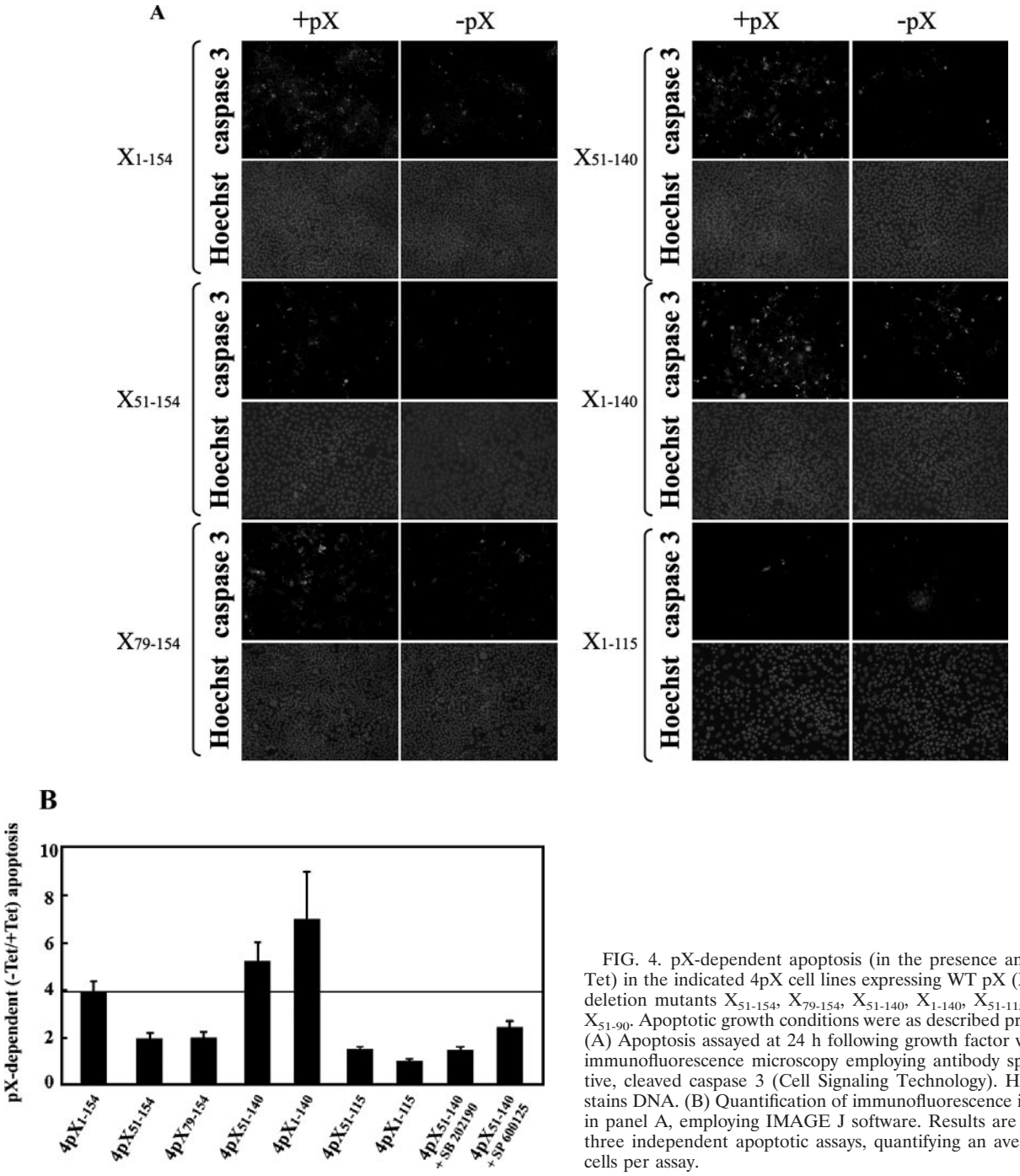


FIG. 4. pX-dependent apoptosis (in the presence and absence of Tet) in the indicated 4pX cell lines expressing WT pX (X_{1-154}) and X deletion mutants X_{51-154}, X_{79-154}, X_{51-140}, X_{1-140}, X_{51-115}, X_{1-115}, and X_{51-90}. Apoptotic growth conditions were as described previously (44). (A) Apoptosis assayed at 24 h following growth factor withdrawal by immunofluorescence microscopy employing antibody specific for active, cleaved caspase 3 (Cell Signaling Technology). Hoechst (blue) stains DNA. (B) Quantification of immunofluorescence images shown in panel A, employing IMAGE J software. Results are from at least three independent apoptotic assays, quantifying an average of 5,000 cells per assay.

of pX (aa 141 to 154) determines the stability of the WT pX. Deletion of this C-terminal region as in pX_{1-140} results in increased protein stability.

Regarding the function of the N-terminal region of pX, in addition to our observations herein, others have demonstrated that deletion of amino acids 1 to 50 increases pX-mediated transactivation (31). Moreover, it was reported that this N-terminal region participates in protein-protein interactions leading to pX oligomerization. To investigate the role of the N-terminal region of pX, we constructed a nonreplicating retrovirus expressing pX_{1-78}, fused to the Myc epitope at its C terminus; the pMSCV-X_{1-78} plasmid was stably integrated in the pT67 retrovirus packaging cell line (Fig. 6A), resulting in retrovirus production. Retroviral titers were quantified at approximately 2.5×10^5 CFU/ml. Infection of 4pX-1 cells with the pX_{1-78}-expressing retrovirus was monitored at 24 to 72 h postinfection by immunofluorescence microscopy using the Myc antibody (Fig. 6B).

Since amino acids 1 to 50 of pX were reported to mediate pX oligomerization (31), we determined the effect of pX_{1-78} on

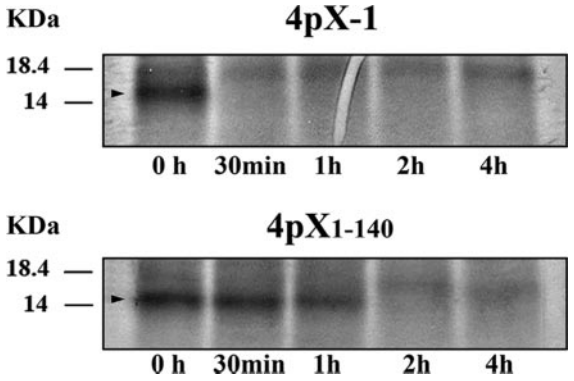


FIG. 5. Pulse-chase study of WT pX and pX_{1-140}. 4pX-1, and $4pX_{1-140}$ cell lines grown without tetracycline for 24 h to express WT pX and pX_{1-140}, respectively, were metabolically labeled with [^{35}S]methionine for 2 h, followed by incubation in [^{35}S]methionine-free medium for various times (0 h, 30 min, and 1, 2, and 4 h). Cellular extracts were immunoprecipitated using a pX-specific antibody and analyzed by 14% SDS-PAGE and autoradiography. A representative assay is shown.

pX-mediated mitogenic pathway activation, employing the 4pX cell lines expressing either WT pX or deletions pX_{1-140} and pX_{51-140}. These pX deletions activate the mitogenic pathways (Fig. 2), induce endogenous cyclin A expression (Fig. 3A), and mediate apoptosis (Fig. 4). Thus, 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines were infected either with the control retrovirus murine stem cell virus (MSCV) or MSCV-X_{1-78}. Transient transreporter assays were performed in retrovirus-infected cells, monitoring activation of the JNK (Fig. 6C) and p38 MAPK (Fig. 6D) pathways as a function of WT pX, pX_{1-140}, or pX_{51-140} expression in the respective cell lines. Infection with the control MSCV retrovirus does not affect pX-dependent activation of either the JNK or p38 MAPK pathway (Fig. 6C and D). Moreover, expression of deletions pX_{1-140} and pX_{51-140} increased pathway activation in comparison to WT pX, as shown above (Fig. 2B and C). Interestingly, infection with MSCV-X_{1-78} retrovirus expressing pX_{1-78} reduces JNK and p38 MAPK pathway activation mediated by WT pX and pX_{1-140} by at least 50% (Fig. 6C and D). By contrast, pX_{1-78} expressed in the $4pX_{51-140}$ cell line does not affect pX_{51-140} in activating the JNK and p38 MAPK pathways (Fig. 6C and D).

To further confirm these observations, we monitored expression of the endogenous cyclin A gene (Fig. 7A). Infection with the control MSCV retrovirus does not affect pX-dependent induction of endogenous cyclin A in the 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines expressing WT pX or the active pX deletions (Fig. 7A). By contrast, infection with MSCV-X_{1-78} retrovirus expressing pX_{1-78} (Fig. 7A) suppresses pX-mediated cyclin A induction to basal levels in 4pX-1 and $4pX_{1-140}$ cell lines, without an effect in the $4pX_{51-140}$ cell line. Similarly, pX_{1-78} expression suppresses apoptosis mediated by WT pX and pX_{1-140} (Fig. 7B). Although the effect of pX_{1-78} on apoptosis mediated by pX_{1-140} is smaller than its effect on WT pX, it is reproducible. By contrast, pX_{1-78} does not rescue apoptosis mediated by pX_{51-140} (Fig. 7B). Infection with MSCV-X_{1-78} further enhances apoptosis in the $4pX_{51-140}$ cell line, suggesting that X_{1-78} acts either as a stress signal acting synergistically with pX_{51-140} or that it interacts with cellular factors involved in apoptosis. We conclude that pX_{1-78} exerts a dominant-negative effect, interfering with pX function only when the N-terminal amino acids 1 to 50 are present.

pX_{1-78} coimmunoprecipitates with WT pX. To determine the mechanism by which pX_{1-78} interferes with WT pX function, we investigated whether pX_{1-78} interacts directly with pX. 4pX-1 cells expressing WT pX were infected with the control MSCV retrovirus or MSCV-X_{1-78}. Cellular extracts were isolated from infected 4pX-1 cells expressing pX and immunoprecipitated either with a Myc antibody recognizing the Myc epitope fused to pX_{1-78} (Fig. 8A, lanes 1 and 2) or a pX antibody (Fig. 8A, lanes 3 and 4). The Myc antibody immunoprecipitates were analyzed by Western blotting using a pX antibody, identifying a 16.5-kDa band only when 4pX-1 cells expressing pX were infected with the MSCV-X_{1-78} retrovirus (Fig. 8A, lane 1). Likewise, the immunoprecipitates with the pX antibody, immunoblotted with the Myc antibody, detect a band comigrating with pX_{1-78} (lane 5) only when 4pX-1 cells expressing pX were infected with the MSCV-X_{1-78} retrovirus (Fig. 8A, lane 3). Importantly, by Western blot analysis, the pX antibody does not detect pX_{1-78} (data not shown). These results conclusively demonstrate that WT pX interacts directly with pX_{1-78}, indicating that this pX region is the site of direct protein-protein interactions of X-protein molecules leading to pX oligomerization.

In Fig. 6B, a time-dependent nuclear translocation of X_{1-78} is observed. Since our results (Fig. 8A) demonstrate that pX_{1-78} interacts with WT pX, to further confirm these observations, we examined by immunofluorescence microscopy the rate of nuclear translocation of X_{1-78} as a function of expression of WT pX, X_{1-140}, or X_{51-140}. The localization of pX_{1-78} was monitored by immunofluorescence microscopy using the Myc antibody in a time course experiment 8 h to 24 h following expression of WT pX, X_{1-140}, or X_{51-140} in the corresponding cell lines infected with MSCV-X_{1-78} retrovirus (Fig. 8B). Expression of WT pX and pX_{1-140} results in the nuclear localization of pX_{1-78} within 12 h of their expression, whereas in the absence of WT pX and pX_{1-140}, nuclear pX_{1-78} is detected by 24 h. Importantly, expression of X_{51-140} does not enhance the rate of nuclear localization of X_{1-78} (Fig. 8B). Taken together (Fig. 6 to 8), the results conclusively demonstrate that pX_{1-78} interacts with pX via the N-terminal amino acids 1 to 50.

DISCUSSION

In this study we demonstrate that the pX region spanning amino acids 50 to 140 is essential for activation of the mitogenic pathways. In contrast to an earlier report (33), we demonstrate that amino acids 116 to 140 of pX are necessary for mitogenic pathway activation and endogenous gene expression and for mediating cell growth outcomes, such as apoptosis. Our analyses have also led to the identification of two regions located at the N terminus and C terminus of the X protein which modulate X-protein function. The C-terminal region spanning aa 141 to 154 determines X-protein stability. The N-terminal region, amino acids 1 to 78 of pX, is the site of direct protein-protein interactions between X molecules.

Construction of tetracycline-regulated cell lines expressing pX deletions. We have earlier demonstrated by systematic deletion analyses of pX that amino acids 51 to 140 are required for pX-dependent CREB/ATF-mediated transcription (4).

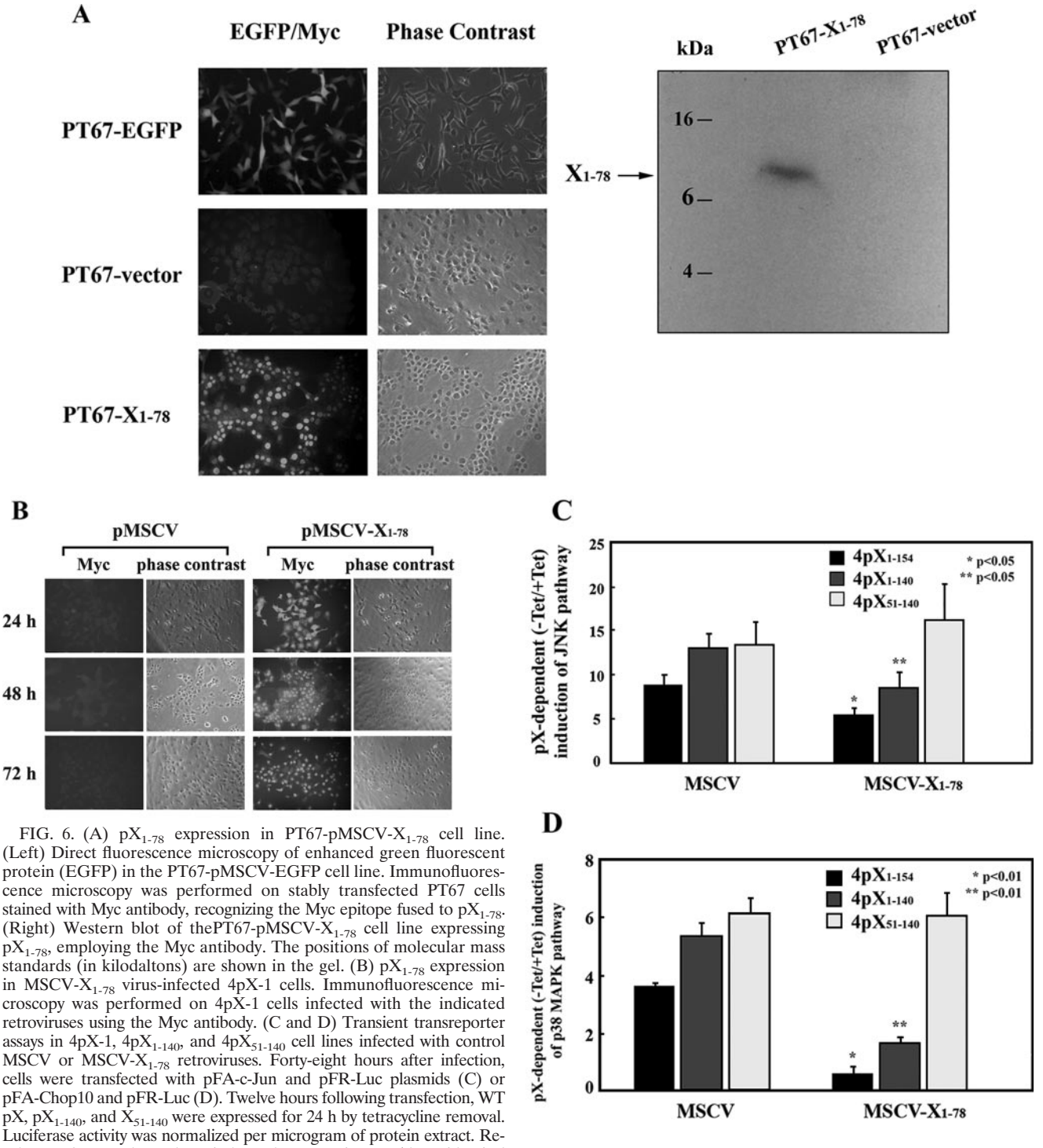


FIG. 6. (A) pX_{1-78} expression in PT67-pMSCV-X_{1-78} cell line. (Left) Direct fluorescence microscopy of enhanced green fluorescent protein (EGFP) in the PT67-pMSCV-EGFP cell line. Immunofluorescence microscopy was performed on stably transfected PT67 cells stained with Myc antibody, recognizing the Myc epitope fused to pX_{1-78}. (Right) Western blot of thePT67-pMSCV-X_{1-78} cell line expressing pX_{1-78}, employing the Myc antibody. The positions of molecular mass standards (in kilodaltons) are shown in the gel. (B) pX_{1-78} expression in MSCV-X_{1-78} virus-infected 4pX-1 cells. Immunofluorescence microscopy was performed on 4pX-1 cells infected with the indicated retroviruses using the Myc antibody. (C and D) Transient transreporter assays in 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines infected with control MSCV or MSCV-X_{1-78} retroviruses. Forty-eight hours after infection, cells were transfected with pFA-c-Jun and pFR-Luc plasmids (C) or pFA-Chop10 and pFR-Luc (D). Twelve hours following transfection, WT pX, pX_{1-140}, and X_{51-140} were expressed for 24 h by tetracycline removal. Luciferase activity was normalized per microgram of protein extract. Results are from at least three independent experiments (three identical experiments). Values for the pX deletion in MSCV-X_{1-78}-infected cells that were statistically significantly different from the value for MSCV-infected cells are indicated by an asterisk(s).

Herein, employing the same pX deletions, we determined the pX region required for activation of the mitogenic pathways. The activity of pX deletions was determined by constructing tetracycline-regulated cell lines expressing these pX deletions in the AML12 clone 4 cell line (40). Expression of WT pX in an AML12 clone 4 4pX-1 cell line mediates sustained activation of the JNK and p38 MAPK pathways and transient activation of the Ras-Raf-MAPK pathway (42). Furthermore, the sustained, pX-dependent activation of the JNK and p38 MAPK pathways in 4pX-1 cells mediates pX-dependent induction of endogenous cyclin A transcription (28, 42) and pX-dependent sensitization to apoptosis (44). Accordingly, on the basis of our earlier studies of the 4pX-1 cell line (28, 40, 42, 44), the AML12 clone 4 cell lines expressing the indicated

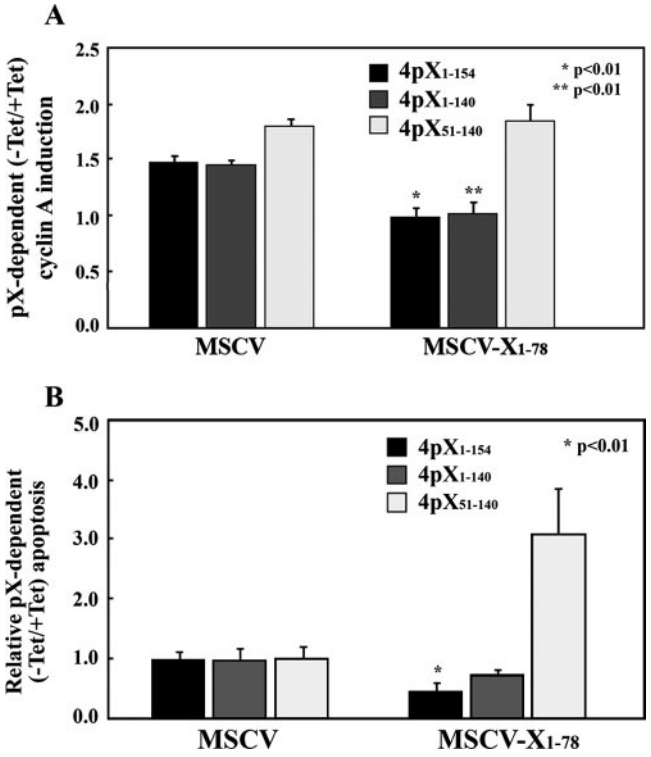


FIG. 7. (A) pX-dependent induction (in the absence of Tet/in the presence of Tet [−Tet/+Tet]) of endogenous cyclin A in 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines infected with control MSCV or MSCV-X_{1-78} retroviruses for 48 h. Endogenous cyclin A mRNA was quantified by real-time PCR, employing RNA isolated from the indicated retrovirus-infected 4pX cells expressing WT pX, pX_{1-140}, and X_{51-140} for 12 h by tetracycline removal. Quantification was performed using endogenous glyceraldehyde-3-phosphate dehydrogenase as an internal control. Results are from at least three independent RNA preparations using identical PCR triplicate experiments. Values for the pX deletion in MSCV-X_{1-78}-infected cells that were statistically significantly different from the value for MSCV-infected cells are indicated by an asterisk(s). (B) Relative pX-dependent (−Tet/+Tet) apoptosis in 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines infected with control MSCV or MSCV-X_{1-78} retroviruses, assayed by immunofluorescence microscopy with the active, cleaved caspase 3-specific antibody. Quantification of immunofluorescence images was by IMAGE J software. Results are from at least three independent apoptotic assays, quantifying an average of 5,000 cells per assay. Values for WT pX in MSCV-X_{1-78}-infected cells that were statistically significantly different from the value for MSCV-infected cells are indicated by an asterisk(s).

pX deletions enabled us to determine not only the direct activation of the mitogenic pathways by transient transreporter assays but also the consequence of their activation, by measuring the effect of each pX deletion on endogenous cyclin A gene expression and on cell growth outcomes, such as apoptosis.

pX_{51-140} mediates sustained JNK pathway activation, inducing endogenous gene expression and apoptosis. Transient transreporter assays measure the direct activation of a specific mitogenic pathway in vivo. In the 4pX cell lines expressing the various pX deletions, the ratio (induction) of transreporter activity, measured in the presence (in the absence of Tet) versus absence (in the presence of Tet) of each pX deletion, can be directly compared. The transreporter induction monitoring activation of the Ras-Raf-MAPK, JNK, and p38 MAPK pathways by pX_{79-154} and pX_{1-140}, containing a deletion of the N-terminal amino acid residues 1 to 78 and C-terminal amino acid residues 141 to 154, respectively, displays a reproducible increase relative to WT pX. Importantly, pX_{51-140} in which both these N- and C-terminal regions have been deleted mediated a statistically significant increase ($P < 0.005$) in transreporter induction for the Ras-Raf-MAPK, JNK, and p38 MAPK ($P < 0.05$) pathways in comparison to WT pX. A larger C-terminal deletion of amino acids 116 to 154 in pX_{1-115} abolished activation of all the mitogenic pathways, demonstrating that amino acids 116 to 140 are necessary for pX-dependent mitogenic pathway activation. We conclude that the pX region containing amino acid residues 51 to 140 is essential for activation of all the mitogenic pathways. In support of this conclusion, we also demonstrate that the MEK-1-, JNK- and p38 MAPK-specific inhibitors inhibit activation of the respective mitogenic pathway by pX_{51-140}. Furthermore, the enhanced activity of pX_{51-140} in comparison to WT pX suggests that these N-terminal and C-terminal regions modulate pX function.

In the 4pX-1 cell line, expression of WT pX induces transcription of the endogenous cyclin A gene via the pX-dependent, sustained activation of the JNK and p38 MAPK pathways (28, 42). pX deletions pX_{51-154}, pX_{79-154}, pX_{51-115}, pX_{1-140} and pX_{51-140} activate all the mitogenic pathways tested by the transient transreporter assay system. However, only 4pX cell lines expressing pX_{1-140} and pX_{51-140} displayed pX-dependent induction of endogenous cyclin A. In vitro JNK assays demonstrated that only pX_{1-140} and pX_{51-140} mediate sustained JNK pathway activation, a requirement for endogenous cyclin A expression (11). Furthermore, similar to WT pX (44), only pX_{1-140} and pX_{51-140} sensitize the respective 4pX cell lines to apoptosis. pX-mediated apoptosis, as we have shown earlier (44), requires sustained activation of the p38 MAPK and JNK pathways. Importantly, neither pX_{1-115} nor pX_{51-115} induces endogenous cyclin A transcription or apoptosis, both assays relying on the potential of the pX deletions to mediate sustained activation of the mitogenic cascades. Thus, these results underscore the significance of the duration of mitogenic pathway activation in altering cell growth outcomes, e.g., progression in the cell cycle, apoptosis, or transformation.

Moreover, our results identifying pX_{51-140} as essential for activation of the mitogenic pathways agree with the activity of the linker scanning mutants of pX, spanning amino acids 53 to 139 and displaying loss of apoptosis (37). However, our results are not in agreement with the conclusions reported by Nijhara et al. (33), namely, that the pX region spanning amino acids 58 to 119 is sufficient for the activation of mitogenic pathways. Similar to our transient transreporter result with pX_{51-115} (Fig. 2), it is likely that residual mitogenic activity in pX_{58-119}, the absence of the modulatory N-terminal region, or overexpression of pX_{58-119} accounts for the observed mitogenic pathway activation (33). In our cellular model system (40, 42, 44), the expression levels of pX and all the pX deletions are very low (Table 1), resembling the pX expression level observed during natural HBV infection (21). Moreover, our conclusion that pX_{51-140} is required for mitogenic pathway activation is derived from both transient transreporter assays and physiologically relevant assays measuring pX-dependent endogenous gene expression and apoptosis.

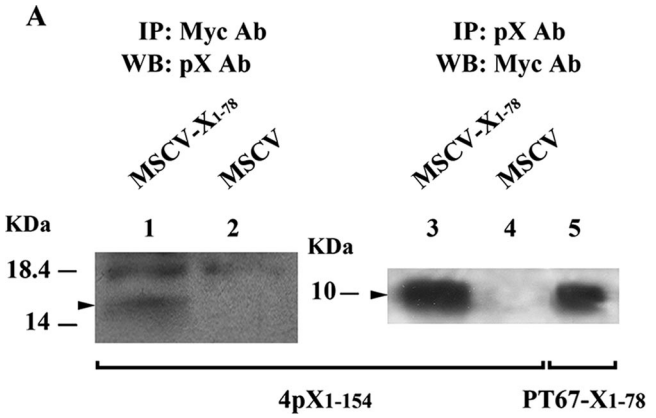
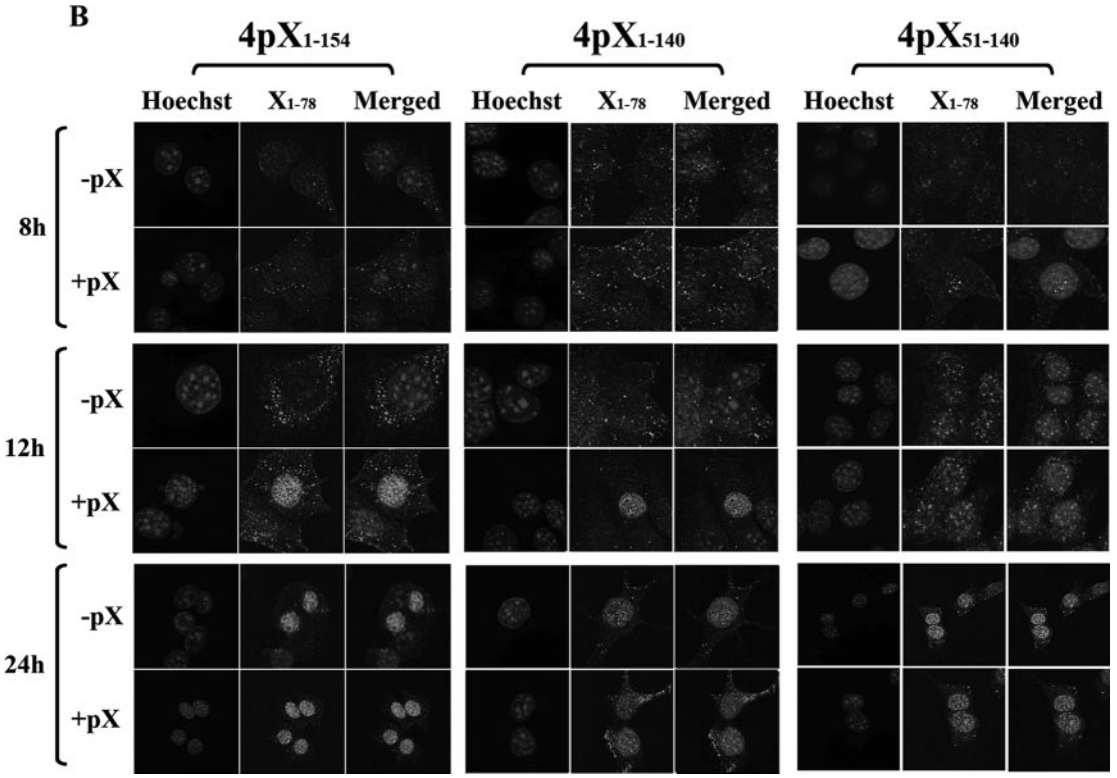


FIG. 8. (A) Cellular extracts were isolated from 4pX-1 cells infected for 48 h with MSCV-X_{1-78} or control MSCV retroviruses and induced to express WT pX for 24 h by tetracycline removal. Whole-cell extract (2 mg) was immunoprecipitated with Myc antibody, recognizing the Myc-pX_{1-78} fusion protein (lanes 1 and 2) or with pX antibody (lanes 3 and 4). Immunoprecipitates (IP) were analyzed by 14% SDS-PAGE and immunoblotted with pX antibody (Ab) (lanes 1 and 2) or Myc antibody (lanes 3 and 4). Lane 5, immunoblot of whole-cell extracts isolated from PT67-X_{1-78} cell line. WB, Western blot. (B) Immunofluorescence microscopy of 4pX-1, $4pX_{1-140}$, and $4pX_{51-140}$ cell lines infected for 48 h with MSCV-X_{1-78} retrovirus followed by expression of pX deletions for the indicated time course, employing the Myc antibody (green). Hoechst (blue) stains DNA.



The comparative analyses of pX_{51-154} and pX_{51-140} suggested that the C-terminal region containing amino acids 141 to 154 modulates pX function. Pulse-chase studies shown herein demonstrate the increased half-life of pX_{1-140} relative to that of WT pX, supporting the hypothesis that the C-terminal 14 amino acids determine pX stability. Interestingly, naturally occurring pX variants found integrated in genomic DNA from liver tumors of patients with HCC contained C-terminal deletions generally lacking amino acids 134 to 154 (36, 43, 46). Thus, the increased protein stability of these pX variants may be a contributing factor in HCC development.

pX_{1-78} has a dominant-negative effect of WT X-protein function. The enhanced mitogenic activity of pX_{51-140} demonstrated herein, together with earlier reports that the N terminus of pX negatively regulates pX-dependent transactivation (31), prompted us to examine whether pX_{1-78} interferes with WT X-protein function. pX_{1-78} expressed via the MSCV-pX_{1-78} retrovirus inhibits mitogenic pathway activation and endogenous cyclin A expression and suppresses apoptosis in 4pX cell lines expressing WT pX and pX_{1-140} without an effect on pX_{51-140} lacking the 50 N-terminal amino acid residues. Coimmunoprecipitations of WT pX with pX_{1-78} established that pX_{1-78} interferes with WT pX function by interacting directly with WT pX. The absence of a dominant-negative effect of pX_{1-78} on the mitogenic activities of pX_{51-140} supports the hypothesis that the N-terminal amino acid residues 1 to 50 constitute the site of direct X-protein-protein interactions. This is the first conclusive evidence of direct protein-protein interactions involving the N terminus of pX. Whether these protein-protein interactions lead to pX dimerization or to formation of more complex oligomers remains to be determined.

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