pp60<sup>v-src</sup> Induction of Cyclin D1 Requires Collaborative Interactions between the Extracellular Signal-regulated Kinase, p38, and Jun Kinase Pathways

A ROLE FOR cAMP RESPONSE ELEMENT-BINDING PROTEIN AND ACTIVATING TRANSCRIPTION FACTOR-2 IN pp60<sup>v-src</sup> SIGNALING IN BREAST CANCER CELLS

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The cyclin D1 gene is overexpressed in breast tumors and encodes a regulatory subunit of cyclin-dependent kinases that phosphorylate the retinoblastoma protein. pp60<sup>v-src</sup> activity is frequently increased in breast tumors; however, the mechanisms governing pp60<sup>v-src</sup> regulation of the cell cycle in breast epithelium are poorly understood. In these studies, pp60<sup>v-src</sup> induced cyclin D1 protein levels and promoter activity (48-fold) in MCF7 cells. Cyclin D1-associated kinase activity and protein levels were increased in mammary tumors from murine mammary tumor virus-pp60<sup>v-src</sup>527F transgenic mice. Optimal induction of cyclin D1 by pp60<sup>v-src</sup> involved the extracellular signal-regulated kinase, p38, and c-Jun N-terminal kinase members of the mitogen-activated protein kinase family. Cyclin D1 promoter activation by pp60<sup>v-src</sup> involved a cAMP response element-binding protein (CREB)/activating transcription factor 2 (ATF-2) binding site. Dominant negative mutants of CREB and ATF-2 but not c-Jun inhibited pp60<sup>v-src</sup> induction of cyclin D1. pp60<sup>v-src</sup> induction of CREB was blocked by the p38 inhibitor SB203580 or by mutation of CREB at Ser<sup>133</sup>. pp60<sup>v-src</sup> induction of ATF-2 was abolished by the c-Jun N-terminal kinase inhibitor JNK-interacting protein-1 or by mutation of ATF-2 at Thr<sup>69</sup> and Thr<sup>71</sup>. CREB and ATF-2, which bind to a common pp60<sup>v-src</sup> response element, are transcriptionally activated by distinct mitogen-activated protein kinases. Induction of cyclin D1 activity by pp60<sup>v-src</sup> may contribute to breast tumorigenesis through phosphorylation and inactivation of the retinoblastoma protein.

The multistep process of tumorigenesis involves the accumulation of genetic defects contributing to the tumor phenotype. In addition to a critical role in the orchestration of orderly cell cycle progression, components of the G<sub>1</sub> phase regulatory apparatus play an important role in tumorigenesis. The protein kinase complexes regulating the G<sub>1</sub> phase include a catalytic subunit, the cyclin-dependent kinase (Cdk),<sup>1</sup> its regulatory activating partner, the cyclin, a Cdk-activating kinase, and cyclin-dependent kinase inhibitors (1). The cyclin D1 gene encodes a regulatory subunit of the Cdk holoenzymes that phosphorylate and thereby inactivate the retinoblastoma tumor suppressor, pRB (2, 3). Immune neutralization and antisense experiments have demonstrated that in the breast cancer cell lines MCF7 and T-47D, the abundance of cyclin D1 is rate-limiting in mitogen-induced G<sub>1</sub> phase progression (4–6).

Cyclin D1 collaborates with oncogenes in cellular transformation and transgenic overexpression of cyclin D1 in the mouse mammary gland induced adenocarcinomas (7, 8). Cyclin D1 protein levels are frequently increased in breast tumor cell lines (9, 10) and human breast cancers (5, 10, 11). Several different factors may contribute to the increased cyclin D1 abundance found in breast tumors. Cyclin D1 abundance is induced transcriptionally, whereas the protein is degraded rapidly upon the withdrawal of growth factors via the ubiquitin-proteasome pathway (12). The human breast cancer cell line MCF7 has been used to examine the regulation of cyclin D1, demonstrating the induction of cyclin D1 protein levels in these cells by estrogens, serum, and epidermal growth factor (6, 13, 14). Cyclin D1 promoter activity and mRNA levels are also

<sup>1</sup> The abbreviations used are: Cdk, cyclin-dependent kinase; pRB, retinoblastoma protein; MAPK, mitogen-activated protein kinase; CREB, cAMP response element-binding protein; MMTV, murine mammary tumor virus; ATF, activating transcription factor; JNK, c-Jun N-terminal kinase; AP, activator protein; JIP, JNK-interacting protein; MEF, mouse embryo fibroblast; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; M KK, MAPK kinase; EMSA, electrophoretic mobility gel shift assay.

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induced by growth factors that are mitogenic to breast cancer cells including epidermal growth factor and insulin-like growth factor-1 (15–17). Activating mutants of pp60src, Rac, and the dbll family of oncogenes induce cyclin D1 promoter activity and protein abundance (16, 18–20). Recent studies suggest that cyclin D1 plays an important role in Ras-induced NIH3T3 cell transformation (21) and in Ras-induced skin tumor formation in vivo (22).

In contrast, relatively little is known of the molecular mechanisms by which activating Src mutations directly affect components of the cell cycle regulatory apparatus (23). The pp60src proto-oncogene encodes a 60-kDa cytoplasmic nonreceptor tyrosine kinase (24) that is sufficient to both initiate and maintain cellular transformation (25). In previous studies, performed in fibroblast cell lines, overexpression of pp60src enhances the rate of G1 phase progression in association with an induction of cyclin D1 protein levels in NIH3T3 cells, implicating cyclin D1 in pp60src action (21). In Rat-1 cells, however, pp60src overexpression did not induce cyclin D1 expression, and a reduction in p27Kip1 levels was observed, suggesting that p27Kip1 transcriptional repression may play a role in pp60src signaling (26). Activation of the pp60src tyrosine kinase has been observed in a large proportion of human breast malignancies (27). Overexpression of a constitutively active mutant of pp60src under control of the murine mammary tumor virus (MMTV) long terminal repeat in transgenic mice induced mammary gland tumor formation (28). The cell cycle regulatory targets of pp60src in mammary epithelial cells are virtually unknown, and the intracellular kinase pathways by which pp60src regulates cell cycle regulatory pathways also remain to be determined.

In fibroblasts and fibroblast-derived cell lines, pp60src induces several downstream signaling pathways including members of the mitogen-activated protein kinase (MAPK) family, the p42 and p44 extracellular signal-regulated kinases (ERKs) (29, 30) and c-Jun N-terminal kinase (JNK) (31). In addition, the phosphatidylinositol 3′-kinase (32) and the signal transducers and activators of transcription (33) play a role in signaling induced by pp60src. The role of MAPKs in pp60src signaling requires reinvestigation as some ten MAPK family members have now been identified in mammalian cells, increasing the potential complexity and allowing for specificity in signaling (34–36). Two of these MAPKs, (MAPK1/ERK1 and MAPK2/ERK2) are strongly activated by polypeptides and growth factors but are poorly induced by stress stimuli. In contrast, the other MAPK family members are strongly activated by stress signals and are frequently referred to as stress-activated protein kinases (SAPKs). Chemical inhibitors of specific MAPKs have aided in identification of downstream signaling pathways. The drug PD98059 suppresses the activation of the MAPK/ERK pathway by preventing activation of the upstream activator MAPK kinase 1 (MKK1 or MEK1). Recent studies have identified distinct members of the SAPK family, with MAP kinase kinase 4 (MKK4, also known as SEK1) and MAP kinase kinase 7 (MKK7) activating JNK, whereas MAP kinase kinase 3 (MKK3) and MAP kinase kinase 6 (MKK6) activate members of the p38 MAPK group (34, 36). The drug SB203580, a specific inhibitor of p38α and p38β MAPK, prevents the activation of MAP kinase-activated protein-K2 and MAP kinase-activated protein-K3 (37).

The induction of gene expression by pp60src involves several different transcription factors and DNA regulatory sequences, including the activator protein-1 (AP-1) site (38–40), the serum response element (41), the TATA box (42), a CRE/activating transcription factor (ATF) site (43) and a unique pp60src-response element (44). A CRE/ATF site that binds members of the ATF/cAMP response element-binding protein (CREB) family is required for pp60src-dependent induction of the prostaglandin synthase (peg2) gene (43). As the ERKs, JNK, and p38 are capable of phosphorylating ATF-2, ATF-2 may play a role in pp60src signaling (45–48). The CREB protein is also a distal target of the MAPK/ERK pathway (49), the p38 pathway (50), or both ERK and p38 pathways (51). These studies suggest that cell type-specific factors mediate the MAPK pathways activating CREB transactivation and raise the possibility that CREB may be a target of pp60src signaling.

Because relatively little is known of the molecular mechanisms by which pp60src engages components of the cell cycle regulatory apparatus in breast cells, we have examined the regulation of cyclin D1 by pp60src in MCF7 cells and determined the expression and activity of cyclin D1 in mammary tumors induced by mammary-targeted overexpression of an activating pp60src mutant in transgenic mice. In these studies, optimal pp60src induction of cyclin D1 required collaboration between the MAPK/ERK, p38, and JNK pathways. We provide evidence that CREB and ATF-2 bind a common DNA element and serve as distal transcriptional targets of pp60src induction of the cyclin D1 gene in MCF7 cells. The induction of CREB by pp60src required a p38 pathway, and induction of ATF-2 by pp60src required the JNK pathway. pp60src activation of distinct transcription factors that bind common DNA sequences of the cyclin D1 promoter occurs through specific MAPK modules.

MATERIALS AND METHODS

**Western Blots and Immune Complex Kinase Assays—**Western blotting analysis was performed as described previously (16, 52). To determine the abundance of cyclin D1 protein the monoclonal cyclin D1 antibody HD-11 (Santa Cruz Biotechnology, Santa Cruz, CA) or DCS-6 (Neomarkers, Fremont, CA) was used; for α-tubulin, the antibody (5H1) (16, 52) was used; and for the Flag epitope, the M2 monoclonal antibody (Kodak Scientific Imaging Systems) was used. Cell homogenates were electrophoresed in an SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (MSI, Westborough, MA). Antibody to α-tubulin; the gel was stained with Comassie Blue to assess transfer efficiency. The blotting membrane was incubated for 2 h at 25 °C in T-phosphate-buffered saline buffer supplemented with 5% (v/v) dry milk, and after a 6 h incubation with primary antibody at a 1:1000 dilution (cyclin D1) or 1:2500 (α-tubulin) in Tween 20 phosphate-buffered saline buffer containing 0.5% (v/v) Tween 20, the membrane was washed with the same buffer. For detection of cyclin D1 and α-tubulin, the membrane was incubated with horseradish peroxidase second antibody (Santa Cruz Biotechnology) and washed again. The enhanced chemiluminescence system (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used to visualize the cyclin D1 protein.

Cyclin D1-immunoprecipitation kinase assays were performed essentially as described previously (52) using saturating amounts of the cyclin D1 antibody, DCS-11 (NeoMarkers, Fremont, CA). The pRB substrate was prepared by transforming *Escherichia coli* with the vector pGEX-Rb (52) (a gift from Dr. E. Harlow). The pG4EKR, pG4ENK, and SAPK/JNK immune complex assays were performed as described previously (52) on cell extracts derived from mammary gland tumor tissue. Staphylococcal protein A-agarose beads (Boehringer Mannheim) were incubated with anti-ERK antibody (C14) (Santa Cruz Biotechnology) or polyclonal SAPK antibody (a gift from Dr. J. Kyriakis) for 1 h at 4 °C. The samples were analyzed by SDS-polyacrylamide gel electrophoresis upon termination of the reaction by boiling in SDS sample buffer. The phosphorylation of myelin basic protein or glutathione S-transferase-c-Jun substrates was quantified by densitometry after exposure to autoradiographic film (LabScientific Inc., Livingston, NJ) using ImageQuant, version 1.11 (Molecular Dynamics Computing Densitometer, Sunnyvale, CA).

**Immunohistochemistry—**Tissues were processed and analyzed as described previously (17) using a monoclonal cyclin D1 antibody DCS-6 (Vector Laboratories, Burlingame, CA) (53). Immunohistochemical analyses were carried out using a biotinylated secondary antibody and an avidin/biotin-linked horseradish peroxidase (the Vectastain ABC

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system) (Vector Laboratories). The complex was stained with diamino-
benzidine tetrahydrochloride (DAB) substrate obtained from Kir-
kegaard & Perry Laboratories Inc. (Gaithersburg, MD). Nuclear staining for cyclin D1 was determined by counting 500 cells within each tumor sample. Tumor samples from four separate animals were examined.

Expression Vectors—A series of 5′ promoter constructions in which the human cyclin D1 promoter was subcloned into the vector pA_LUC was described previously (16, 22). The constructions of CD1LUC and CD1ATFmutLUC were created by polymerase chain reaction from the −141 CD1LUC plasmid using specific primers and the region including the CRE/ATF site was mutated from 5′-T AAT GTC ACA CGG ACT-3′ to 5′-GAT CCA CAG CCA CGG ACT-3′. The sequences resembling a CRE/ATF site located between −66 and −40 were cloned into the reporter TK81p_LUC to form the reporter pCD1ATF/TKLUC. The reporter pTLPR contains a trimeric collagenase AP-1 site, c-fosLUC contains the c-fos promoter sequences from −361 to +157, and the serum response element TATA-LUC reporter encodes the c-fos serum response element linked to the luciferase reporter gene (52). The expression vectors encoding pp60 
src (pMr-src), an activating mutant of pp60 
src (pSRC527F) and pp60 
src (pMv-src) (28, 55), the dominant negative mutants of MKK3 (pBSV-Flag-MKK3 Ala) and MKK6 (pCDNA3-Flag-MKK6 K28A) (48), the vector glutathione S-transferase c-Jun, and the dominant negative MEK1 (Ala-218/Ala222) were previously described (56). The CREB dominant negative expression vector pCREB (57) was a gift from Dr. R. Goodman. The ATF-2 dominant negative mutant ATF-2 M2 (58) was a gift from Dr. L. Glimcher. The ATF-2 dominant negative pECE-ATF-2 (Ala 69/71) was described previously (46). The c-Jun dominant negative mutant TAM-67 (59) was a gift from Dr. M. Birrer. The human ATF-2 cDNA was cloned into pGEM3 for in vitro translation. The GAL4-CREB, GAL4-CREB 
src (Ser133mut) (60), GAL4-ATF2 (C-19-X), c-Fos (K-25-X) (Santa Cruz Biotechnology), CREB (HM93) (a generous gift from Dr. J. Habener) (64), and a Jun antibody resembling a CRE/ATF site located between 5′-9 and 3′-11 were used. The antibody against ATF-2 (C-19-X), c-Fos (K-25-X) (Santa Cruz Biotechnology), CREB (HM93) (a generous gift from Dr. J. Habener) (64), and a Jun antibody (Upstate Biotechnology) were previously described (61).

Cell Culture, DNA Transfection, Luciferase Assays, and Chemicals—Cell culture, DNA transfection, and luciferase assays were performed as described previously (16). The MCF7 cells were maintained in DME containing 5% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were transfected with DNA plasmids using calcium phosphate precipitation, the medium was changed 16 h after transfection and luciferase activity was determined after a further 24–36 h. Cell culture, DNA transfection, and luciferase assays were performed essentially as described previously (16). The MCF7 cells were transfected with an expression vector encoding pp60 
src or empty vector cassette, and Western blotting was performed for cyclin D1 abundance and normalized by probing of the same blots with an α-tubulin antibody. B, increasing amounts of pp60 
src (pSRC527F) (representative experiment), or mean data using 300–600 ng of expression vector for pp60 
src were transfected with 2.4 μg of −1745 CD1LUC. C and D, comparison was made with the effect of pp60 
src on −1745 CD1LUC or pA_LUC (C) or c-fosLUC and serum response element TATA-LUC reporters (D). The data are the mean ± S.E. for n separate transfactions as indicated in parentheses.

RESULTS

pp60 
src Induction of Cyclin D1—Previous studies had shown that cyclin D1 protein levels were increased in NIH3T3 cells overexpressing pp60 
src (21). To extend this analysis, we examined the role of pp60 
src in expression of cyclin D1 in mammary epithelial cells. Cyclin D1 protein levels are regulated by growth factors and mitogens in MCF7 cells (6, 13, 14); however, the effect of pp60 
src on cyclin D1 abundance in MCF7 cells had not been determined. MCF7 cells were transfected with the expression vector encoding pp60 
src, and Western blotting was performed. Cyclin D1 protein levels were increased 8.1-fold by overexpression of pp60 
src compared with the effect of pp60 
src in NIH3T3 cells (Fig. 1A). To extend this analysis, we examined the role of pp60 
src in expression of cyclin D1 in mammary epithelial cells. Cyclin D1 protein levels are regulated by growth factors and mitogens in MCF7 cells (6, 13, 14); however, the effect of pp60 
src on cyclin D1 abundance in MCF7 cells had not been determined. MCF7 cells were transfected with the expression vector encoding pp60 
src, and Western blotting was performed. Cyclin D1 protein levels were increased 8.1-fold by overexpression of pp60 
src (Fig. 1A). As a form of internal control, the blot was probed with the structural protein α-tubulin, the abundance of which was unchanged by the overexpression of pp60 
src in the MCF7 cells (Fig. 1A, lower panel).

In order to determine whether the cyclin D1 gene promoter was a direct transcriptional target of pp60 
src, transient expression studies were conducted with the human cyclin D1 promoter linked to the luciferase reporter gene (−1745 CD1LUC) and the pp60 
src expression vector. pp60 
src induced
the cyclin D1 promoter with a mean of 6.1-fold (± 1.7) in MCF7 cells (Fig. 1B). Comparison was also made with the pp60<sup>src</sup> expression vector, which induced the full-length 1745 CD1LUC reporter a mean of 48-fold (± 9.0) (Fig. 1C). In contrast, control plasmids including the promoterless pA<sub>LUC</sub> vector were not regulated by pp60<sup>src</sup> (Fig. 1C). As a form of positive control, promoters for genes previously shown to be induced by pp60<sup>src</sup> in fibroblast cell lines were examined in MCF7 cells. The c-fos LUC and serum response element TATA-LUC reporters were induced 142-fold and 62-fold, respectively (Fig. 1D).

**Fig. 2.** Inhibitors of Src kinase, p38, ERK, and JNK inhibit pp60<sup>src</sup>-induced induction of the cyclin D1 promoter activity in MCF7 cells. In order to examine the intracellular signaling pathways involved in pp60<sup>src</sup>-activation of the cyclin D1 promoter, the 1745 CD1LUC reporter was introduced into MCF7 cells with the pp60<sup>src</sup> expression vector as described under "Materials and Methods." The effect of the chemical inhibitors of the tyrosine kinase pathway induced by Src, chemotherapeutic agents, and expression vectors encoding dominant interfering mutants of intracellular signaling pathways was employed. The chemical inhibitor genistein was previously shown to inhibit tyrosine kinases with a preferential inhibition of Src kinase (65). Genistein inhibited pp60<sup>src</sup> activation of cyclin D1 promoter activity in a dose-dependent manner between 36 and 200 μM (Fig. 2A). Herbimycin A was also used as a Src kinase inhibitor (63) and blocked cyclin D1 promoter activation by pp60<sup>src</sup> in a dose-dependent manner (Fig. 2B).

Both the JNK and ERK families of MAPKs have been implicated in pp60<sup>src</sup> signaling. In our previous studies, the cyclin D1 promoter was induced by growth factors via the ERK pathway in adrenal and trophoblast cell lines and in tracheal myocytes (16, 56, 66). The MEK1/ERK inhibitor PD98059 (10–20 μM) suppresses the activation of the MAPK/ERK pathway by preventing activation of the upstream activator MAPK kinase 1 (MEK1) (67). pp60<sup>src</sup> induction of the cyclin D1 promoter was reduced 31% by the addition of PD98059 (Fig. 2C). In previous studies the p38 kinase pathway was identified as an inhibitor of cyclin D1 expression. The drug SB203580 is a specific inhibitor of p38α and p38β MAPK (68). The addition of SB203580 (10–20 μM) reduced pp60<sup>src</sup> induction of the cyclin D1 promoter activity by 38% (Fig. 2C). To investigate the role of the Jun kinase pathway in pp60<sup>src</sup>-induction of cyclin D1 promoter activity, we employed an expression vector encoding JIP-1. Through cytoplasmic retention of JNK, JIP-1 functions as a powerful dominant negative of JNK-dependent activation of both c-Jun and ATF-2 transcriptional activities (61). Overexpression of JIP-1 reduced pp60<sup>src</sup>-induced cyclin D1 promoter activity by 80–90% (Fig. 2D). Inhibition of pp60<sup>src</sup>-induced cyclin D1 promoter activity by JIP-1 occurred in a dose-dependent manner (Fig. 2D). Consistent with a role for Jun kinase activity in pp60<sup>src</sup>-induction of an AP-1 reporter, JIP-1 overexpression also inhibited pp60<sup>src</sup>-induction of the collagenase AP-1 reporter, p<sub>3TPLUX</sub> (Fig. 2E) in a dose-dependent manner.

The role of the MKK3 and MKK6 pathways in oncogene signaling or in regulation of cyclin D1 remained to be determined. To further discriminate the signaling pathways involved in pp60<sup>src</sup>-induction of the cyclin D1 gene, we used plasmids encoding dominant negative mutants of MEK1 (K82A). Comparison was made between the effect of the dominant negative mutant and equal amounts of empty expression vector cassette (control). In G, the effect of increasing concentration of the MKK6 dominant negative mutant on pp60<sup>src</sup>-induced cyclin D1 promoter activity was determined. In the inset, a Western blot is shown of MCF7 cell extracts transfected with increasing amounts of the dominant negative mutants for MKK3 (MKK3 Ala) or MKK6 (MKK6 K82A). The amount of transfected expression vector is shown above each lane. Western blotting was performed after 48 h using an antibody to the FLAG epitope. The expression level of the MKK6 vector was 20% of the MKK3 vector in MCF7 cells.
(MEKC), MKK3, and MKK6. Dominant negative mutants of MEK1 have been shown to specifically inhibit induction of the downstream targets, ERK1 and ERK2. MKK3 and MKK6 govern activity of the p38 kinase group of MAP kinases (48, 68). Expression plasmids encoding dominant negative mutants of MKK3, MKK6, or MEK1 were co-expressed with pp60v-src and the −1745 cyclin D1 promoter reporter in MCF7 cells. Overexpression of the MEK1 dominant negative mutant (MEKC) reduced pp60v-src-induced cyclin D1 promoter activity by >50% (Fig. 2F).

Overexpression of the dominant negative mutant of MKK3 (MKK3 Ala) reduced pp60v-src induction of −1745 CD1LUC reporter activity by 73%. The dominant negative mutant of MKK6 (MKK6 K82A) did not inhibit pp60v-src induction of cyclin D1 (Fig. 2F). The expression levels of the MKK3 and MKK6 dominant negative expression plasmids were assessed in transfected 293T and MCF7 cells. The Flag epitope-tagged MKK3 dominant negative vector (pRSV-Flag-MKK3 Ala) and MKK6 dominant negative expression plasmids were transfected into cells with increasing doses of expression plasmid from 5 to 30 μg. Western blotting was performed of cell extracts using the Flag antibody (M2). In 293T cells, protein expression was identical by Western blotting (data not shown). In MCF7 cells, however, in three separate experiments, the relative abundance of Flag-tagged MKK6 protein was 5–6-fold less than Flag-tagged MKK3 protein when equal amounts of supercoiled plasmid was transfected (Fig. 2G, inset). Further comparison was therefore made using up to 10-fold larger amounts of transfected kinase dead MKK6 expression vector; however, MKK6 K82A did not inhibit pp60v-src induction of cyclin D1 (Fig. 2G).

Together, these findings suggest that pp60v-src-induced cyclin D1 promoter activity requires several signaling pathways, including the MEK1, JNK, and MKK3 pathways but not the MKK6 pathway. In contrast with previous studies in which the pp38 (69) or JNK pathways (56) inhibited basal cyclin D1 expression, in the presence of the transforming pp60v-src, ERK1 and ERK2, MKK3 and MKK6, or MEK1 were co-expressed with pp60v-src and the −1745 cyclin D1 promoter reporter in MCF7 cells. Overexpression of the MEK1 dominant negative mutant (MEKC) reduced pp60v-src-induced cyclin D1 promoter activity by >50% (Fig. 2F).

A CRE/ATF Site Is Required for pp60v-src Activation of the Cyclin D1 Promoter—In order to determine the minimal region of the cyclin D1 promoter required for regulation by pp60v-src, co-transfection experiments were conducted with a series of 5′ promoter deletion constructions in MCF7 cells. pp60v-src responsiveness of the promoter was preserved with deletion from −1745 to −141 (Fig. 3B). Deletion from −141 to −66 reduced induction from 46- to 17-fold. Deletion from −66 to −22 reduced induction from 17- to less than 3-fold (Fig. 3B). Enhancer elements resembling a CRE/ATF site are located within the pp60v-src-responsive region between −66 and −22 (56). As the minimal pp60v-src-responsive sequences were located between −66 and −22, the role of the CRE/ATF-like sequences at −58 were initially assessed. Clustered point mutation of the ATF site was performed in the context of the −66 bp promoter fragment. Direct comparison was made between the −66 CD1LUC and the −66 CD1ATFmutLUC reporters in the presence of pp60v-src. Induction of the −66 bp reporter fragment was reduced 80% by mutation of the CRE/ATF site (Fig. 3C).

In order to determine whether the CRE/ATF sequences were sufficient to convey induction in response to overexpression by pp60v-src, these sequences were linked to an heterologous reporter to form pCD1ATF/TKLUC. The pCD1ATF/TKLUC reporter was induced 16-fold by pp60v-src compared with empty expression vector control (Fig. 3D). Thus, the cyclin D1 CRE/ATF site responds to induction by pp60v-src.

ATF-2/CREB Proteins Bind the Cyclin D1 −58 Region in MCF7 Cells—In previous studies we observed the −58 region

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** The cyclin D1 promoter CRE/ATF site is required for induction of the cyclin D1 promoter by pp60v-src in MCF7 cells. A, schematic representation of the cyclin D1 promoter deletion constructs. B, the pp60v-src expression vector or cassette was transfected with cyclin D1 5′ promoter constructs. * represents significant difference from the adjacent 5′ deletion construct for p < 0.05. Data are shown as the mean ± S.E. for n separate transfections as indicated in parentheses, comparing the effect of pp60v-src expression vector with empty vector cassette. C, comparison was made between the −66 CD1LUC and −66 CD1ATFmutLUC reporters in the presence of 300–600 ng of expression vector. Results are shown as percentage of relative activity for the effect of the transfected pp60v-src expression vector on −66 CD1ATFmutLUC versus −66 CD1LUC. D, the pp60v-src expression vector or empty cassette was transfected with either the heterologous construction encoding the CRE/ATF region of the cyclin D1 promoter linked to the minimal TK promoter (P1/CD1ATF) TKLUC or the minimal TK promoter (TKLUC).
KCREB expression plasmid encodes a CREB cDNA that contains a mutation of a single amino acid in the DNA binding domain and blocks the ability of wild type CREB to bind to the CRE of the somatostatin promoter (57). The KCREB expression plasmid was previously shown to inhibit cAMP-induced expression of the somatostatin CRE by 55%. The pp60src-induced cyclin D1 promoter activity was reduced 70% by the KCREB mutant (Fig. 5A). The ATF-2 dominant negative expression plasmid ATF-2 M2 contains an alanine to arginine substitution, which abolishes DNA binding to the CRE/ATF site and abolished induction of the α-subunit CRE reporter (58). The pp60src-induced cyclin D1 promoter activity was reduced 25% by the ATF-2 dominant negative expression plasmid ATF-2 M2 (Fig. 5A).

Because c-Jun was implicated in pp60src signaling to the psg2 gene (70), and we had previously shown that c-Jun induced cyclin D1 expression in JEG-3 cells (16), we examined the effect of the c-Jun dominant negative mutant TAM-67 on pp60src induction of cyclin D1. The TAM-67 mutant, which lacks the transactivation domain of c-Jun, was previously shown to convey potent dominant negative function and prevented AP-1 mediated transcriptional activation and transformation in breast cancer cell lines (59). The pp60src-induced cyclin D1 promoter activity was further induced 2.4-fold (n = 14) by TAM-67 (Fig. 5A), although this mutant effectively inhibited Ras induction of the collagenase AP-1 site reporter p2TPLUX (data not shown). Overexpression of TAM-67 also induced basal cyclin D1 promoter activity 2.2-fold (n = 10) (data not shown). These findings suggest that, in contrast with the findings in fibroblasts and JEG-3 cells, the cyclin D1 promoter may be under basal repression by c-Jun in MCF7 cells.

pp60src Induction of CREB and ATF-2 Transactivation Domains in MCF7 Cells—These studies suggested that ATF-2 and CREB were involved in the regulation of cyclin D1 by pp60src in MCF7 cells. We hypothesized that pp60src could either enhance the binding affinity of proteins at the cyclin D1 CRE/ATF site or increase transactivation function of transcription factors bound to the site. We examined the possibility that pp60src may directly induce the activity of CREB or ATF-2. The transactivation domains of these proteins linked to the GAL4 DNA binding domain were introduced into MCF7 cells with a heterologous DNA binding site for the GAL4 DNA binding sequence. The (UAS)6E1BTATALUC reporter consists of multimeric GAL4 DNA binding sites linked to a luciferase reporter gene (Fig. 5B). The CREB and ATF-2 transactivation domains conveyed basal enhancer activity in MCF7 cells (Fig. 5B). Overexpression of pp60src enhanced CREB activity 9-fold and point mutation of Ser133 in CREB abolished induction by pp60src (Fig. 5B). Overexpression of pp60src enhanced ATF-2 activity 3-fold. Mutation of the important threonine phosphorylation sites in the transactivation domain of ATF-2 (Thr69 and Thr73) to alanine (46) reduced both basal and pp60src-induced activity (Fig. 5B).

The CREB and the ATF-2 proteins have been shown to function at the distal end of signal transduction pathways involving MKK3 and MKK6 (48, 50, 51, 68, 71). The ERK, p38, and JNK pathways were involved in pp60src induction of the psg2 gene (70), and we had previously shown that pp60src induction of the collagenase AP-1 site reporter p2TPLUX (data not shown). Overexpression of pp60src enhanced CREB activity 9-fold and point mutation of Ser133 in CREB abolished induction by pp60src (Fig. 5B). Overexpression of pp60src enhanced ATF-2 activity 3-fold. Mutation of the important threonine phosphorylation sites in the transactivation domain of ATF-2 (Thr69 and Thr73) to alanine (46) reduced both basal and pp60src-induced activity (Fig. 5B).

The CREB and the ATF-2 proteins have been shown to function at the distal end of signal transduction pathways involving MKK3 and MKK6 (48, 50, 51, 68, 71). The ERK, p38, and JNK pathways were involved in pp60src induction of the cyclin D1 promoter and both ATF-2 and CREB bound the pp60src response element. We therefore assessed the independent contribution of the ERK, p38 and JNK pathways to induction of CREB and ATF-2 transactivation function in MCF7 cells. pp60src induction of CREB was inhibited 50% by SB203580, whereas JIP-1 and PD98059 did not affect CREB function (Fig. 5C). The pp60src induction of ATF-2 was inhibited by JIP-1 more than 80%; however, the MEK/ERK inhibitor, PD98059 and the p38 inhibitor, SB203580, did not affect

**Fig. 4. Binding of ATF-2 to the cyclin D1 CRE/ATF site in MCF7 cells.** A, the γ32P-labeled cyclin D1 CRE/ATF probe was incubated with MCF7 nuclear extracts either alone (lane 1), with 100-fold excess of cold wild type competitor (lane 2), with an equimolar amount of double-stranded mutant competitor (lane 3), with ATF-2 antibody (Ab) (lane 4), with c-Fos antibody (lane 5), or with preimmune serum (lane 6). The probe was incubated with in vitro translated ATF-2 alone (lane 7) and in the presence of ATF-2 antibody (lane 8). B, the γ32P-labeled cyclin D1 CRE/ATF probe was incubated either with MCF7 nuclear extracts alone (lane 1), with preimmune serum (lane 2), or with antibodies to Jun (lane 3), cAMP response element-binding protein/ cAMP response element modulator (lane 4), or ATF-2 (lane 5).

| WT competitor | Mut competitor | ATF-2 Ab | c-Fos Ab | preimmune | ATF-2 | ATF-2+ATF-2 Ab |
|---------------|---------------|---------|---------|-----------|------|---------------|
| S              | A             | B       | SS      | SS        | SS   | SS            |

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|

**pp60src Induction of Cyclin D1 Is Inhibited by Dominant Negative Expression Plasmids for ATF-2 and CREB but Not c-Jun—**The EMSA studies indicated that CREB/ATF-2 complexes bound the cyclin D1 promoter region involved in pp60src induction of the cyclin D1 promoter in MCF-7 cells. In order to examine the role of CREB and ATF-2 proteins in pp60src induction of the cyclin D1, transient expression studies were conducted with expression plasmids encoding dominant negative mutants of these transcription factors. The
Kinase Activities in the Mammary Gland Tumors of MMTV-pp60\textsuperscript{527F} Transgenic Mice—We had previously shown that overexpression of pp60\textsuperscript{c-src} (MMTV-pp60\textsuperscript{SRC527F}) under transcriptional control of the MMTV long terminal repeat induced mammary gland tumors with increased levels of pp60\textsuperscript{c-src} activity (28). To determine whether cyclin D1 levels were induced in these mammary gland tumors, samples from the MMTV-pp60\textsuperscript{c-src} transgenic mice were analyzed and compared with mammary gland tissue from strain-matched nontumorous mammary tissue. Cyclin D1 protein levels were increased in 14 of 15 mammary gland tumors examined when compared with mammary gland from nontransgenic mice (Fig. 6A). Infrequent immunostaining for cyclin D1 was observed in the adjacent normal mammary gland tissue or mammary tissue from control animals (2–5%).

In order to examine the activity of cyclin D1 in the mammary gland tumors, immune-precipitation kinase assays were performed using pRB protein as substrate. The phosphorylated pRB band in Fig. 6C was dependent upon the addition of pRB substrate and inhibited by the addition of p16\textsuperscript{INK4a} protein (data not shown), consistent with the specificity of the kinase assays. An increase in cyclin D1-dependent kinase (CD1K) activity was observed in each of the tumors examined (Fig. 6C). The mean CD1K activity of the tumors was increased 5.26 ± 0.8-fold (n = 15) compared with kinase activity from equal amounts of protein derived from the mammary gland tissue of three virgin mammary glands.

In order to determine the ERK and JNK activity in the mammary tumors of pp60\textsuperscript{c-src} transgenic mice, we performed immunoprecipitation kinase assays. As a substrate for activation of the ERK pathway, myelin basic protein was used, whereas for the JNK pathway, a synthetic fusion protein for the MEK/ERK pathway (PD98059), the p38 pathway (SB203580), and the JNK pathway (JIP-1) were assessed for their role in pp60\textsuperscript{c-src} activity of CREB or ATF-2. The heterologous reporters GAL4-CREB and GAL4-ATF-2 were transfected with the reporter (UAS)\textsubscript{5E1BTATALUC} (2.4 μg) in the presence of pp60\textsuperscript{c-src} (300 ng). The data are shown for the percentage of change in activity. The mean induction by pp60\textsuperscript{c-src} in these experiments was 9-fold for CREB and 3-fold for ATF-2. The data are shown as mean ± S.E. for n separate experiments as indicated.

ATF-2 activity (Fig. 5C). These results indicate that pp60\textsuperscript{c-src} induction of CREB occurs at least in part through the p38 pathway and pp60\textsuperscript{c-src} induction of ATF-2 occurs primarily through the JNK pathway.

Kinase Activities in the Mammary Gland Tumors of MMTV-pp60\textsuperscript{arc527F} Transgenic Mice—We had previously shown that overexpression of pp60\textsuperscript{arc} (MMTV-pp60\textsuperscript{SRC527F}) under transcriptional control of the MMTV long terminal repeat induced mammary gland tumors with increased levels of pp60\textsuperscript{arc} activity (28). To determine whether cyclin D1 levels were induced in these mammary gland tumors, samples from the MMTV-pp60\textsuperscript{arc} transgenic mice were analyzed and compared with mammary gland tissue from strain-matched nontumorous mammary tissue. Cyclin D1 protein levels were increased in 14 of 15 mammary gland tumors examined when compared with mammary gland from nontransgenic mice (Fig. 6A).

Immunostaining of the mammary gland tumors of the transgenic animals was performed to determine whether the increase in cyclin D1 protein observed was localized to the nucleus or cytoplasm of the cell. In each tumor, 500 cells were examined and scored for nuclear cyclin D1 staining. Tumors from four separate animals were examined. Cyclin D1 protein was found in the nucleus and increased nuclear abundance of cyclin D1 was found in all tumors examined (15, 33, 46, and 47%) (Fig. 6B).
The N terminus of c-Jun was used. Equal amounts of protein were used and the induction of immune-complex kinase activity was normalized to the activity determined in the mammary gland tissue of virgin nontransgenic animals. ERK activity was induced in each tumor. The induction varied from 3- to 60-fold (Fig. 6D). JNK activity was either unchanged or reduced in every tumor. The reduction in JNK activity was between 30 and 50% below basal (Fig. 6D). Although ERK activity and cyclin D1 protein levels were increased in most mammary gland tumors, the increase in cyclin D1 protein abundance by Western blotting did not correlate significantly with ERK activity or JNK activity. These results are consistent with a model in which additional pathways to ERK/JNK are involved in the sustained induction of cyclin D1 protein in these tumors. Alternatively, it may be that heterogeneity within the tumor confounds interpretation, and analysis of kinase activity at the single cell level within the tumor may be more informative.

**DISCUSSION**

In these studies, we demonstrate that cyclin D1 is induced by pp60<sup>src</sup> in mammary tumor cells and identify the intracellular signaling pathway responsible for induction of the cyclin D1 gene by pp60<sup>src</sup>. Induction of the cyclin D1 gene by pp60<sup>src</sup> required the MEK1/ERK, MKK3/p38, and JNK pathways in mammary epithelial cells. Consistent with our previous results in which ERK induced cyclin D1 promoter activity and dominant negative mutants of ERK reduced growth factor-regulated promoter activity (16, 52, 56), we found that the dominant negative mutants of MEK1 and the MEK inhibitor PD98059 reduced pp60<sup>src</sup> activation of the cyclin D1 promoter. The ERKs functioned downstream of pp60<sup>src</sup> signaling to the vascular endothelial growth factor gene (72). In contrast with previous studies in which p38 was an inhibitor of cyclin D1 in CCL39 fibroblasts (69), we observed that the p38 inhibitor SB203580 reduced pp60<sup>src</sup> induction of cyclin D1 by 38%. In addition, the use of the JIP-1 expression plasmid, which selectively retains Jun kinase in the cytoplasm thereby inhibiting Jun kinase signaling, allowed us to identify an important role for Jun kinase in pp60<sup>src</sup> signaling to cyclin D1. These results are consistent with a previous study in which pp60<sup>src</sup> induced JNK/SAPK activity using glutathione S-transferase-c-Jun as substrate (31). The finding that cyclin D1 is induced by pp60<sup>src</sup> through a JNK signaling pathway is consistent with previous correlative analysis in which activating Rac mutants, which induce JNK activity, also induced the cyclin D1 promoter in a p21-activated kinase-dependent manner (18) and that Dbl family members, which induce transformation and JNK activity, also induced cyclin D1 expression (20). The current studies are the first to directly demonstrate the requirement for JNK in oncogene signaling to cyclin D1.

The cyclin D1 promoter CRE/ATF site was required for optimal induction by pp60<sup>src</sup>. Additional pp60<sup>src</sup>-responsive sequences were also present within the region between −141 and −66; however, the minimal sequences required for induction remained within the −66 bp fragment. Mutation of the CRE/ATF site in the context of the native promoter significantly reduced induction by pp60<sup>src</sup> and the CRE/ATF sequence was induced 16-fold by pp60<sup>src</sup> when linked to a heterologous promoter, consistent with an important role for this sequence in pp60<sup>src</sup> signaling to the cyclin D1 gene. These studies identified the ATF-2 and CREB transcription factors as the primary proteins binding these DNA sequences in MCF7 and activity is shown for each tumor on the y axis, with cyclin D1 abundance expressed as fold induction compared with wild type mammary tissue shown on the x axis. In E, ERK and JNK activity is shown with the CDK activity for each tumor.
cells, and overexpression of pp60src increased the amount of total complexes bound at this site (data not shown). We had previously shown that the cyclin D1 CRE/ATF site binds CREB in human choriocarcinoma (JEG-3) cells (56). The CRE/ATF site contributed to induction of the cyclin D1 promoter by the SV40 small t antigen (56) and was important in serum-induced cyclin D1 expression in MEFs (54). In MEFs, we observed a serum-inducible complex consisting primarily of FOS/CREB proteins binding this site (54). In MEFs derived from mice homozygously deleted of the c-fos and fosB genes (c-fos<sup>−/−</sup>/fosB<sup>−/−</sup>), the serum-induced proliferation rate was reduced in association with a loss of serum-induced cyclin D1 mRNA and protein (54). Together with the observation that cyclin D1<sup>−/−</sup> MEFs are reduced in their proliferative response to serum (54), these studies were consistent with a role for FOS proteins and the cyclin D1 CRE/ATF site in serum responsiveness of the cyclin D1 gene in MEFs. The cyclin A promoter contains a CRE/ATF binding site that bound ATF1, CREB1, and ATF2 in HeLa cell extracts (73). This region of the cyclin A promoter was important in activation by TAF250. Cells mutant in TAF<sub>250</sub> are defective in cell cycle progression and have reduced cyclin D1 and cyclin A transcription, suggesting an important link between cellular proliferation and the CREB/ATF family of proteins (73).

The dominant negative mutant of CREB reduced pp60src-induced cyclin D1 expression by 70%. CREB activity was induced by pp60src, and the induction was abolished by mutation of Ser<sup>133</sup>. CREB phosphorylation at Ser<sup>133</sup> is induced by protein kinase A (60), by calcium ions (74), by calmodulin-dependent kinases II and IV (75), through a growth factor-induced MAPK/ERK cascade (which was prevented by PD98059 (49)), and by FGF through a p38/MAP kinase-activated protein pathway (50). In the current studies, SB203580 reduced pp60<sup>src</sup> induction of CREB activity by 50%, suggesting a role for the p38 pathway. Unlike previous studies in glial cell progenitors in which growth factor-induced phosphorylation of CREB was sensitive to PD98059 (49), inhibition of the MAPK/ERK pathway enhanced pp60<sup>src</sup> induction of CREB activity in MCF7 cells. As with the induction of CREB phosphorylation by FGF (50), our studies suggest that CREB activation by pp60<sup>src</sup> at Ser<sup>133</sup> is at least in part dependent upon the p38 pathway.

ATF-2 binds to and is phosphorylated by JNKs and p38, and both the ERKs and JNK enhance ATF-2 transcriptional activity (45, 46, 48, 61). ERK phosphorylates ATF-2 in vitro, promoting ATF-2 DNA binding (45, 47). JNK phosphorylation of ATF-2 enhances its transcriptional activity (45, 46) and protects it from JNK-mediated ubiquitination and degradation, thereby extending its half-life (76). The signaling pathways regulating ATF-2 function in breast cancer cells were not known. In the current studies, pp60<sup>src</sup> induced ATF-2 activity and mutation of threonines 69 and 71 dramatically inhibited both basal and pp60<sup>src</sup>-induced activity. Recently, the JIP-1 protein was identified and shown to bind JNK in the cytoplasm, interfering with JNK nuclear translocation and activity (61). JIP-1 binds several components of the JNK signaling pathway, including hematopoietic progenitor kinase-1 and JNK, thereby functioning as a scaffold to promote interactions between these components of the JNK signal transduction module (77). In the present studies, JIP-1 blocked induction of ATF-2 transactivation by pp60<sup>src</sup>. Neither the MEK nor p38 inhibitors reduced pp60<sup>src</sup>-induced ATF-2 activity. These studies provide further evidence that ATF-2 is a target of pp60<sup>src</sup> through a JNK-dependent pathway in breast cancer cells.

In the current studies, c-Jun did not contribute to the induction of cyclin D1 by pp60<sup>src</sup>. The dominant negative of c-Jun, TAM-67, which efficiently inhibited AP-1 activity in MCF7 cells (59, 78), further induced the cyclin D1 promoter, consistent with a role for c-Jun as an inhibitor of cyclin D1 in MCF7 cells. pp60<sup>src</sup> has been shown to induce components of the AP-1 signaling pathway in fibroblasts. In the current studies, the results from the 5′ promoter deletion analysis demonstrated the minimal region required for induction by pp60<sup>src</sup> was located within the CRE/ATF site at −58. Our laboratory previously identified sequences in the cyclin D1 promoter capable of binding AP-1 proteins, located at −953 (16, 52, 56). The induction of cyclin D1 by the mitogen angiotensin II in human adrenal cells was mediated through a c-Fos/c-Jun AP-1 binding site located at −953 (52) and the AP-1 site was also important in activation of the promoter by p21ras in trophoblast cells (16). The sequences at −953 were not involved in pp60<sup>src</sup>-induced induction. Furthermore, c-Jun/c-Fos did not form part of the complex binding to the pp60<sup>src</sup> response element at −58 in MCF7 cells. Together these results indicate that distinct components of the AP-1/CREB protein family can bind either to the −953 or −58 site in the cyclin D1 promoter to convey signaling by different mitogenic-oncogenic signaling pathways in a cell-type specific manner.

In the current studies, cyclin D1 was induced by pp60<sup>src</sup> in MCF7 cells. These results contrast with the findings in fibroblast cells. In Rat-1 cells, cyclin D1 expression was not induced by a temperature-sensitive pp60<sup>src</sup> (26). We also observed, in a detailed time course using the Src dominant negative cell line 3T3SrcRF<sup>−</sup> cells (72) in the presence or absence of isopropyl-1-thio-β-p-galactopyranoside (5 mM) for 24 h, that cyclin D1 levels were unchanged despite a 10-fold induction of dominant negative Src protein levels (data not shown). Furthermore, in Rat-1 cells, pp60<sup>src</sup>-inhibited p27Kip1 levels (26), whereas in murine mammary tumors induced by transgenic overexpression of pp60<sup>src</sup> inhibited p27Kip1 levels (26), whereas in murine mammary tumors induced by transgenic overexpression of pp60<sup>src</sup> and p27Kip1 levels were uniformly increased (see below). These studies support the notion that pp60<sup>src</sup> utilizes distinct signaling pathways in mammary compared with fibroblast cells and imply important differences may exist in the mechanisms by which pp60<sup>src</sup> regulates the cell cycle in different cell types.

The mammary gland tumors from the pp60<sup>src</sup> mice exhibited increased cyclin D1 protein levels, CD<sub>4</sub>K activity, and an induction of ERK activity but unchanged or reduced JNK activity. The process of tumorigenesis involves multiple events, and cellular heterogeneity within the tumor mass limits detailed correlative analysis; however, the induction of both cyclin D1 levels and ERK activity in the tumors provides supportive data for a role for cyclin D1 and ERK in the pathogenesis of the pp60<sup>src</sup> mammary tumors. Cell lines derived from MMTV-Neu transgenic mice were recently shown to exhibit increased ERK but unchanged JNK activity (79). Induction of JNK and p38 kinases play an important role in apoptosis (80, 81). It is possible, therefore, that an induction of JNK activity in the mammary gland tumors may not have been detected due to apoptosis of cells containing activated JNK activity. CD<sub>4</sub>K activity is determined by the abundance and subcellular distribution of the cyclin D1/cdk4 proteins and the cyclin-dependent kinase inhibitor proteins, including members of the INK4 family and the CIP/KIP family (3). p27Kip1 increases the association of cyclin D1 with cdk4 and targets this complex to its nuclear site of action (82, 83). In contrast with studies in Rat-1 cells in which overexpression of pp60<sup>src</sup> inhibited p27Kip1 levels (26), our studies demonstrated that nuclear p27Kip1 was increased in the majority of mammary gland tumors examined from the MMTV-pp60<sup>src</sup> transgenic mice (data not shown). p16<sup>INK4A</sup> protein was not detected in
most of these tumors. As deletion of the CDKI p16 locus occurs in many breast tumors and breast cell lines (84), and reduced p16INK4A abundance is associated with increased CDK activity (85), loss of p16INK4A may also contribute to the multistep tumorigenesis in these tumors.

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