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A Role for Both Ets and C/EBP Transcription Factors and mRNA Stabilization in the MAPK-dependent Increase in p21 $^{Cip\text{-}1/WAF1/mda6}$ Protein Levels in Primary Hepatocytes

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In primary hepatocytes and HepG2 hepatoma cells, prolonged activation of the p42/44 mitogen-activated protein kinase (MAPK) pathway is associated with a reduction in DNA synthesis, mediated by increased expression of the cyclin-dependent kinase inhibitor protein p21 $^{Cip\text{-}1/WAF1/mda6}$ (p21). This study was performed to evaluate the contribution of transcriptional and post-transcriptional regulation in this response. Prolonged activation of the MAPK pathway in wild-type or p21 null hepatocytes caused a large decrease and increase, respectively, in DNA synthesis. Prolonged activation of the MAPK pathway in either wild-type or p21 antisense HepG2 cells also caused large decreases and increases, respectively, in DNA synthesis. MAPK signaling increased the phosphorylation of the transcription factors Ets2, C/EBPα, and C/EBPβ, and rapidly increased transcription from the p21 promoter via multiple Ets- and C/EBP-elements within the enhancer region. Eight hours after MAPK activation, loss of C/EBPβ or Ets2 function significantly reduced MAPK-stimulated transcription from the p21 promoter and abolished increased p21 protein expression. At this time, MAPK signaling increased both p21 mRNA and p21 protein stabilities that were also demonstrated to be essential for a profound increase in p21 protein levels. Thirty-six hours after MAPK activation, transcription from the p21 promoter was still significantly reduced in cells without either C/EBPβ or Ets2 function; however, these cells were now capable of exhibiting a partial increase in p21 protein expression. In contrast, loss of C/EBPα function modestly reduced MAPK-stimulated transcription from the p21 promoter but strongly inhibited the ability of prolonged MAPK activation to increase protein levels of p21. This data suggested that prolonged enhancement of p21 protein levels may be under posttranscriptional control. In agreement with this hypothesis, prolonged MAPK signaling further increased p21 mRNA stability at 36 h, compared with the 8-h time point. Our data argue that MAPK signaling increased p21 promoter activity *via* multiple transcription factors, which alone were insufficient for a robust prolonged increase in p21 protein levels in primary hepatocytes, and that to increase p21 protein levels also required enhanced stabilization of p21 mRNA and p21 protein. Collectively, these data suggest that loss of transcription factor and mRNA/protein stabilization functions correlates with an inability of MAPK signaling to cause growth arrest versus proliferation in primary hepatocytes.

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Abbreviations used: cdk, cyclin-dependent kinase; C/EBP, CCAATT enhancer binding protein; DMEM, Dulbecco's modified Eagle's medium; ΔB-Raf:ER, estrogen receptor-B-Raf fusion protein; EGF, epidermal growth factor; GST, glutathione-S-transferase; GTPase, guanine nucleotide trisphosphate (γ-phosphate) hydrolase; HGF, hepatocyte growth factor; JNK, c-Jun NH_2-terminal kinase; MAPK,mitogen-activated protein kinase; MBP, myelin basic protein; MEF, mouse embryonic fibroblast; MEK, mitogen/extracellular regulated protein kinase; null mouse, a mouse in which a specific gene has been embryonically deleted; PHX, two-thirds partial hepatectomy; Rb, retinoblastoma gene product; RK, reactivating kinase; SAP kinase, stress-activated protein kinase; TAM, 4-hydroxytamoxifen

INTRODUCTION

Partial hepatectomy or dissociation followed by primary culture triggers hepatocyte entry into the cell cycle (Michalopoulos and DeFrances, 1997; Diehl and Rai, 1996; Loyer *et al.*, 1996). Maximal DNA synthesis in vivo occurs between 12 and 36 h post partial hepatectomy (PHX). Primary culture in vitro in the presence of growth factors such as insulin, epidermal growth factor (EGF) or hepatocyte growth factor (HGF) stimulates hepatocyte DNA synthesis which is maximally observed between 40 and 70 h post isolation (Loyer *et al.*, 1996; Spector *et al.*, 1997; Westwick *et al.*, 1996; Talarmin *et al.*, 1999). Hepatocytes do not terminally differentiate and can enter and exit the cell cycle during cycles of liver regeneration. This is in contrast to other cell types, e.g., intestinal epithelial cells, chondrocytes, and keratinocytes, which undergo irreversible terminal differentiation processes (Chinery *et al.*, 1997; Missero *et al.*, 1995).

Recently, several signaling pathways leading to increased DNA synthesis in primary hepatocytes have been shown to be the c-Jun NH_2-terminal kinase (JNK), the p38-reactivating kinase (p38-RK) pathways and the p42/44 mitogen-activated protein kinase (MAPK) pathway (Spector *et al.*, 1997; Auer *et al.*, 1998a; Talarmin *et al.*, 1999). In addition, prolonged signaling by the MAPK pathway was shown to play a prominent role in causing cell cycle arrest in these cells (Tombes *et al.*, 1998; Auer *et al.*, 1998b). The ability of MAPK to cause cell cycle arrest in these studies was correlated with increased expression of the cyclin-dependent kinase inhibitor proteins p21 $^{Cip\text{-}1/WAF1/mda6}$ (p21), and to a lesser extent p16 ^{INK4a} and p27 $^{Kip\text{-}1}$.

Hepatoma cells which are incapable of increasing p21 expression are known to be more tumorigenic in vivo than hepatoma cells which still retain the ability to express p21 (Pu *et al.*, 1997). In part, this may be due to loss of p53 function, although several studies have reported that childhood hepatomas and early stage adult liver cancers express functional p53 (Imai *et al.*, 1996; Chen *et al.*, 1995; Teramoto *et al.*, 1994). A reduction in the ability of many cell types to increase p21 expression has also been suggested to be important in the process of transformation and differentiation (Sherr and Roberts, 1999; Chellappan *et al.*, 1998). This may be due to a loss of transcription factor function(s), or a consequence of altered signaling via other pathways which regulate p21 expression (Olson *et al.*, 1998; Hirai *et al.*, 1998; Serfas *et al.*, 1997). In further agreement with the importance of p21 expression in hepatocyte cell cycle control, it was recently shown that MAPK signaling had a reduced ability to increase p21 expression in hepatoma cells and that inducible overexpression of p21 could blunt liver regeneration (Albrecht *et al.*, 1997; Wu *et al.*, 1996; Timchenko *et al.*, 1997). Together, these data suggest that regulation of p21 expression and function may play a pivotal role in both the regulation of liver regeneration and in hepatocellular transformation.

Regulation of the p21 promoter appears to be complex, consisting of both potential positive and negative regulatory elements. Multiple transcription factor binding sites within the promoter have also been noted. Potential regulatory transcription factors include p53, the glucocorticoid receptor family, Ets family, C/EBP family, Stat family, and Sp family (Sugikawa *et al.*, 1999; Chinery *et al.*, 1997a; Timchenko *et al.*, 1997; Timchenko *et al.*, 1996; Hendricks-Taylor and Darlington 1995; Buck *et al.*, 1994; Chinery *et al.*, 1997; Cram *et al.*, 1998; Auer *et al.*, 1998b; Park *et al.*, 1999; Olson *et al.*, 1998; Park *et al.*, 2000; Mitchell and El-Deiry 1999; Johannessen *et al.*, 1999; Beier *et al.*, 1999). C/EBP transcription factors have been proposed to play key roles in the acute phase responses of hepatocytes (Yamada *et al.*, 1997; Alam *et al.*, 1992; Michalopoulos and DeFrances, 1997; Diehl and Rai, 1996). In hepatocytes, we and others have defined important roles for C/EBP transcription factors in the regulation of p21 protein levels, although the involvement of these factors in regulating the promoter via MAPK signaling has not been described. Using different cell types, it has also been argued that MAPK signaling regulates the p21 promoter, and thereby p21 protein levels, via the transcription factor Ets2 (Beier *et al.*, 1999). Other studies have implicated phorbol ester signaling in post-transcriptional stabilization of p21 mRNA and protein, thereby increasing p21 protein levels (Johannessen *et al.*, 1999; Akashi *et al.*, 1999). Thus, it is likely that multiple MAPK-dependent events may play a cell type- and growth status-specific role in modulating p21 expression, at the levels of transcription and post-transcriptional stabilization.

These studies were initiated to provide further insight into the transcriptional and post-transcriptional mechanism(s) by which MAPK signaling increases both p21 mRNA and protein expression in primary hepatocytes and in weakly tumorigenic HepG2 hepatoma cells (Auer *et al.*, 1998b; Auer *et al.*, 1998c).

MATERIALS AND METHODS

Materials

Male C57BL/6J wild-type and p21 null mice (30 g) had access to food and water ad libidum. HepG2 hepatoma cells were from the ATTCC (Bethesda, MD). Antip42/44 $^{MAP\ kinase}$ (sc-154AC), anti-C/EBPβ (sc-150), anti-C/EBPα (sc-61), anti-Ets2 (sc-351), antihuman p53 (sc-126), antirodent p53 (sc-100), anti-β-actin (sc-1616), anti-HuR (sc-5261, sc-5481); anti-PCNA (sc-56), anticdk2 (sc-163AC), anticdk4 (sc-601AC), antip16 ^{INK4a} (sc-1207) antip27 $^{Kip\text{-}1}$ (sc-528) and antip21 (sc-397-G and sc-817), Anti-Cyclins A- (sc-596), D- (sc-753) and E- (sc-481) were from Santa Cruz Biotechnology (Santa Cruz, CA). Radiolabeled [γ - ^{32} P]-ATP and ^{3} H-thymidine were from NEN (Wilmington, DE). Western immunoblotting was performed using the Enhanced Chemi-Luminescence (ECL) system (Amersham, Arlington Heights, IL). Protein preparations and other reagents were as noted in (Spector *et al.*, 1997; Auer *et al.*, 1998a; Auer *et al.*, 1998b; Auer *et al.*, 1998c). Plasmids containing full length (-2326) and truncated (-1458 and -883) p21 promoter linked to the firefly luciferase gene were as described by Chinery *et al.* (1997). Plasmids containing a portion of the p21 promoter (-1383/-1184) with or without the mutated C/EBP binding site were as described by Cram *et al.* (1998). Plasmids containing antisense C/EBPα and antisense C/EBPβ were generated from sense plasmids described in Chinery *et al.* (1997), Timchenko *et al.* (1997), and Cram *et al.* (1998). Antisense phosphothio-oligonucleotides toward Ets2 were generated using published sequences (Watson *et al.*, 1986). Dominant negative p53(R175H) was a gift from Dr. B. Vogelstein (Sugikawa *et al.*, 1999). The specific MEK1/2 activation inhibitors PD98059 and U0126 (Alessi *et al.*, 1995; Park *et al.*, 2000) were gifts from Parke-Davis (Ann Arbor, MI) and DuPont Pharmaceuticals (Wilmington, DE).

Methods: Recombinant Adenoviral Vectors; Generation and Infection In Vitro

The studies herein were performed using 2 adenoviral technologies. Replication defective adenovirus was conjugated to poly-L-lysine as

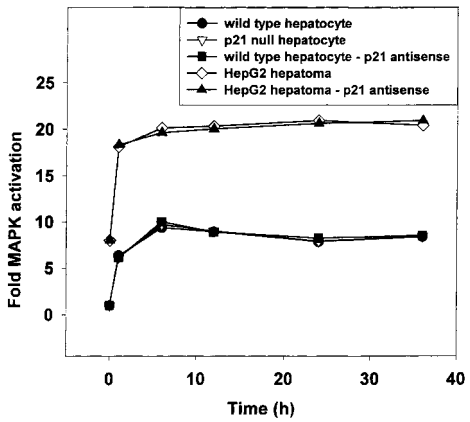


Figure 1. Treatment of hepatocytes and hepatoma cells expressing ΔB-Raf:ER with 4-hydroxytamoxifen causes activation of MAPK. Cells were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (at 250 multiplicity of infection (m.o.i.), followed by culture as in Methods. As indicated, and in addition to ΔB-Raf:ER infection, cells were also infected with either null recombinant adenovirus or a recombinant adenovirus expressing p21 antisense mRNA, (at 100 m.o.i. each). After 24 h to allow protein/mRNA expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen for 36 h (total time in culture 60 h). Cells were assayed for MAPK activity before 4-hydroxytamoxifen addition (0 min), 1 h, 6 h, 12 h, 24 h, and 36 h after addition of 4-hydroxytamoxifen. Data are the means of triplicate determinations from 3 separate experiments/animals (to the nearest 50 cpm ± SEM) and are expressed as cpm incorporated into myelin basic protein above background (250 ± 50 cpm) in the standard MAPK assay (Methods). No activation of MAPK was observed in cells infected with kinase inactive ΔRaf:ER (301) treated with 4-hydroxytamoxifen (our unpublished observations). No activation of MAPK was observed in cells infected with ΔB-Raf: ER treated with matched vehicle control (DMSO) (our unpublished observations). Addition of 50 μM PD98059 to the culture media abolished the activation of MAPK by ΔB-Raf:ER in all of the cells examined above (our unpublished observations), in agreement with Auer *et al.* (1998b).

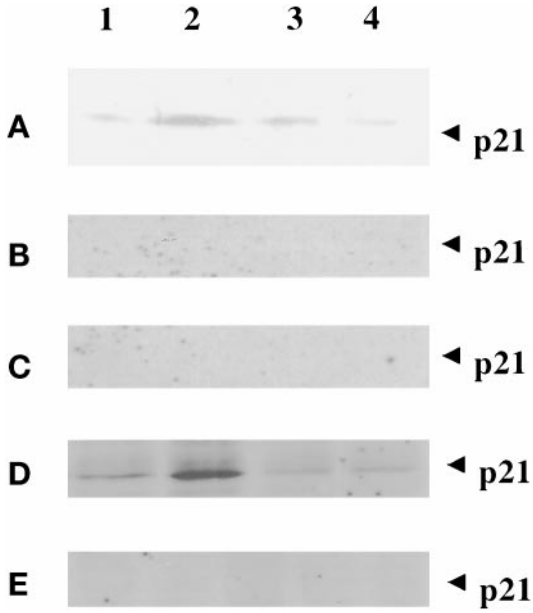


Figure 2. Prolonged activation of the MAPK cascade results in increased expression of the cyclin dependent kinase inhibitor p21. (A) Wild-type hepatocytes. (B) p21 null hepatocytes. (C) Wild-type hepatocytes with p21 antisense mRNA. (D) Hep G2 cells. (E) Hep G2 cells with p21 antisense mRNA. Hepatocytes or HepG2 hepatoma cells were infected with kinase active ΔB-Raf: ER poly-L-lysine adenovirus (250 m.o.i), followed by culture as in Methods. In some experiments and in addition to ΔB-Raf:ER infection, cells were also infected with either a null recombinant adenovirus or a p21 antisense mRNA recombinant adenovirus (at 100 m.o.i. each). After 24 h to allow protein expression, cells were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen for 36 h (total time in culture 60 h). Protein expression of p21 was determined by immunoblotting. Equal protein loading (200 μg) per lane; exposures of x-ray film for the ECL immunoblots shown were from 30 s (Panel A) to 5 min (Panels B and C). Lane 1: ΔB-Raf:ER + vehicle control. Lane 2: ΔB-Raf:ER + 4-hydroxytamoxifen. Lane 3: ΔB-Raf:ER + 4-hydroxytamoxifen + 50 μM PD98059. Lane 4: vector control + 50 μM PD98059 + 4-hydroxytamoxifen. A representative experiment for each cell type/condition is shown (n = 6).

described (Auer *et al.*, 1998a; Auer *et al.*, 1998b; Auer *et al.*, 1998c, Cristiano *et al.*, 1993). The DNA conjugated virus was added to hepatocytes at a multiplicity of infection (m.o.i.) of 250, and the cells incubated for 4 h at 37°C. The cells were washed with media to remove unadsorbed virus. Cells expressed transduced gene products from 10 to 24 h after infection. Using a plasmid to express β-galactosidase under control of the *CMV*-promoter, we determined that 1 μg of plasmid conjugated to virus particles and infected into mouse hepatocytes before plating at an m.o.i. of 250 gave 100% infection as judged by blue coloration after 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) incubation 24-h post infection. Second, we generated recombinant adenoviruses (Auer *et al.*, 1998a; Auer *et al.*, 1998b). To assess the effectiveness of recombinant adenoviral infection, we generated a recombinant virus containing the gene for β-galactosidase. Mouse hepatocytes were infected with this virus after isolation in vitro (m.o.i. 250), and incubated at 37°C for a further 24 h; cells were fixed and incubated with X-Gal (Auer *et al.*, 1998b).

Preparation of Mouse Hepatocytes

Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg), and the lower thorax and abdomen was shaved to remove fur. A small (3 cm) vertical midline incision was made in the abdominal wall from just below the costal margin/xiphoid process. Hepatocytes were prepared by cannulation of the portal vein, collagenase perfusion of the liver, and washing in DMEM containing 5% (vol/vol) fetal calf serum as described (Spector *et al.*, 1997).

Primary Culture, Hormonal Treatment, and Assay for DNA Synthesis in Cultures of Hepatocytes

Mouse hepatocytes were cultured on rat-tail collagen (Vitrogen)-coated plastic dishes (12 × 20 mm, 2×10^5 cells) in 1 ml phenol red free DMEM in 5% (vol/vol) CO_2 supplemented with [50 nM insulin, 0.1 nM dexamethasone, 1 nM thyroxine]. At this time, cells were infected with various adenoviruses according to the experimental protocol. For cells undergoing acute exposure, treatments occurred 90 min after plating. For adenoviral infected cells, 4 h after infection, media was replaced and hepatocytes were cultured in the same supplemented DMEM for 24 h. Hormonal treatments, and/or pro-

Table 1. Prolonged activation of the MAPK pathway inhibits DNA synthesis in cells expressing p21, but not in cells where p21 protein expression has been blocked

Infection/treatment	Wild-type hepatocyte	p21 null hepatocyte	Wild-type p21 antisense	Hep G2 hepatoma	Hep G2 hepatoma + p21 antisense
ΔB-Raf:ER + vehicle	10,150 ± 2,250	9,700 ± 1,600	9,950 ± 1,850	72,000 ± 5,100	94,700 ± 6,700
ΔB-Raf:ER + TAM	2,050 ± 300[b]	26,600 ± 2,100[c,a]	25,250 ± 1,650[c,a]	6,650 ± 850[b]	124,100 ± 8,500[c]
ΔB-Raf:ER + TAM + PD98059	7,300 ± 850[b,c]	10,450 ± 1,550[a]	9,240 ± 1,100[a]	41,450 ± 3,050	52,800 ± 4,000
ΔB-Raf:ER + vehicle + PD98059	8,100 ± 950[b,a]	8,200 ± 1,300[b,a]	8,450 ± 1,200[b,a]	45,250 ± 2,900	47,650 ± 4,200

Cells were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (at 250 m.o.i.), followed by culture as in Methods. In some experiments and in addition to ΔB-Raf:ER infection, cells were also infected with either null recombinant adenovirus or a p21 antisense recombinant adenovirus (at 100 m.o.i. each). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen (TAM) for 36 h (total time in primary culture, 60 h). In some experiments, prior to 4-hydroxytamoxifen addition, cells were treated with 50 μM PD98059. Hepatocytes under all conditions were continually incubated with 4 μCi/ml ^{3}H-thymidine throughout the final 36 h of culture, after which they were lysed and ^{3}H-thymidine incorporation into DNA determined as in Methods. Addition of 4-hydroxytamoxifen did not alter the rate of DNA synthesis (data not shown). Data are the means (counts per minute ^{3}H-thymidine incorporated into DNA ± SEM) of sextuplicate experiments from 4 separate animals.

[a] $p < 0.05$ increase compared to wild-type hepatocyte ΔB-Raf:ER + 4-hydroxytamoxifen cells.

[b] $p < 0.05$ decrease compared with ΔB-Raf:ER + vehicle control.

[c] $p < 0.05$ increase compared to ΔB-Raf:ER + vehicle control.

tein kinase inhibitors were added 24 h after the media change (protein kinase inhibitors were added 30 min before further treatment). Twenty-four hours after infection, hepatocytes were treated for 36 h with 100 nM 4-hydroxytamoxifen. The activity of $p42/44_{MAP\ kinase}$ was determined before 4-hydroxytamoxifen addition, 6 h after the start of treatment, and then after a further 36 h. Twenty seconds before terminating the experiment, media was aspirated followed by immediate homogenization. Cells were homogenized in 1 ml ice-cold homogenization buffer A [25 mM HEPES, pH 7.4 at 4°C, 5 mM EDTA, 5 mM EGTA, 5 mM benzamidine, 1 mM phenylmethyl sulfonylfluoride, 40 μg/ml pepstatin A, 1 μM microcystin-LR, 0.5 mM sodium vanadate, 0.5 mM sodium pyrophosphate, 0.05% (wt/vol) sodium deoxycholate, 1% (vol/vol) triton ×100, 0.1% (vol/vol) 2-mercaptoethanol], with trituration using a P1000 pipette to lyse the cells. Homogenates were stored on ice before clarification by centrifugation (4°C), and clarified aliquots were subjected to immunoprecipitation. For DNA synthesis assays, hepatocytes were isolated from wild-type and p21 null mice and infected with ΔB-Raf:ER. Twenty-four hours after infection, hepatocytes were treated with 100 nM 4-hydroxytamoxifen. Hepatocytes were cultured in the presence of 4 μCi ^{3}H-thymidine for a further 36 h, after which time cells were lysed with 0.5 M NaOH and DNA precipitated with 12.5% (wt/vol) TCA (final). Acid precipitable material was transferred to glass fiber filters, washed with 5% (wt/vol) TCA, and ^{3}H-thymidine incorporation into DNA was quantified by liquid scintillation spectrometry (Spector *et al.*, 1997).

Culture and Adenoviral Infection of HepG2 Cells

HepG2 cells were cultured in phenol red-free DMEM supplemented with [50 nM insulin, 0.1 nM dexamethasone, 1 nM thyroxine], in an identical manner to primary hepatocytes. In experiments assessing DNA synthesis, cells were cultured in phenol red-free DMEM supplemented with 5% (vol/vol) fetal calf serum (FCS). Cells were infected with adenoviruses as described for primary hepatocytes.

Immunoprecipitations from Homogenates

Fifty microliters of protein A agarose (Ag) slurry (25 μl bead volume) was washed twice with 1 ml PBS containing 0.1% (vol/vol) Tween 20, and resuspended in 0.1 ml of the same buffer. Antibodies (2 μg, 20 μl) or serum (20 μl) were added to each tube and incubated (3 h, 4°C). Clarified hepatocyte homogenates (1.0 ml, 1 mg total protein) were mixed with protein A-Ag-conjugated antibody in duplicate using gentle agitation (2.5 h, 4°C). Protein A-Ag was recovered by centrifugation, the supernatant discarded, and washed (10 min) sequentially with 0.5 ml buffer A (twice), PBS and buffer B [25 mM HEPES, pH 7.4, 15 mM $MgCl_2$, 0.1 mM Na_3VO_4, 0.1% (vol/vol) 2-mercaptoethanol].

Assay of p42/44 $^{MAP\ kinase}$ Activity

Immunoprecipitates were suspended in a final volume of 50 μl of buffer B containing 0.2 mM [γ-^{32}P]ATP (2000 cpm/pmol), 1 μM microcystin- LR, 0.5 mg/ml myelin basic protein (MBP), which initiated reactions and incubated at 37°C. After 20 min, 40 μl of the reaction mixtures were spotted onto 2-cm circles of P81 phosphocellulose paper (Whatman, Maidstone, England) and immediately placed into 180 mM phosphoric acid. Papers were washed four times (10 min each) with phosphoric acid, and once with acetone, and ^{32} P-incorporation into MBP was quantified by liquid scintillation spectroscopy. Preimmune controls were performed to ensure MBP phosphorylation was dependent upon specific immunoprecipitation of MAPK.

Luciferase Assay

For luciferase assays, hepatocytes were isolated from wild-type and p21 null mice and infected with various constructs. In cells expressing ΔB-Raf:ER, 24 h after infection, hepatocytes were treated with 100 nM 4-hydroxytamoxifen. Hepatocytes were cultured for a further 6 to 36 h, after which time cells were lysed with manufacturers lysis buffer and luciferase assays performed on equal protein amounts of lysate as per manufacturers instructions using a Bertold Luminometer (Promega Luciferase assay kit, Madison, WI) (Auer *et al.*, 1998a). Infection with a construct to express β-galactosidase was used as a transfection efficiency internal control.

Chloramphenicol Acteyltransferase (CAT) Assay

Hepatocytes were isolated from wild-type and p21 null mice and infected with constructs -1383/-1184 containing a functional C/EBP

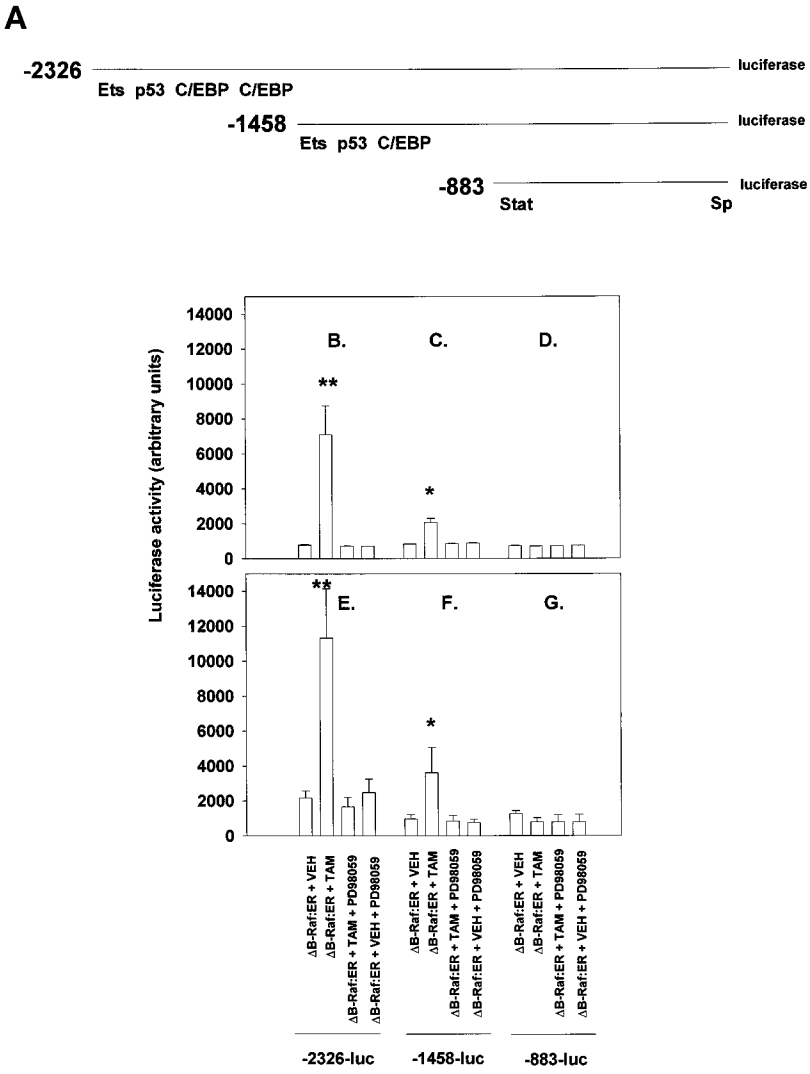


Figure 3. MAPK-dependent modulation of p21-promoter activity in primary hepatocytes and Hep G2 cells. (A) Schematic diagram showing the p21 promoter, the deletions used in this study, and putative p53, Ets, and C/EBP transcription factor binding sites within the promoter. MAPK-dependent modulation of p21-promoter activity in primary hepatocytes and Hep G2 cells. (B) -2326 full-length p21-promoter in primary hepatocytes. (C) -1458 truncated p21-promoter in primary hepatocytes. (D) -883 truncated p21-promoter in primary hepatocytes. (E) -2326 full-length p21-promoter in Hep G2 cells. (F) -1458 truncated p21-promoter in Hep G2 cells. (G) -883 truncated p21-promoter in HepG2 cells; hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. In some experiments and in addition to ΔB-Raf:ER infection, cells were also infected with plasmids containing either full-length p21-promoter or a truncated p21-promoters (containing the initiating ATG proximal 883 or 1458 bp) poly-L-lysine adenovirus (at 100 m.o.i. each). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen, and with or without 50 μM PD98059, for 480 min and luciferase activity determined as in Methods ($n = 8 \pm$ SEM). ** $p < 0.001$ greater than non-TAM treated control value; *$p < 0.05$ greater than non-TAM treated control value.

site (at -1270/-1256) or -1383/-1184 with a mutated C/EBP site linked to CAT. CAT assays were performed on equal protein amounts of lysate as described in Cram *et al.* (1998) and Taher *et al.* (1999) using chloramphenicol and ^{14}C-acetyl CoA. The enzyme activity was determined after TLC as a function of ^{14}C-acetylated chloramphenicol produced per microgram protein in the cell lysate. Infection with a construct expressing β-galactosidase was used as a transfection efficiency internal control.

Western Blotting

Twenty-four hours after infection, in cells expressing ΔB-Raf:ER, hepatocytes were treated with 100 nM 4-hydroxytamoxifen. Hepatocytes were cultured for a further 6 to 36 h, after which the cells were lysed with either 10% (wt/vol) TCA or ice-cold homogenization buffer. TCA precipitated protein was collected by centrifugation, washed once with cold acetone, and resuspended in SDS PAGE sample buffer before resolution on SDS PAGE using 10–12% gels. Cells lysed in homogenization buffer were subjected to immunoprecipitation as described in the previous sections, prior resuspension in SDS PAGE sample buffer and resolution on SDS PAGE using 10–12% gels. Gels were transferred to a 0.22 μ nitrocellulose filter and immunoblotting performed using the ECL system (Amersham) (Auer *et al.*, 1998b).

Determination of p21 mRNA Levels by Reverse Transcriptase-PCR (RT-PCR)

For determination of p21 mRNA levels, hepatocytes were isolated from wild-type and p21 null mice and infected with various constructs. In cells expressing ΔB-Raf:ER, 24 h after infection, hepatocytes were treated with 100 nM 4-hydroxytamoxifen. Hepatocytes were cultured for a further 6 to 36 h, after which time cells were lysed and total RNA prepared. RNA was subjected to 35 cycles of RT-PCR using specific oligonucleotides for mouse p21 [5′CCG CAC AGG AGC AAA GTG TGC; 3′CTT GCA GAA GAC CAA TCT GCG] and for β-actin (internal control) as previously described (Cale *et al.*, 1998; Gao and Kunos, 1998; Nozawa *et al.*, 1999). Equal loading of total RNA was used for gel electrophoresis; quantitated values were plotted after normalization to β-actin (internal control). Following agarose gel electrophoresis, RNA bands were visualized using propidium iodide staining and quantification of p21 and β-actin UV-flourescent band intensity determined using SigmaScan (Regina, SK, Canada) software.

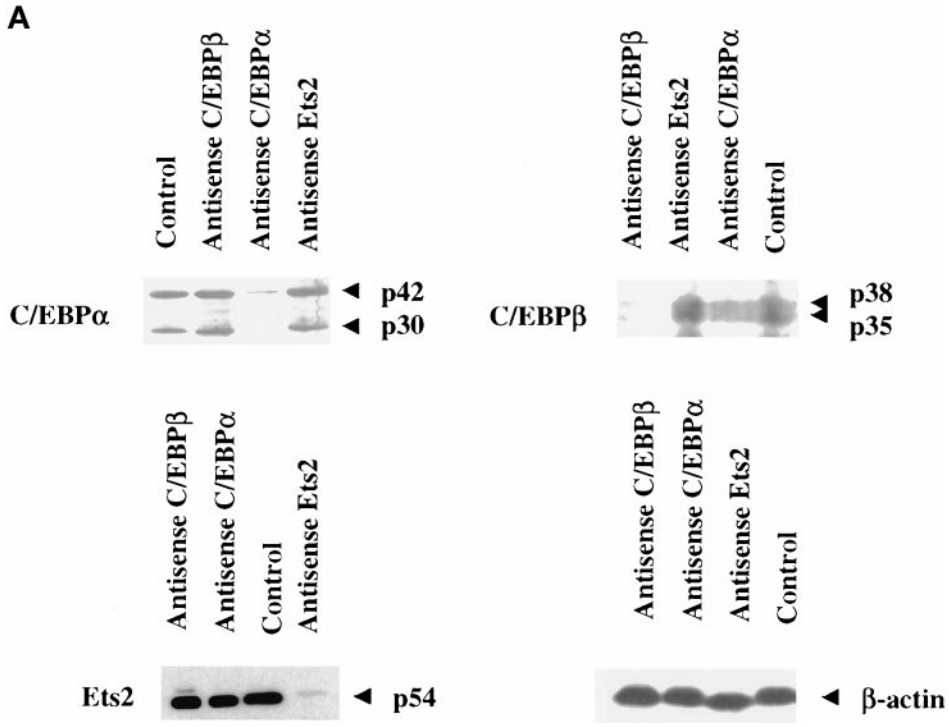


Figure 4. MAPK-dependent modulation of p21-promoter activity in primary hepatocytes depends upon the function of multiple transcription factors. (A) Antisense C/EBPα, antisense C/EBPβ, and antisense Ets2 reduce protein levels of their respective proteins in primary hepatocytes. (B) Antisense C/EBPα partially reduces MAPK-induced promoter activity from -2326 full length and -1458 truncated p21 promoter constructs. (C) Antisense C/EBPβ considerably reduces MAPK-induced promoter activity from -2326 full length and -1458 truncated p21 promoter constructs. (D) Antisense Ets2 considerably reduces MAPK-induced promoter activity from -2326 full length and -1458 truncated p21 promoter constructs in primary hepatocytes. (E) Time course (0 to 36 h) of -2326 full-length promoter activation in cells infected with either antisense C/EBPα, antisense C/EBPβ or antisense Ets2. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. In addition to ΔB-Raf:ER infection, cells were also infected with plasmids containing either -2326 full-length or -1458 truncated p21-promoter constructs (100 m.o.i.) where indicated. In panels B and C, in addition to ΔB-Raf:ER infection, cells were also infected with plasmids to express either antisense C/EBPα or antisense C/EBPβ (200 m.o.i.). In panel D, cells were transfected with an antisense oligonucleotide to Ets2 (10 μM, using Superfect reagent as per manufacturer's instructions). (A-D) After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen, and with or without 50 μM PD98059, for 480 min and luciferase activity determined as in Methods (n = 8 $\pm$ SEM). (E) After 24 h to allow protein expression, hepatocytes were treated with 100 nM 4-hydroxytamoxifen and luciferase activity determined at the indicated times as in Methods (n = 3 $\pm$ SEM). **$p < 0.001$ greater than non-TAM treated control value; *$p < 0.05$ greater than non-TAM treated control value; $^{\&}$ $p < 0.05$ less than corresponding control (nonantisense) value.

Determination of Protein Half-life/Radiolabeling of p21

For determination of p21 protein stability, hepatocytes were isolated from wild-type and p21 null mice and infected with various constructs. Twenty-four hours after infection, media was replaced with methionine/cysteine free media containing 1 μCi/10 μl [^{35}S]methionine. In cells expressing ΔB-Raf:ER, 24 h after infection, hepatocytes were also treated with 100 nM 4-hydroxytamoxifen. In studies examining MAPK-mediated alterations in stability, 36 h after MAPK activation, media was replaced with media containing nonradioactive methionine/cysteine and 20 μg/ml cycloheximide. Portions of cells were treated with 50 μM PD98059, which abolished MAPK activity. The rate at which ^{35}S-radioactivity was lost from p21 was determined following immunoprecipitation, SDS PAGE, transfer to nitrocellulose, and autoradiography in control cells; MAPK active cells; and MAPK active + PD98059 cells over the following 6 h (Johannessen *et al.*, 1999; Schreiber *et al.*, 1999). Quantification of band intensity used Molecular Dynamics software (Sunnyvale, CA).

Data Analysis

Comparison of the effects of various treatments was performed using one-way analysis of variance and a two-tailed *t* test. Differences with a p value of < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points from multiple separate experiments (using hepatocytes from different animals) ($\pm$ SEM).

RESULTS

Prolonged Activation of the MAPK Cascade Increases p21 $^{Cip-1/WAF1/mda6}$ (p21) Expression in Primary Hepatocytes and HepG2 Hepatoma Cells

Hepatocytes were isolated from wild-type and p21 null mice and infected with a construct expressing an inducible estrogen receptor-B-Raf fusion protein (ΔB-Raf:ER). Cells were treated with 4-hydroxytamoxifen as in Methods, and the activity of MAPK was determined. Treatment of wild-type hepatocytes expressing ΔB-Raf:ER with 4-hydroxytamoxifen increased MAPK activity ~10-fold after 6 h, which was maintained for 36 h (Figure 1). MAPK activation was blocked by incubation of cells with the specific inhibitor of MEK1/2, PD98059 (50 μM), and no activation was observed in cells infected with vector control treated with 4-hydroxytamoxifen [our unpublished results, in agreement with Auer *et al.* (1998b) and Auer *et al.* (1998c)]. Prolonged MAPK activation caused an increase in p21 protein expression in hepatocytes from wild-type, but not p21 null, mice (Figures

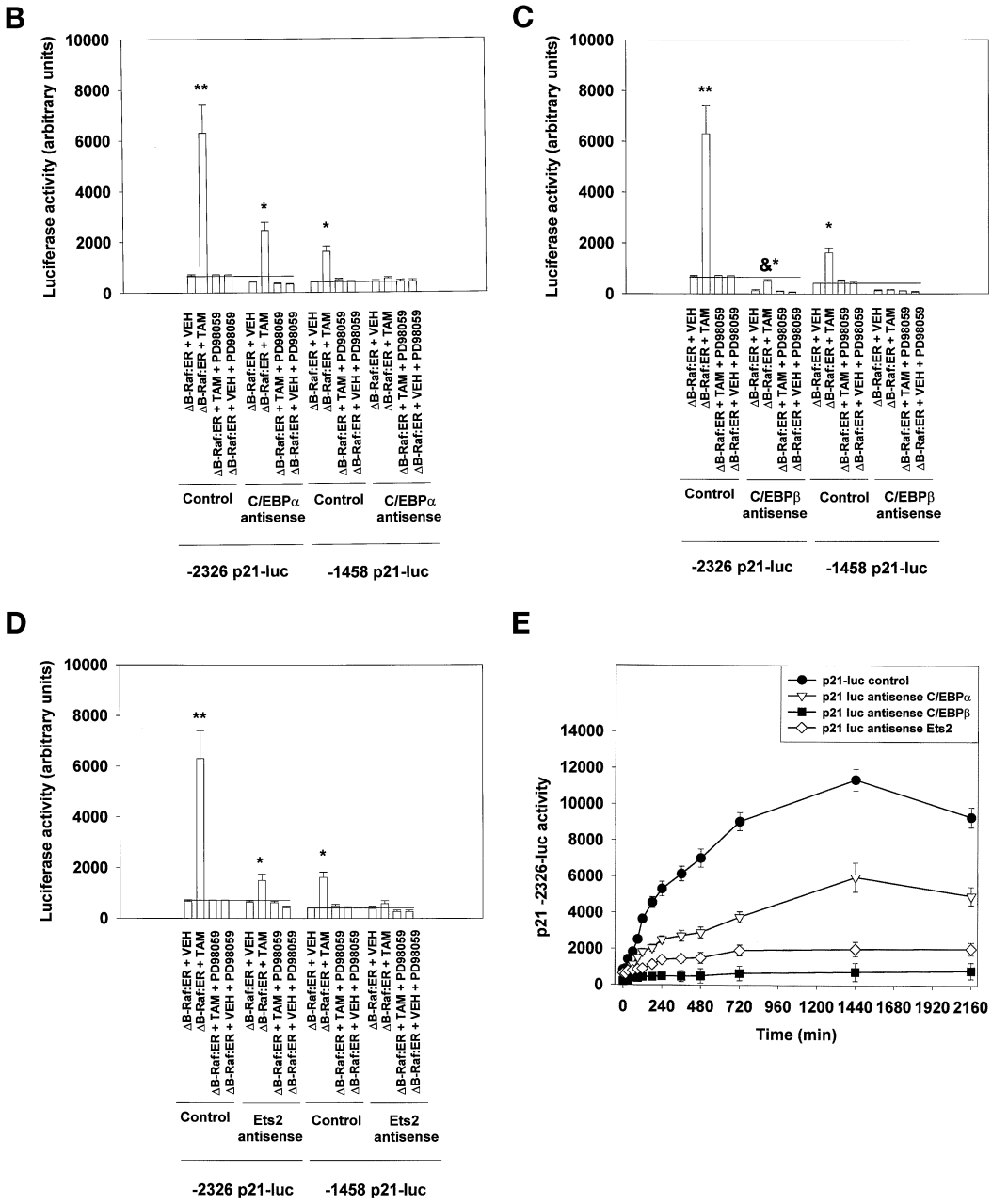


Figure 4 (legend on facing page).

2A-2C). No increase in MAPK activity or in p21 protein expression was observed in 4-hydroxytamoxifen treated hepatocytes cultured in the presence of PD98059.

In a similar manner, HepG2 cells were infected with either a control recombinant adenovirus or a virus expressing antisense p21 mRNA, together with a construct expressing ΔB-Raf:ER. Cells were treated with 4-hydroxytamoxifen as in Methods, and the activity of MAPK was determined. Treatment of HepG2 cells expressing ΔB-Raf:ER with 4-hydroxytamoxifen increased MAPK activity ~4-fold after 6 h, which was also maintained for the following 36 h (Figure 1). MAPK activation was blocked by incubation with the specific inhibitor of MEK1/2, PD98059 (50 μM). Inhibition of basal MAPK signaling in HepG2 cells caused a small reduction in basal p21 protein levels (Figure 2D, compare lanes 1 and 5). Prolonged MAPK activation increased p21 protein expression in control virus infected cells, but not in cells expressing antisense p21 mRNA (Figures 2D and 2E). No increase in MAPK activity or in p21 protein expression was observed in 4-hydroxytamoxifen treated cells in the presence of PD98059, and no activation was observed in cells infected with vector control treated with 4-hydroxytamox-

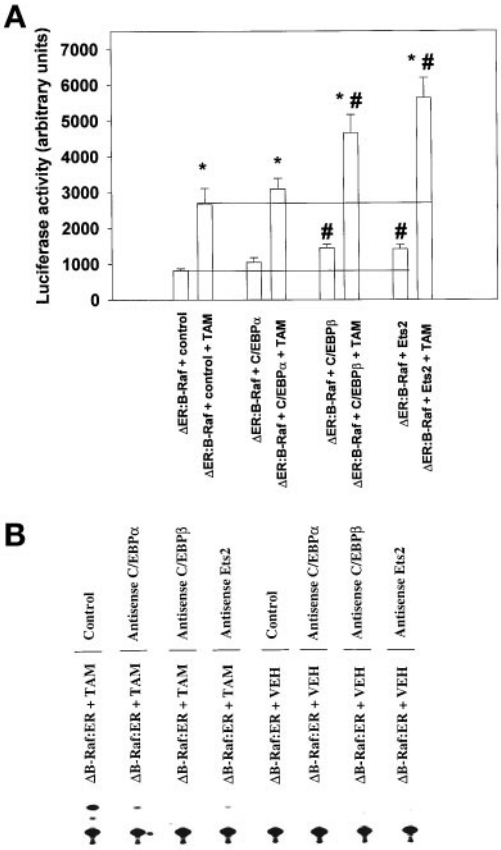


Figure 5. Overexpression of transcription factors enhances MAPK-dependent activation of the p21 promoter. (A) Overexpression of either C/EBPβ or Ets2 proteins enhances basal and MAPK-stimulated -2326 full-length p21-luciferase promoter activity. (B) Antisense C/EBPβ, and to a lesser extent antisense Ets2 and antisense C/EBPα, reduce MAPK-induced activation of the -1384/-1184 portion of the p21 promoter. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. In panel A, in addition to ΔB-Raf:ER infection, cells were also infected with a plasmid containing the full-length p21-promoter (100 m.o.i.). In panel A, portions of cells were also transfected with constructs to express either p42 C/EBPα, p35 C/EBPβ, or p53 Ets2; half the protein amount was used in panel A compared with data panels in Figures 4 and 5. In panel B, addition to ΔB-Raf:ER infection, cells were also infected with plasmid containing a small portion of the p21 promoter (-1383/-1184) in which exists a putative binding site (-1270/-1256) for C/EBP transcription factors; using poly-L-lysine adenovirus (at 100 m.o.i.). In panel B, cells were also infected with plasmids to express either anti sense C/EBPα or antisense C/EBPβ. In panels B and C, cells were transfected with an antisense oligonucleotide to Ets2 (10 μM, using Superfect reagent as per manufacturer's instructions; Qiagen, Valencia, CA). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen, and with or without 50 μM PD98059, for 480 min and luciferase activity or CAT activity determined as in Methods. For luciferase studies (n = 8 $\pm$ SEM), $^{*}p < 0.05$ greater than non-TAM treated control value; $^{\#}p < 0.05$ greater than corresponding control cell value. For CAT assays, representative experiments are shown (n = 10).

ifen (our unpublished results). Collectively, the data in Figures 1 and 2 demonstrate that prolonged MAPK signaling can increase p21 protein levels in primary hepatocytes and HepG2 hepatoma cells.

Prolonged MAPK Activity Promotes DNA Synthesis in the Absence of p21 Expression

Hepatocytes were isolated from wild-type and p21 null mice and infected with ΔB-Raf:ER. The ability of 4-hydroxytamoxifen treatment to alter ^{3}H-thymidine incorporation into DNA was determined. Prolonged MAPK activity reduced DNA synthesis in wild-type hepatocytes and increased DNA synthesis in p21 null hepatocytes (Table 1). These data demonstrate that the ability of prolonged MAPK signaling to inhibit DNA synthesis in primary hepatocytes requires p21 expression. Inhibition of MAPK activity caused a 50% reduction in basal DNA synthesis in HepG2 cells and prolonged MAPK activity reduced DNA synthesis in control virus-infected cells (Table 1). Expression of p21 antisense mRNA in HepG2 hepatoma cells blocked the ability of prolonged MAPK activation to increase p21 expression, but permitted this stimulus to increase DNA synthesis (Table 1). These data support the view that the molecular mechanism by which prolonged MAPK signaling inhibits DNA synthesis in primary cultures of hepatocytes and in hepatoma cells is via increasing protein levels of p21.

Regulation of the p21 Promoter by MAPK Signaling in Primary Hepatocytes and HepG2 Hepatoma Cells

Primary hepatocytes and HepG2 cells were coinfected with ΔB-Raf:ER and a series of constructs containing portions of the p21 promoter linked to the luciferase gene product (Figure 3A). Twenty-four hours after infection, cells were treated with 4-hydroxytamoxifen, followed by processing 8 h later to determine p21 promoter activity.

Activation of the MAPK pathway increased luciferase activity in primary hepatocytes (Figure 3B) and hepatoma cells (Figure 3E) from the -2326 bp full-length p21 promoter. Loss of the distal p53, Ets, and C/EBP binding sites significantly reduced, by ~70%, the ability of MAPK signaling to increase luciferase activity, in both primary hepatocytes (Figure 3C) and hepatoma cells (Figure 3F). However deletion of the proximal p53, Ets, and C/EBP binding sites, with the exception of the initiation-ATG proximal 883 bp, abolished MAPK-mediated stimulation of luciferase promoter activity in these cells (Figures 3D and 3G). This data suggests that the MAPK-responsive p21 promoter elements exist within the -2326 to -883 bp portion of the p21 promoter.

MAPK-mediated Regulation of the p21 Promoter by Transcription Factors in Primary Hepatocytes

To further investigate the role of transcription factors in the MAPK-dependent regulation of the p21 gene, we made use of sense and antisense constructs targeted toward C/EBPα, C/EBPβ, and Ets2 (Figure 4A). Antisense C/EBPβ, antisense C/EBPα, and antisense Ets2 reduced protein levels of their respective gene products by > 90%. Antisense C/EBPα also caused a nonspecific ~30% reduction in C/EBPβ protein

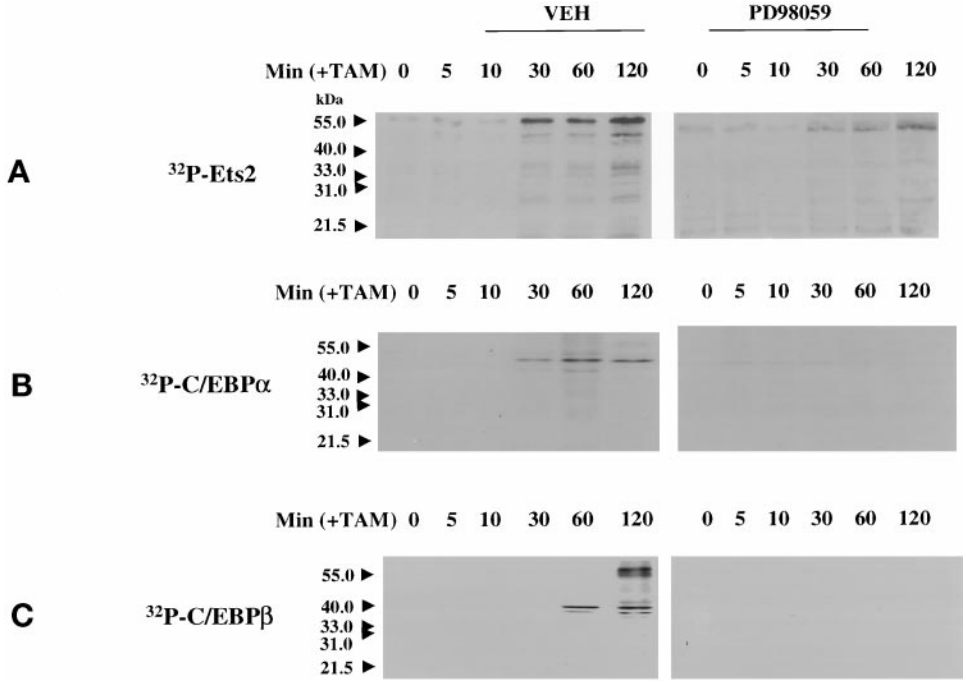


Figure 6. MAPK signaling increases the phosphorylation of Ets2, C/EBPα, and C/EBPβ in primary hepatocytes. (A) MAPK activation increases the phosphorylation of Ets2. (B) MAPK activation increases the phosphorylation of C/EBPα. (C) MAPK activation increases the phosphorylation of C/EBPβ. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen (TAM), and with or without 50 μM PD98059, for 0 to 120 min at 37°C. In experiments using ^{32}P-labeling of cells, phosphate free media was used and cells were preincubated with ^{32}P for 2 h before the start of 4-hydroxytamoxifen treatment. No significant alteration in the immunoblotting amounts of Ets2, C/EBPα, or C/EBPβ occurred during the 2-h time course of the experiment (our unpublished data). The MAPK-dependent incorporation of ^{32}P into either Ets2, C/EBPα, or C/EBPβ was determined following immunoprecipitation, SDS PAGE, and transfer to nitrocellulose. Incorporation of ^{32}P into Ets2 and C/EBP proteins was determined after immunoblotting by autoradiography.

levels. No alteration was observed in the protein levels of control blotted proteins, MEK1 and β-actin.

Loss of either C/EBPα, C/EBPβ, or Ets2 expression/function lowered both basal and reduced MAPK-induced luciferase activity in primary hepatocytes versus both the -2326 full-length and -1458 truncated p21 promoter constructs 8 h after 4-hydroxytamoxifen addition (Figures 4B, 4C, and 4D). Loss of either C/EBPβ or Ets2 function reduced MAPK-dependent p21 promoter activation from the -2326 p21-luc construct by ~90%. Loss of C/EBPα modestly reduced MAPK-dependent p21 promoter activation from the -2326 p21-luc construct by ~30%. Loss of either C/EBPα, C/EBPβ, or Ets2 function abolished MAPK-dependent p21 promoter activation from the -1458 p21-luc construct. In addition, we examined MAPK-dependent p21 promoter activation from the -2326 p21-luc construct over the full 36-h time course of our studies (Figure 4E). Similar to our previous observation at the 8-h time point, loss of either C/EBPβ or Ets2 reduced p21 promoter function by ~80% 36 h after MAPK activation. These results suggest that C/EBP and Ets-family transcription factors play a prominent role in the MAPK-dependent regulation p21 promoter function in hepatocytes, which is in agreement with Hill and Treisman, (1995), Hunter (1995), and Beier *et al.* (1999).

Since loss of C/EBP and Ets function altered p21 promoter activity and MAPK activation (Figure 4), we next determined whether overexpression of these transcription factors could also modify the ability of MAPK signaling to increase p21 promoter function. Overexpression of either C/EBPβ or Ets2 significantly enhanced both basal and MAPK-stimulated full-length -2326 p21 promoter activity 8 h after activation, whereas overexpression of C/EBPα caused a marginal nonsignificant stimulatory effect (Figure 5A).

As shown above, deletion of bases -2326 to -1458 reduced MAPK-induced p21 promoter activity by ~70%, and deletion of bases -1458 to -883 within the promoter abolished the residual MAPK-induced p21-promoter activity. This also correlated with the loss of one C/EBP binding site at position -1270/-1256 as well as an Ets binding site at position -1352/-1340. To determine whether MAPK signaling could regulate this portion of the p21 promoter, we infected cells with a construct containing this portion of the promoter (-1383/-1184) linked to the chloramphenicol acetyl transferase gene (CAT). Activation of the MAPK pathway in primary hepatocytes increased the transcriptional activity from this portion of the p21-CAT promoter within 8 h. Specific mutation of the C/EBP binding site within this portion of the promoter

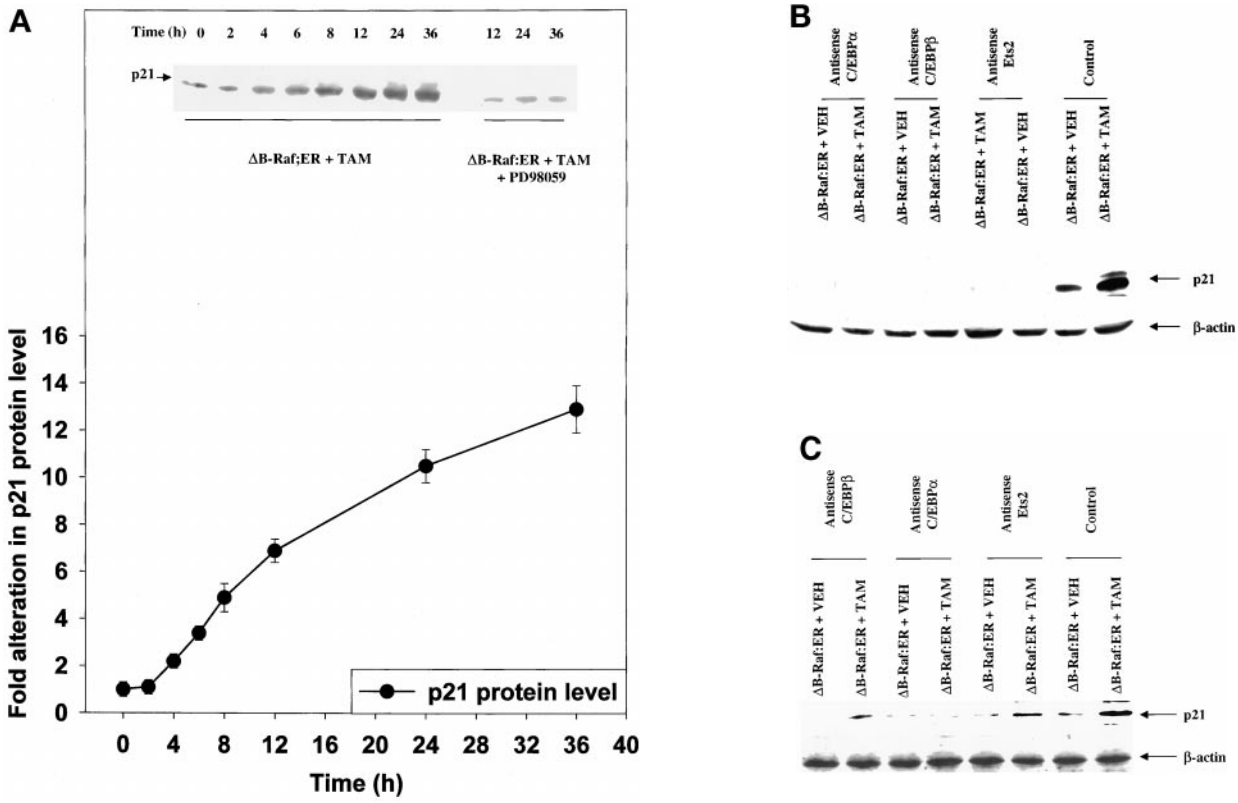


Figure 7. MAPK signaling increases p21 protein levels via multiple transcription factors. (A) Time course and quantitation of p21 protein induction by ΔB-Raf:ER. (B) Expression of antisense C/EBPα, antisense C/EBPβ, and antisense Ets2 modify both basal and ΔB-Raf:ER-mediated p21 protein induction 8 h after MAPK activation. (C) Expression of antisense C/EBPα, antisense C/EBPβ, and antisense Ets2 modify both basal and ΔB-Raf:ER-mediated p21 protein induction 36 h after MAPK activation. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. In some experiments and in addition to ΔB-Raf:ER infection, cells were also infected with either an additional null plasmid poly-L-lysine adenovirus or with either antisense C/EBPα or antisense C/EBPβ poly-L-lysine adenoviruses (at 200 m.o.i. each). In some experiments and in addition to ΔB-Raf:ER infection, cells were also transfected with antisense oligonucleotides toward Ets2. Transfection with a scrambled antisense Ets2 oligonucleotide did not alter basal or stimulated p21 protein levels (our unpublished observations). After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen for either 8 h or 36 h. Protein expression of p21 was determined by immunoblotting. Equal protein loading (Panels A and C: 200 μg. Panel B: 400 μg) per lane; exposures of x-ray film for the ECL immunoblots shown were from 30 s to 1 min. (A) Time course 0 to 36 h after either ΔB-Raf:ER + 4-hydroxytamoxifen or ΔB-Raf:ER + 4-hydroxytamoxifen + 50 μM PD98059. (B and C) Lanes with either ΔB-Raf:ER + vehicle control (VEH) or ΔB-Raf:ER + 4-hydroxytamoxifen (TAM). Equal protein amounts were loaded, and the internal control immunoblot was β-actin. A representative experiment for each cell type/condition is shown (panel B: n = 3. panel C: n = 6).

abolished this MAPK-dependent promoter activation (our unpublished observations).

We further investigated whether loss of C/EBP and Ets transcription factor functions altered the regulation of the C/EBP site within this portion of the promoter. Hepatocytes were infected with the -1383/-1184 p21-CAT promoter construct. MAPK signaling increased the activity of the -1383 to -1184 portion of the p21-promoter which was abolished following antisense ablation of C/EBPβ function and considerably reduced following antisense ablation of either Ets2 or C/EBPα, in general agreement with data of Cram *et al.* (1998) (Figure 5). A portion of the antisense C/EBPα effect may be due to this antisense construct causing a modest reduction in the expression of C/EBPβ. Since specific mutation of the C/EBP binding site within this portion of the promoter also abolished promoter activation by MAPK signaling, this data argues that the ability of Ets2 to modulate this portion of the p21 promoter is dependent upon C/EBP function. Collectively, the data in Figures 4 and 5 suggest that MAPK signaling, via transcription factors of the C/EBP and Ets families, can regulate p21 promoter activity at multiple sites.

MAPK-mediated Regulation of Transcription Factor Functions in Primary Hepatocytes

Since the data in Figures 4 and 5 implicated Ets2, C/EBPβ, and to a lesser extent C/EBPα in the MAPK-dependent regulation of the p21 promoter, we next investigated whether MAPK signaling altered the phosphorylation states of these proteins in hepatocytes. Activation of MAPK en-

hanced the phosphorylation of Ets2, C/EBPα, and of C/EBPβ within 60 min (Figures 6A, 6B, and 6C), without significantly altering their protein levels during this time course (our unpublished results). This increase in transcription factor phosphorylation correlated with the MAPK-dependent p21 promoter activation from the -2326 p21-luc construct, as observed in Figure 4E. No increases in phosphorylation were observed in either p18 C/EBPα or p21 C/EBPβ (our unpublished results); this was surprising since p21 C/EBPβ is know to contain some of the putative MAPK regulatory sites found in p38 and p35 C/EBPβ. Of particular note, MAPK also enhanced the phosphorylation of an unknown ~55 kDa protein which coimmunoprecipitated with C/EBPβ 120 min after MAPK activation (Figure 6C). Thus MAPK increases the phosphorylation of Ets2, C/EBPα, and C/EBPβ, which correlates with increased MAPK-dependent activation of the p21-promoter, and further argues that MAPK signaling may increase p21-promoter activity through the functions of multiple transcription factors.

Prolonged Activation of the MAPK Cascade Increases p21 Expression via Transcriptional and Posttranscriptional Mechanisms in Primary Hepatocytes

Because it has been suggested that MAPK signaling may regulate p21 protein levels via posttranscriptional mechanism(s), we further investigated how MAPK signaling increases p21 protein levels at both early (8 h) and later (36 h) times. Measurable elevation of p21 expression was first observed ~4 h after MAPK activation, which was increased and maintained over the following 8 to 36 h (Figure 7A). Antisense ablation of either C/EBPα, C/EBPβ, or Ets2 protein expression abolished both basal and stimulated p21 protein levels 8 h after MAPK activation (Figure 7B). Antisense ablation of either C/EBPβ protein or Ets2 protein also caused variable weak partial reductions in MAPK-induced p21 expression 36 h after MAPK activation (Figure 7C). Surprisingly, and in contrast to our data examining transcriptional control of the p21 promoter, loss of C/EBPα function largely abolished MAPK-induced p21 protein expression (Figures 7B and 7C). Expression of these transcription factors did not alter the ability of ΔB-Raf:ER to activate the MAPK pathway (our unpublished results) or alter levels of a control protein β-actin. Of particular note, the increase in p21 promoter activity which occurred within the first 2 h (Figure 4E) was not reflected in enhanced p21 protein levels until at least 4 h, which suggests that additional MAPK-dependent posttranscriptional mechanisms may play a role in controlling p21 protein expression. Collectively, our data suggest that a portion of the MAPK-induced p21 expression may be under transcriptional control, but that other posttranscriptional mechanisms may be recruited by MAPK signaling to increase p21 protein levels.

Because our findings in Figure 7 suggested that p21 protein levels were being regulated at a posttranscriptional level, we next attempted to determine potential additional downstream mechanism(s) by which MAPK signaling increases p21 protein levels. Inhibitors of transcription (actinomycin D and DRB) caused a large reduction in the ability of MAPK signaling to induce p21 protein expression after 8 h by $>$ 80% (Figure 8A, upper panel). Inhibitors of translation (cycloheximide), however, completely blocked enhancement of p21 protein expression by this stimulus. When this experiment was repeated 36 h after MAPK activation, little decrease was observed in the p21 protein levels when transcription was inhibited by either actinomycin D or DRB (Figure 8A, lower panel). MAPK-signaling enhanced the levels of p21 mRNA 0 to 8 h after MAPK activation (Figures 8B and 8C), and MAPK signaling maintained p21 mRNA levels at near control in the presence of actinomycin D. In contrast, in control cells which had their transcription inhibited, basal levels of p21 mRNA had significantly declined within 3 h by 48 $\pm$ 2.1%. In agreement with this data, 8 h after MAPK activation we determined that the half-life of *stimulated* p21 mRNA was ~3 h without MAPK activity and ~12 h with MAPK activity (Figure 8D), i.e., MAPK signaling increases p21 mRNA stability ~4-fold. During this time, no effect was observed on the levels of control β-actin mRNA (our unpublished observations). We performed an identical experiment to determine p21 mRNA stability 36 h after MAPK activation and determined that the half-life of stimulated p21 mRNA was ~3 h without MAPK activity and ~30 h with MAPK activity (Figure 8E). This data demonstrates that MAPK-induced p21 protein expression may be due to a combination of both an increased rate of transcription from the p21 promoter as well as MAPK signaling increasing p21 mRNA stability in primary hepatocytes.

To determine whether MAPK signaling also increased the stability of p21 protein, pulse-chase experiments were performed in which either hepatocytes or HepG2 cells were incubated with [^{35}S]methionine in the presence of active MAPK. After induction of p21 expression, cells were incubated in media containing nonradioactive methionine. Cells were also incubated with cycloheximide and with the either MEK1/2 inhibitor PD98059 or vehicle control. The rate at which ^{35}S-radioactivity was lost from immunoprecipitated p21 protein was determined (Figure 9). Within 8 h, activation of MAPK increased the half-life of radiolabeled p21 protein ~2.5-fold, from less than ~30 to 45 min to ~60 to 90 min in primary hepatocytes (Figure 9A). After 36 h, MAPK signaling caused a similar enhancement in the half-life of radiolabeled p21 protein at ~2.5-fold above basal (Figure 9B). Activation of MAPK increased the half-life of radiolabeled p21 protein from less than ~30 min to ~60 min in HepG2 cells (Figure 9C). Incubation of cells with PD98059 blunted the MAPK-dependent increase in protein stability. Treatment with cycloheximide before addition of [^{35}S]methionine abolished radio-label incorporation into p21 protein, but did not alter the observed increase in stability of radiolabeled p21 protein (our unpublished observations). Furthermore, cycloheximide treatment by itself did not increase p21 protein levels (our unpublished results). Thus MAPK signaling increases p21 protein stability in both primary hepatocytes and HepG2 cells.

Loss of C/EBPα and C/EBPβ Function Reduces the Ability of Prolonged MAPK Activation To Reduce DNA Synthesis and To Increase Protein Levels of p21 in Primary Hepatocytes

Because MAPK signaling appeared to regulate p21 expression through the transcription factors Ets2, C/EBPα, and C/EBPβ, and p21 expression modulates the growth re-

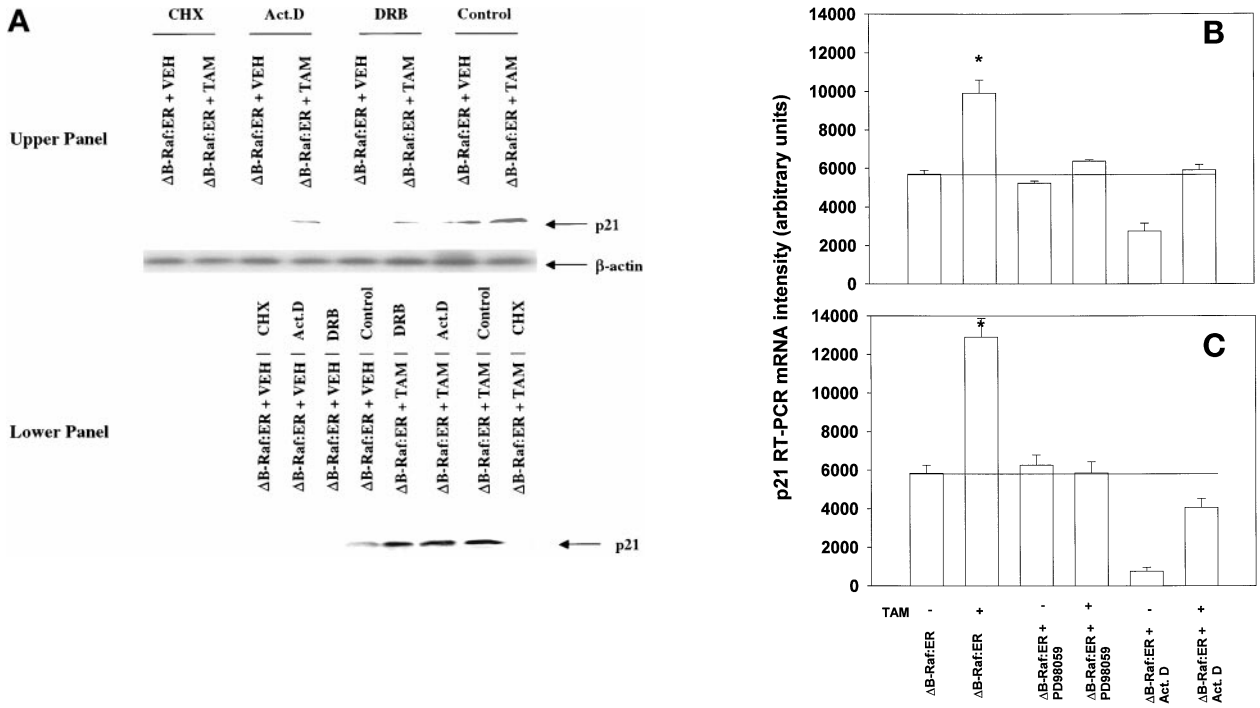


Figure 8. Prolonged MAPK signaling increases p21 protein expression via posttranscriptional mechanisms in primary hepatocytes. (A) MAPK-dependent increases in p21 protein are blunted by inhibitors of transcription and translation. (B and C) MAPK-induced an increase in p21 mRNA levels which is blocked by inhibitors of transcription. (D and E) MAPK enhances the mRNA stability of stimulated levels of p21 mRNA. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen for the indicated times. (A, upper section) Cells were treated for 8 h with 4-hydroxytamoxifen in the presence or absence of either 5 μM actinomycin D, 30 μM DRB, 20 μg/ml cycloheximide. (A, lower section) Cells were treated for 28 h with 4-hydroxytamoxifen and then for an additional 8 h with 4-hydroxytamoxifen in the presence or absence of either 5 μM Actinomycin D, 30 μM DRB, 20 μg/ml cycloheximide. The expression of p21 determined by immunoblotting. Lanes with either ΔB-Raf:ER + vehicle control (VEH) or ΔB-Raf:ER + 4-hydroxytamoxifen (TAM). A representative experiment for each condition is shown (n = 4). (B and C) Cells were treated for (B) 3 h and (C) 8 h with 4-hydroxytamoxifen in the presence or absence of 5 μM actinomycin D. RT-PCR determination of p21 mRNA was performed using specific oligonucleotides as in Methods (n = 3 ± SEM). RT-PCR was simultaneously performed on β-actin as an internal standard; p21 mRNA levels shown are normalized to the internal β-actin standard. During this time, no effect was observed on the levels of control β-actin mRNA (our unpublished data). (D and E) Cells were treated for (D) 8 h and (E) 36 h with 4-hydroxytamoxifen followed by treatment with either PD98059, Actinomycin D or both drugs and samples were taken at various times for a further 2 h, 4 h, and 6 h to determine p21 mRNA levels. RT-PCR determination of p21 mRNA was performed using specific oligonucleotides as in Methods (n = 3 ± SEM). RT-PCR was simultaneously performed on β-actin as an internal standard; p21 mRNA levels shown are normalized to the internal β-actin standard. During this time, no effect was observed on the levels of control β-actin mRNA (our unpublished data) *$p < 0.05$ greater than non-TAM treated control value.

sponse of hepatocytes, we investigated whether functional loss of either Ets2, C/EBPα, or C/EBPβ altered the proliferative response of wild-type and p21 null hepatocytes after prolonged MAPK activation.

Loss of Ets2 expression/function reduced basal and MAPK-stimulated DNA synthesis by $>90\%$ in wild-type and p21 null hepatocytes (Figure 10A). This data suggests that Ets2 function plays a key role in the MAPK-dependent regulation of DNA synthesis in hepatocytes. Loss of C/EBPα expression caused a small increase in basal DNA synthesis and largely abolished the ability of prolonged MAPK signaling to lower DNA synthesis in wild-type hepatocytes (Figure 10B). However in p21 null cells, ablation of C/EBPα expression did not significantly alter basal DNA synthesis and surprisingly reduced MAPK-stimulated DNA synthesis. This data argues for both MAPK-, C/EBPα-, and p21-dependent inhibition of DNA synthesis in wild-type cells as well as a MAPK- and C/EBPα-dependent stimulation of DNA synthesis in cells which cannot express p21.

Loss of C/EBPβ expression significantly reduced basal DNA synthesis in both wild-type and p21 null hepatocytes (Figure 10C). However, reduced C/EBPβ expression did not alter the ability of MAPK signaling to lower DNA synthesis in wild-type hepatocytes (compare Figures 10B and 10C). Reduced C/EBPβ expression abolished MAPK-stimulated DNA synthesis in p21 null cells, in contrast to the partial reduction in stimulation found with loss of C/EBPα expression. This data argues that C/EBPβ plays a small role in the MAPK- and p21-dependent inhibition of DNA synthesis in wild-type cells. The data also demonstrate that the MAPK-dependent stimulation of DNA synthesis in p21 null hepatocytes requires C/EBPβ.

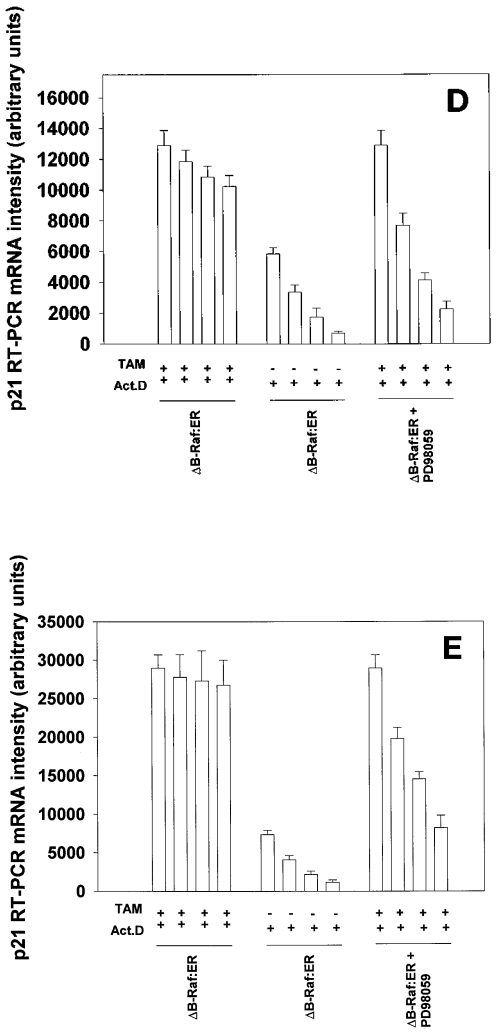


Figure 8 (legend on facing page).

DISCUSSION

Expression of the cyclin-dependent kinase inhibitor is purported to play an important role in the control of the cell cycle in many cell types, including hepatocytes in vivo and in vitro (Tombes *et al.*, 1998; Auer *et al.*, 1998b; Serfas *et al.*, 1997; Albrecht *et al.*, 1997; Wu *et al.*, 1996; Timchenko *et al.*, 1997). We recently suggested that prolonged activation of the MAPK pathway increases expression of p21 in primary hepatocytes and hepatoma cells, leading to a reduction in DNA synthesis (Tombes *et al.*, 1998; Auer *et al.*, 1998b; Auer *et al.*, 1998c). The central question posed at the initiation of these studies was to determine the mechanism by which MAPK signaling increases expression of p21.

We demonstrated that prolonged activation of the MAPK pathway inhibited reduced DNA synthesis in both wild-type hepatocytes and HepG2 hepatoma cells. However, in p21 null hepatocytes or HepG2 cells expressing antisense p21 mRNA, prolonged activation of the MAPK pathway stimulated DNA synthesis. These data indicate that in response to prolonged MAPK activation, increased expression of p21 plays a key role in blunting hepatocyte/hepatoma DNA synthesis. That p21 null primary hepatocytes did not growth arrest in response to prolonged MAPK signaling was unexpected, since they also possess a functional retinoblastoma (Rb) protein and can express p16 ^{INK4a} (Tombes *et al.*, 1998; Auer *et al.*, 1998b; Auer *et al.*, 1998c; Park *et al.*, 2000). Increased expression of p16 ^{INK4a} would be expected to inhibit cdk4 activity, blocking both Rb phosphorylation and its inactivation. Our data suggest that p21 plays a more prominent role in regulating RB function and cell cycle progression in hepatocytes (Park *et al.*, 2000).

The molecular mechanisms by which MAPK signaling increases p21 protein levels are under investigation by many laboratories. We found that MAPK signaling rapidly increased p21-promoter activity from a full-length promoter. Promoter deletion analysis revealed that the MAPK sensitive elements are within the -2.346 kb to -0.883 kb portion of the promoter. Very similar data were observed in both primary hepatocytes and hepatoma cells, suggesting that MAPK-dependent regulatory mechanisms exist in both normal and transformed hepatocytes. However, it should be noted that while basal promoter activity per μg cell protein in both cell types was similar, basal MAPK activity per μg cell protein was 8-fold greater in HepG2 cells as compared with primary hepatocytes. This finding suggests that the relative ability of MAPK signaling to enhance p21-promoter activity is diminished in HepG2 hepatoma cells compared with primary hepatocytes.

Based on our promoter deletion analysis data, we next examined whether transcription factors of the C/EBP and Ets families play a role in the MAPK-dependent regulation of the p21 promoter. MAPK signaling increased the phosphorylation of Ets2 and C/EBPβ, as well as their DNA binding abilities in EMSA assays (our unpublished results), in agreement with Auer *et al.*, (1998b), Hill and Treisman (1995), and Hanlon and Sealy (1999). Phosphorylation of Ets2 and C/EBPβ correlated with the increase in p21 promoter function, and loss of either Ets2 or C/EBPβ function significantly reduced both basal and MAPK-dependent stimulation of p21 promoter activity. Loss of either C/EBPβ function or Ets2 function also abolished MAPK-stimulated p21 protein expression 8 h after activation and caused variable, partial, reductions in MAPK-stimulated p21 protein expression 36 h after activation. This data argues that from 0 to 8h following MAPK activation, increased transcription from the p21 promoter plays an essential role in enhancing p21 protein levels. However our data also suggest that Ets2 and C/EBPβ are not essential mediators of MAPK signaling toward increasing p21 protein levels 36 h after MAPK activation in hepatocytes.

In contrast to our observations with Ets2 and C/EBPβ, we found that loss of C/EBPα function partially reduced the ability of prolonged MAPK signaling to increase p21 promoter activity but caused a significant $>$ 90% reduction in the MAPK-dependent enhancement in p21 protein expression. This suggested that C/EBPα function plays a minor role in controlling the function of the p21 promoter in response to MAPK signaling, but plays a major role in regulating p21 protein levels, potentially at a posttranscriptional level.

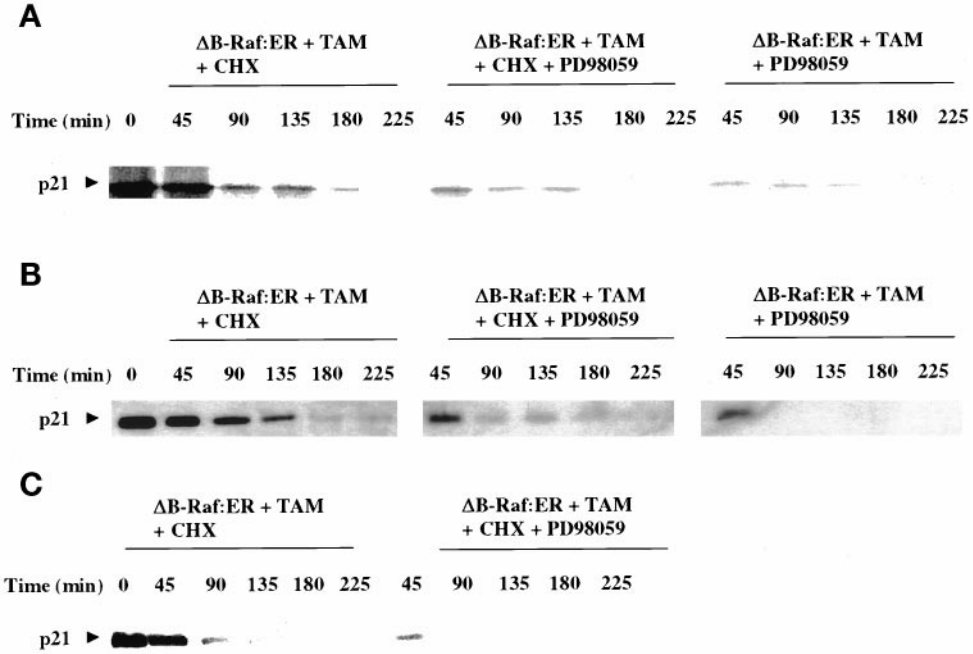
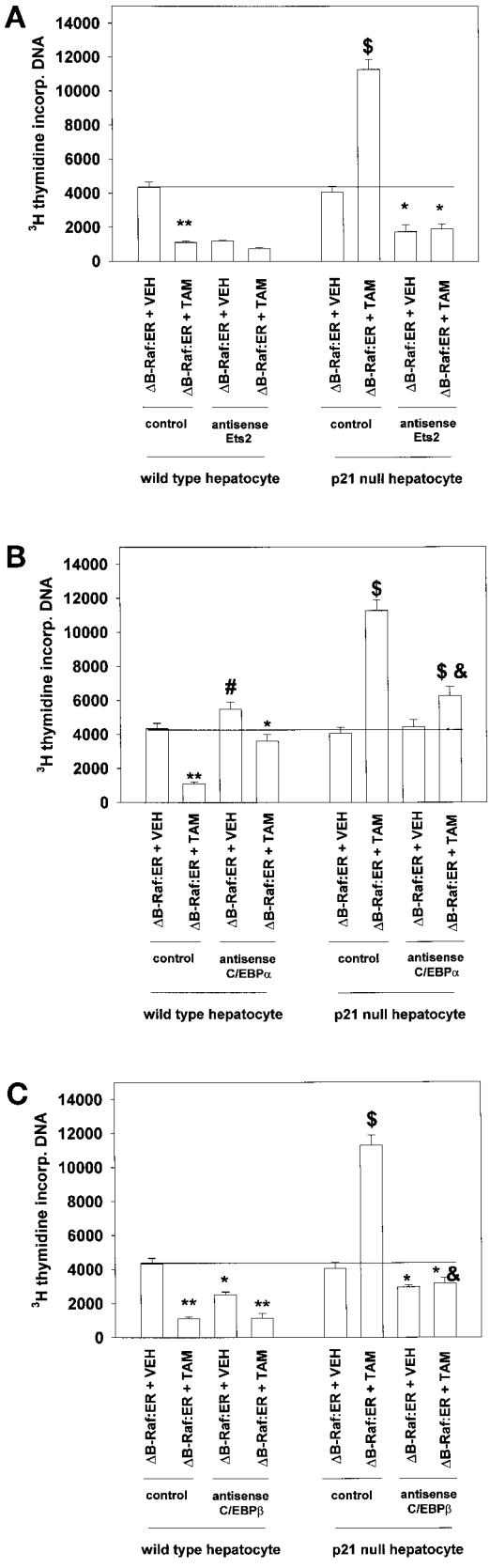


Figure 9. MAPK activation increases the protein stability of p21 in primary hepatocytes and HepG2 cells. Primary hepatocytes (panels A and B) and HepG2 cells (panel C) were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture as in Methods. (A and C) After 24 h to allow ΔB-Raf:ER protein expression, cells were incubated in methionine/cysteine-free culture media containing 0.1 μCi/10 μl [^{35}S]methionine. Cells were treated for 480 min with 4-hydroxytamoxifen. After 480 min, [^{35}S]methionine-containing media was replaced with media containing methionine/cysteine, still containing 4-hydroxytamoxifen, and where indicated including MAPK (50 μM PD98059)/translation inhibitors (20 μg/ml cycloheximide). (B) After 24 h to allow ΔB-Raf:ER protein expression, cells were treated for 32 h with 4-hydroxytamoxifen, and then incubated in methionine/cysteine-free culture media for a further 4 h containing 0.1 μCi/10 μl [^{35}S]methionine and 4-hydroxytamoxifen. After 36 h (total time), [^{35}S]methionine-containing media was replaced with media containing methionine/cysteine, still containing 4-hydroxytamoxifen, and where indicated including translation inhibitor. The incorporation of [^{35}S]methionine into p21 protein was determined following p21 immunoprecipitation over a time course following removal of [^{35}S]methionine. Equal protein loading (200 μg) per lane; p21 protein was detected by autoradiography of radiolabeled p21-bands and immunoblotting. Exposures of x-ray film for immunoblots shown were 30 s to 1 min. A representative experiment for each condition is shown (n = 3).

Comparing data for transcriptional regulation of the p21 promoter and data showing increased p21 protein levels (e.g., Figures 4E and 7A), it seemed probable that p21 protein levels were under MAPK-dependent control at a post-transcriptional level. Inhibition of either transcription or protein synthesis significantly reduced MAPK-stimulated p21 protein levels 8 h after MAPK activation. However, loss of transcriptional function at 36 h did not significantly reduce p21 protein levels, suggesting that MAPK-mediated transcription at this time may not be required for the maintenance of stimulated p21 protein levels. One reason for this difference between these time points may be that MAPK signaling enhanced p21 mRNA levels and p21 mRNA stability in primary hepatocytes. MAPK signaling enhanced p21 mRNA stability ~4-fold and ~10-fold, 8 h and 36 h, respectively, after MAPK activation. This data demonstrates that MAPK signaling causes a large enhancement in p21 mRNA stability in primary hepatocytes, in general agreement with data of Akashi *et al.* (1999) using a protein kinase C activator (phorbol ester). More recently, it was demonstrated that UV irradiation could stabilize p21 mRNA via the mRNA binding protein HuR and inhibiting basal MAPK activity did not alter HuR binding to p21 mRNA (Wang *et al.*, 2000; Taher *et al.*, 1999). Nevertheless, we have found that MAPK signaling can stimulate snRNP localization in the cytoplasm (our unpublished observations).

In addition to our data examining p21 mRNA stability, we also demonstrated that MAPK signaling impacted upon p21 protein stability, increasing the half-life of p21 protein ~2.5-fold in hepatocytes and HepG2 cells. In contrast to our findings for p21 mRNA stability, no increase in relative degree of p21 protein stabilization was observed between the 8 and 36 h time points. Collectively, our data demonstrate that there is a MAPK-dependent increase in p21 protein stability, mRNA stability and transcriptional control at both 8 h and 36 h. At 8 h, MAPK had increased p21 promoter function 6-fold, p21 mRNA stability 4-fold, and p21 protein stability 2.5-fold. At 36 h, MAPK signaling had increased p21 promoter function 9-fold, p21 mRNA stability 10-fold, and p21 protein stability 2.5-fold (Table 2). Overall, our data suggest that increased transcription and mRNA stability play the most important roles in the MAPK-dependent increase in p21 protein levels; increased transcription and mRNA stability are important shortly following MAPK activation at 8 h, whereas increased mRNA stability may be the most important mechanism at 36 h. Other studies outside the scope of this manuscript will be required to deter-



mine the mechanism(s) by which MAPK signaling enhances p21 mRNA and p21 protein stabilities.

Some studies provide evidence suggesting that increases in p21 protein levels in hepatocytes can stem from enhanced protein stability (Timchenko *et al.*, 1997; Timchenko *et al.*, 1996; Hendricks-Taylor and Darlington 1995; Park *et al.*, 2000). In these studies, it was suggested that the direct interaction of p21 protein with C/EBPα plays an important role in maintaining this stability (Timchenko *et al.*, 1997; Timchenko *et al.*, 1996). Other investigators have shown that p21 expression and nuclear C/EBPα protein expression co-localize in hepatocytes in vivo (Serfas *et al.*, 1997; Timchenko *et al.*, 1997; Timchenko *et al.*, 1996). In agreement with this concept, we found that loss of C/EBPα function reduced MAPK-induced p21 protein expression, but had a lesser effect at modulating promoter activity. Thus, data from several laboratories suggest that C/EBPα may play a role in mediating MAPK signaling toward p21 protein stability in hepatocytes. Ongoing studies are currently defining the precise mechanism by which MAPK signaling, potentially via C/EBPα, may increase p21 protein stability.

Transcription factors of the Ets and C/EBP families have been implicated in both proliferative and growth arrest/differentiation responses (Taub *et al.*, 1999; Muller *et al.*, 1999; Hanlon and Sealy 1999; Timchenko *et al.*, 1999; Diehl 1998; Beier *et al.*, 1999). We found that modulation of Ets2, C/EBPα, or C/EBPβ expression had a profound effect upon MAPK-mediated regulation of DNA synthesis in hepatocytes. Ets2 function was essential for both basal- and MAPK-stimulated DNA synthesis (Le Gallic *et al.*, 1999). Loss of C/EBPα expression enhanced basal DNA synthesis in wild-

Figure 10. Loss of C/EBPα function blocks the ability of prolonged MAPK activation to reduce DNA synthesis and to increase protein levels of p21 in wild-type hepatocytes. Loss of C/EBPβ function abolishes the ability of prolonged MAPK activation to increase DNA synthesis in p21 null hepatocytes. (A) Antisense Ets2 modulation abolishes MAPK-induced proliferation in hepatocytes. (B) Antisense C/EBPα modulation alters the ability of MAPK signaling to cause cell cycle arrest and proliferation in wild-type and p21 null cells, respectively. (C) Antisense C/EBPβ modulation alters the ability of MAPK signaling to cause cell cycle arrest and proliferation in wild-type and p21 null cells, respectively. Hepatocytes were infected with kinase active ΔB-Raf:ER poly-L-lysine adenovirus (250 m.o.i.), followed by culture, as in Methods. In addition to ΔB-Raf:ER infection, cells were also infected with plasmids to express either antisense C/EBPα or antisense C/EBPβ. (A) Cells were transfected with an antisense oligonucleotide to Ets2. After 24 h to allow protein expression, hepatocytes were treated with either vehicle control or with 100 nM 4-hydroxytamoxifen (TAM) for 36 h (total time in primary culture, 60 h). In some experiments, before 4-hydroxytamoxifen addition, cells were treated with 50 μM PD98059. Hepatocytes under all conditions were continually incubated with 2 μCi ^{3}H-thymidine throughout the final 36 h of culture, after which they were lysed and ^{3}H-thymidine incorporation into DNA determined as in Methods. Addition of 4-hydroxytamoxifen alone did not alter the rate of DNA synthesis (our unpublished results). Data are the means (cpm ^{3}H-thymidine incorporated into DNA ± SEM) of sextuplicate experiments from 4 separate animals. $^{**}p < 0.001$ less than corresponding non-TAM treated control value; $^{*}p < 0.05$ less than corresponding non-TAM treated control value. $^{\#}p < 0.05$ greater than corresponding value without antisense C/EBPα. $^{\$}p < 0.05$ greater than corresponding control value. $^{\&}p < 0.05$ less than corresponding control infected ΔB-Raf:ER + TAM value.

Table 2. Fold alterations in p21 promoter activation, p21 mRNA stability, p21 protein stability, and p21 protein levels following MAPK activation

Variable	-Fold alteration, 8 h after MAPK activation	-Fold alteration, 36 h after MAPK activation
MAPK activation	10-fold	9-fold
p21 full-length −2326 promoter activation	6-fold	9-fold
p21 mRNA stabilization	4-fold	10-fold
p21 protein stabilization	2.5-fold	2.5-fold
p21 protein expression	5-fold	13-fold

The -fold increases in p21 promoter activation, p21 mRNA stability, p21 protein stability, and p21 protein levels were derived from data presented in Figures 1–9.

type hepatocytes but not in p21 null cells, arguing that in cells which express p21, C/EBPα plays an important role in blunting DNA synthesis via p21. Loss of C/EBPα expression also reduced the ability of MAPK signaling to inhibit DNA synthesis in wild-type cells, in agreement with data showing C/EBPα function is required for MAPK-stimulated p21 expression. Surprisingly, loss of C/EBPα expression reduced the MAPK-dependent increase in DNA synthesis in p21 null cells. Collectively, these data suggest that in wild-type hepatocytes C/EBPα function is biased toward a MAPK- and p21-dependent inhibition of DNA synthesis, whereas in p21 null cells C/EBPα function is biased toward a MAPK- and C/EBPα-dependent stimulation of DNA synthesis.

In contrast to C/EBPα, loss of C/EBPβ expression reduced basal DNA synthesis in both wild-type and p21 null cells, implying that C/EBPβ is an essential transcription factor for normal hepatocyte proliferation. Loss of C/EBPβ function did not prevent prolonged MAPK signaling inhibition of DNA synthesis in wild-type cells, an inhibition which was identical to that observed in control cells with normal C/EBPβ function. This data is in agreement with our finding that loss of C/EBPβ function has a marginal effect on MAPK-induced p21 protein levels. Loss of C/EBPβ function abolished the ability of MAPK signaling to increase DNA synthesis in p21 null cells. This data suggests that C/EBPβ is an essential mediator of the MAPK-dependent stimulation of DNA synthesis in p21 null cells. Further studies will be required to determine the precise mechanism(s) by which MAPK signaling regulates p21-promoter/transcription factor function and hepatocyte cell growth in vitro and in vivo.

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