Dominant-Negative Activator Protein 1 (TAM67) Targets Cyclooxygenase-2 and Osteopontin under Conditions in which It Specifically Inhibits Tumorigenesis

Connie P. Matthews,[1] Alysia M. Birkholz,[1] Alyson R. Baker,[1] Christine M. Perella,[2] George R. Beck, Jr.,[3] Matthew R. Young,[1] and Nancy H. Colburn[1]

[1]Laboratory of Cancer Prevention, Center for Cancer Research, [2]Science Applications International Corporation-Frederick, National Cancer Institute, Frederick, Maryland and [3]Emory University School of Medicine, Atlanta, Georgia

Abstract

Activation of activator protein 1 (AP-1) and nuclear factor κB (NFκB)–dependent transcription is required for tumor promotion in cell culture models and transgenic mice. Dominant-negative c-Jun (TAM67) blocks AP-1 activation by dimerizing with Jun or Fos family proteins and blocks NFκB activation by interacting with NFκB p65. Two-stage [7,12-dimethylbenz(*a*)anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)] skin carcinogenesis experiments in a model relevant to human cancer risk, transgenic mice expressing human papillomavirus 16 E7 oncogene (K14-HPV16-E7), show E7-enhanced tumor promotion. A cross to K14-TAM67–expressing mice results in dramatic inhibition of tumor promoter–induced AP-1 luciferase reporter activation and papillomagenesis. Epithelial specific TAM67 expression inhibits tumorigenesis without affecting TPA- or E7-induced hyperproliferation of the skin. Thus, the mouse model enriches for TAM67 targets relevant to tumorigenesis rather than to general cell proliferation or hyperplasia, implicating a subset of AP-1– and/or NFκB-dependent genes. The aim of the present study was to identify target genes responsible for TAM67 inhibition of DMBA-TPA–induced tumorigenesis. Microarray expression analysis of epidermal tissues revealed small sets of genes in which expression is both up-regulated by tumor promoter and down-regulated by TAM67. Among these, cyclooxygenase-2 (Cox-2/Ptgs2) and osteopontin (Opn/Spp1) are known to be functionally significant in driving carcinogenesis. Results identify both Cox-2 and Opn as transcriptional targets of TAM67 with CRE, but not NFκB sites important in the Cox-2 promoter and an AP-1 site important in the Opn promoter. [Cancer Res 2007;67(6):2430–8]

Introduction

Tumorigenesis is a multistage process involving the transformation of genetically altered "initiated" cells to benign lesions that progress to malignant carcinomas with distant metastases. A single mutagenic event, however, does not produce a cancer. Additional genetic and epigenetic events are required for tumorigenesis and tumor progression. Tumors are produced by chronic exposure of the initiated epithelial and normal stromal cells to tumor-promoting compounds. In the case of human cancer, there is generally chronic exposure to a combination of tumor promoters. Cervical cancer, for example, is initiated in young adults by infection with a high-risk human papilloma virus (HPV), such as HPV16. Cervical cancer development may then be promoted by decades of exposure to estrogen, as well as the HPV16-E7 oncoprotein, which has been shown in *in vivo* mouse models to act during the promotion stage to enhance tumorigenesis (1–3). Tumor promoters induce inflammation, cell proliferation, and other reversible cellular effects. The rate-limiting steps of carcinogenesis occur during promotion and progression. Understanding the molecular basis of these rate-limiting steps is important for the prevention of cancer. Studies by our laboratory (4–9) and others (10–12) using *in vitro* and *in vivo* models have indicated that activator protein 1 (AP-1) and nuclear factor κB (NFκB) activation are driving forces during tumor promotion and progression, although skin carcinogenesis seems to be unique in not requiring NFκB (13).

The AP-1 transcription factor is composed of structurally related Jun and Fos family proteins that form DNA-binding dimers and control the transcription of certain target genes. The AP-1 family members (c-Jun, JunB, JunD, cFos, FosB, Fra1, and Fra2) can form a variety of Jun homodimers and Jun/Fos heterodimers that bind similar DNA elements. A basal level of AP-1 activity is required for normal cellular function (14–16). Elevated AP-1 activity levels, however, can drive tumor progression (7, 9, 10, 17, 18). A dominant-negative transactivation deletion mutant of c-Jun (TAM67) inhibits AP-1 activity by dimerizing with wild-type AP-1 proteins to yield low-activity dimers containing only one transactivation domain. Transgenic expression of TAM67 in the mouse skin inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced but not basal AP-1 transactivation and protects against both TPA-induced and E7-enhanced two-stage skin carcinogenesis (7, 9).

Expression of TAM67 inhibits not only AP-1, but also NFκB activity. NFκB is a transcription factor composed of dimers of REL family proteins: RelA/p65, RelB, c-Rel, NFκB1/p105, and NFκB2/p100 (19, 20). p100 and p105 are processed to release p50 and p52, respectively. The REL family proteins form homo- and heterodimers capable of binding DNA elements in the promoter regions of NFκB target genes (19, 20). NFκB is a key mediator of many cellular processes, including apoptosis, cell growth, and differentiation. NFκB is activated by a wide variety of extracellular stimuli, including known tumor promoters such as TPA, tumor necrosis factor α, UV, and stress. Inappropriate transactivation of NFκB is associated with neoplastic transformation (21), whereas specific inhibition of NFκB blocks tumorigenesis (22, 23). TAM67 was previously shown to bind RelA/p65 and to specifically inhibit the expression of target genes responsive to NFκB in human keratinocytes (8).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Connie P. Matthews, Laboratory of Cancer Prevention, Center for Cancer Research, National Cancer Institute-Frederick, Frederick, MD 21702. Phone: 301-846-6858; Fax: 301-846-6907; E-mail: cmatthews@mail.ncifcrf.gov.

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AP-1 and NFκB synergize during tumorigenesis. TAM67 expression blocks the activities of both AP-1 and NFκB. TAM67 inhibits TPA-induced and HPV16-E7–enhanced tumorigenesis without blocking hyperproliferation or cell survival (9). The biology of the mouse model thus enriches for genes that are functionally significant for tumor promotion or progression. In this study, we sought to identify genes that are important targets of TAM67 when it specifically inhibits tumorigenesis. We examined the gene expression profiles of 7,12-dimethylbenz(*a*)anthracene (DMBA)–initiated, TPA-induced K14-HPV16-E7 transgenic and wild-type mouse skin in the presence or absence of TAM67 expression. We hypothesized that a limited number of genes are targets for regulation by TAM67 under conditions known to inhibit skin carcinogenesis. The analysis identified several genes important for tumor promotion and progression, including cyclooxygenase-2 (Cox-2), osteopontin (Spp1/Opn), Cxcl1/Gro α, Alox5AP, matrix metalloproteinase 10 (MMP-10), and urokinase plasminogen activator as targets of TAM67. The well-recognized molecular target for chemoprevention, cyclooxygenase-2 (Ptgs2/Cox-2), was among only six genes downregulated by TAM67 expression under all of the tumor promotion conditions tested. This is the first report of *in vivo* suppression of Cox-2 and Opn transcription by TAM67 under conditions in which TAM67 specifically inhibits tumorigenesis.

Materials and Methods

Mice. The K14-TAM67/B6D2 mouse expresses dominant-negative c-Jun (TAM67) in squamous epithelia under the control of the human K14 promoter (7). The K14-HPV16-E7/FVB mouse expresses the HPV16-E7 gene under the control of the K14-promoter (24). K14-TAM67 mice were crossed with K14-HPV16-E7 mice. The offspring from the four possible genotypes, K14-TAM67/K14-HPV16-E7, K14 TAM67, K14-HPV16-E7, and wild type, were used in gene expression studies and isolation of primary keratinocytes. Transgene-positive mice were determined by PCR of tail DNA, as described by Young et al. (7). Mice were housed and cared for in accordance with the recommendations for the Guide for the Care and Use of Laboratory Animals.

DMBA/TPA treatment. Groups of 8-week-old E7/TAM67, E7, TAM, and wild-type sibling mice were initiated with DMBA (400 nmol per 200 μL of acetone) and, 2 weeks later, treated with a single dose of TPA (10 nmol in 200 μL of acetone) as described by Young et al. (7). Tissues for RNA and protein expression were harvested from the animals after cervical dislocation.

Total RNA extraction. Full thickness dorsal skin was harvested 6 h after TPA treatment and snap frozen in liquid nitrogen. Epidermis was separated from the dermis as previously described (25). The epidermal tissue was homogenized in a guanidinium-based lysis solution and extracted sequentially with phenol/chloroform/isoamyl alcohol and acid-phenol/chloroform. Total RNA was isolated according to manufacturer's directions for the ToTALLY RNA kit from Ambion (Austin, TX). Total RNA isolated from the epidermis was then subjected to microarray analysis.

Microarrays, labeling, and hybridization of RNA samples. Gene expression patterns were determined using spotted microarrays containing 22,000 Compugen (San Jose, CA) oligonucleotides representing mouse genes manufactured by the National Cancer Institute/Laboratory of Molecular Technology. Fluorescence-labeled cDNA was generated by reverse transcription of 20 μg of total RNA according to standard protocols using SuperscriptIII indirect cDNA labeling system (Invitrogen, Carlsbad, CA) and Cy3/Cy5-monoreactive dyes (GE Healthcare Biosciences Corp., Piscataway, NJ). Cy3-labeled test samples and Cy5-labeled universal control (Stratagene, La Jolla, CA) cDNA samples were combined and concentrated using Microcon YM-30 filter units (Millipore, Billerica, MA). Samples were diluted in F-hybridization buffer (25% formamide, 5× SSC, 0.1% SDS containing 10 μg mouse Cot-1 DNA, 10 μg polydA, and 4 μg tRNA) and hybridized to 22K oligonucleotide array slides overnight at 42°C. The slides were then washed at room temperature with (*a*) 2× SSC and 0.1% SDS; (*b*) 1× SSC and 0.1% SDS for 2 min; (*c*) 0.2× SSC for 2 min; and (*d*) 0.05× SSC for 10 to 20 s, and finally dried by centrifugation at 50 × *g* for 10 min. Three to four independent epidermal RNA samples were analyzed for each set of conditions.

Data acquisition and analysis. Microarrays were scanned and processed using a GenePix 4000A microarray scanner in combination with GenePix Pro 4.0 software (Molecular Devices Corporation, Sunnyvale, CA). Data analysis was done using the MADb program suite[4] and Microsoft Excel. For each hybridization, mean intensity minus median background ratios were normalized by the median ratio of all genes found in that experiment. Data sets representing differentially expressed genes were selected according to the following filtering criteria: (*a*) data obtained from at least 50% of all arrays analyzed in this study (i.e., 15 of 29 arrays); (*b*) mean intensities beyond the threshold set at 500 units and at least 2 SD above background; and (*c*) mean signal differences >1.5-fold for ratio comparisons of cDNAs derived from three independent mouse epidermal tissue samples harvested from TAM67 transgenic and nontransgenic litter mates for each treatment group. Further filtering was done to identify genes induced by tumor promoter and more than 4-fold inhibited by TAM67 with induction completely blocked by TAM67. A pooled *t* test was applied to this completely blocked set of genes. Supplementary Fig. S1 shows the flow diagram used for sequential filtering, statistical analysis, and quantitative PCR confirmation.

Quantitative reverse transcription-PCR analysis. Genes to be analyzed for status as transcriptional targets of TAM67 were first subjected to quantitative PCR to confirm tumor promoter induction and TAM67 inhibition of induction. The same RNA samples (at least three per set of variables) used for hybridization were applied to real-time quantitative reverse transcription-PCR for confirmation of the microarray data. cDNA synthesis was done according to standard protocols using SuperscriptIII (Invitrogen) with oligodT as primer. Production of gene-specific fragments was measured real time based on standard TaqMan (Molecular Beacons, Newark, NJ) protocols, with β2-microglobulin (B2m) serving as a standardization control.

Western analysis. For isolation of protein from mouse epidermis, full thickness dorsal skin was harvested 6 h after TPA treatment and snap frozen in liquid nitrogen. The epidermis was dissected away from the dermis and homogenized in ice-cold lysis buffer [20 mmol/L Tris-HCl (pH, 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1% Triton X-100, and complete miniprotease inhibitor cocktail (Roche, Indianapolis, IN)]. The lysate was incubated at 4°C for 10 min, centrifuged at 12,000 × *g* for 20 min, and supernatant containing total protein was processed for Western analysis. Protein concentrations were assayed with the bicinchoninic acid reagent (Pierce Biotechnology, Inc., Rockford, IL). Equal amounts of protein were separated on 10% SDS polyacrylamide gels and transferred onto nitrocellulose membranes for immunodetection using the requisite primary and secondary antibodies. Antibodies were purchased from Santa Cruz Biotechnolgy (Santa Cruz, CA).

Plasmids. A series of mouse Cox-2 promoter reporter plasmids, including −963/+70, −547/+70, −203/+70, −203 Ebox mutant, −203 NFIL6 mutant, and −203 Ebox/NFIL6 double mutant, was kindly provided by Susan M. Fischer (M.D. Anderson Cancer Center, Houston, Texas; ref. 26). The −203/+70 construct was used as a template to generate site-directed mutants of AP-1 like sites at positions −69 (AGAGTCA to AGAGTTG) and +34 (TCAGTCA to TCAGTTG). Quick Change II Site Directed Mutagenesis (Stratagene) methodology was employed to produce these mutations. Site-specific mutations were confirmed by sequencing. A deletion series of mouse Opn promoter reporter plasmids including −772/+79, −88/+79, and −50/+79 subcloned from pSDKlacZpA Opn promoter constructs (kindly provided by David Denhardt, Rutgers University, Piscataway, NJ) by PCR. The PCR products were cloned into Topa-TA 2.1 (Invitrogen), then cut with *Kpn*I/*Xho*I and cloned into pGL3 (Promega, Madison, WI).

Cell culture: mouse JB6 cells and primary epidermal cells. Transformation-sensitive (P+) mouse epidermal JB6 cells were grown as

[4] http://nciarray.nci.nih.gov

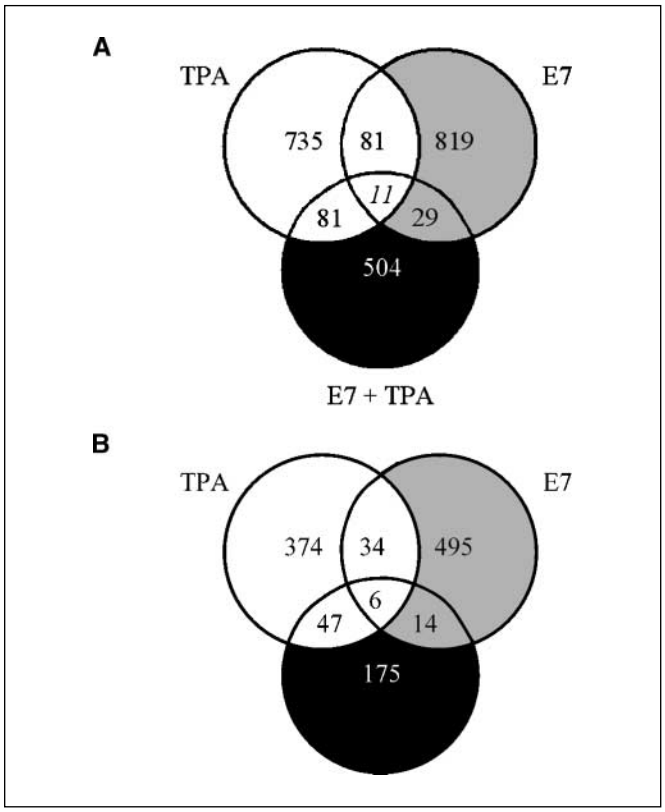

Figure 1. Genes are oppositely regulated by tumor promoter and TAM67. Genes in which expression is oppositely regulated by tumor promoter and inhibitor TAM67 constitute ~9% of informative genes, whereas the subset of oppositely regulated genes down-regulated by TAM67 constitutes ~5%. Comparative gene expression profiling of DMBA-initiated, TPA-induced K14-HPV16-E7 transgenic or wild-type epidermal tissue in the presence or absence of transgenic K14-TAM67 expression was carried out using three to four independent samples of epidermal RNA for each set of conditions. Changes in gene expression were analyzed by cohybridization of differentially labeled test (mouse epidermal) and control (mouse universal) cDNAs derived from these RNAs onto 20,000 oligonucleotide microarrays. Genes of interest were required to be oppositely regulated by the tumor promoter (TPA or E7 or both) in the absence of TAM67 and by TAM67 in the presence of tumor promoter (*A*) and exhibit a minimum of 1.5-fold increase in expression with tumor promoter and 1.5-fold inhibition by TAM67 (*B*). *A,* the overlap pattern of genes found to be oppositely regulated by TAM67 and each of the three tumor promotion regimens. *B,* the subset of genes in which mRNA expression was down-regulated by TAM67.

described previously (4). Epidermal keratinocytes were isolated from newborn mice as previously described (27), with minor modifications. Primary cultures were isolated in EpiLife media (Cascade Biologics, Portland, OR) containing 10% chelexed fetal bovine serum (FBS), 1.8 mmol/L $CaCl_2$, bovine pituitary extract (0.2% v/v), bovine insulin (5 μg/mL), hydrocortisone (0.18 μg/mL), bovine transferrin (5 μg/mL), human epidermal growth factor (0.2 ng/mL), 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 0.25 μg/mL amphotericin B. Keratinocytes were initially plated in fully supplemented serum-free EpiLife media (Cascade Biologics) adjusted to 0.275 mmol/L $CaCl_2$. After attachment occurred, primary cells were maintained in fully supplemented serum-free EpiLife media (Cascade Biologics) adjusted to 0.05 mmol/L $CaCl_2$ at 37°C in a humidified atmosphere of 7% CO_2 in air.

Transfection and luciferase activity assay. Transformation-sensitive (P+) JB6 cells were transiently cotransfected with 200 ng of luciferase reporter and 20 ng pcDNA-TAM67 (or vector control) per well (as indicated in the figure legends). Subconfluent primary keratinocytes were transfected with a total of 200 ng of luciferase reporter DNA per well (as described in the figure legends) of 24-well dish using a 1:3 DNA/Fugene 6 (Roche Applied Science) ratio. After 24 h, the medium was replaced with 0.5 mL fresh EpiLife medium containing 0.05 mmol/L $CaCl_2$ and 50% supplements. After 18 h, the cells were treated with 10 nm TPA or DMSO (as described in the figure legends). After 24 h, the cells were lysed with 1× passive lysis buffer (Promega) and assayed for luciferase activity as previously described (28).

Results

A limited number of genes are targets for regulation by dominant-negative c-Jun (TAM67) under conditions in which two-stage skin carcinogenesis is inhibited. To identify genes regulated by AP-1 or NFκB that play a critical role in tumor promotion, we did comparative gene expression profiling of DMBA-initiated, TPA-induced K14-HPV16-E7 transgenic or wild-type epidermal tissue in the presence or absence of transgenic K14-TAM67 expression. Previous studies have shown that papillomagenesis and subsequent progression to carcinomas is markedly inhibited by the expression of TAM67 (7, 9). To study the differences in gene expression in response to tumor promoters in the presence and absence of TAM67, total RNA was collected from mouse epidermis of each genotype (wild type, TAM67 transgenic, E7 transgenic, or double transgenic) that had been initiated with DMBA and treated with a single dose of TPA or solvent control for 6 h. Changes in gene expression were analyzed by cohybridization of differentially labeled test and universal control cDNAs derived from these RNAs onto spotted microarrays containing 20,000 Compugen oligonucleotides. Because TAM67 expression shows little or no effect on basal AP-1–luciferase activation (5, 7), but markedly inhibits tumor promoter–induced AP-1–luciferase, we sought to identify TPA-, E7-, and E7 plus TPA-induced genes that were suppressed by TAM67 expression. The genes of interest had to meet the following criteria. They must (*a*) be informative on 50% of all arrays; (*b*) be oppositely regulated by the tumor promoter (in the absence of TAM67) and TAM67 (in the presence of tumor promoter); and (*c*) exhibit a minimum of 1.5-fold increase in expression with tumor promoter and 1.5-fold inhibition by TAM67 in TPA-treated samples. The flow diagram for sequential filtering is

Table 1. Quantitative PCR confirmation of tumor promoter-induced TAM67-inhibited gene expression

A	Fold change by tumor promoter			
	Microarray		Quantitative PCR	
	TPA	HPV E7	TPA	I HPV E7
Ptgs2/Cox-2	4.99	2.07	393.89	271.47
Spp1/Opn	5.30	NA	15.72	NA
B	**Fold change by TAM67**			
	Microarray		Quantitative PCR	
	TPA	HPV E7	TPA	HPV E7
Ptgs2/Cox-2	−2.04	−1.79	−4.24	−2.84
Spp1/Opn	−6.60	NA	−14.97	NA

Abbreviation: NA, not applicable.

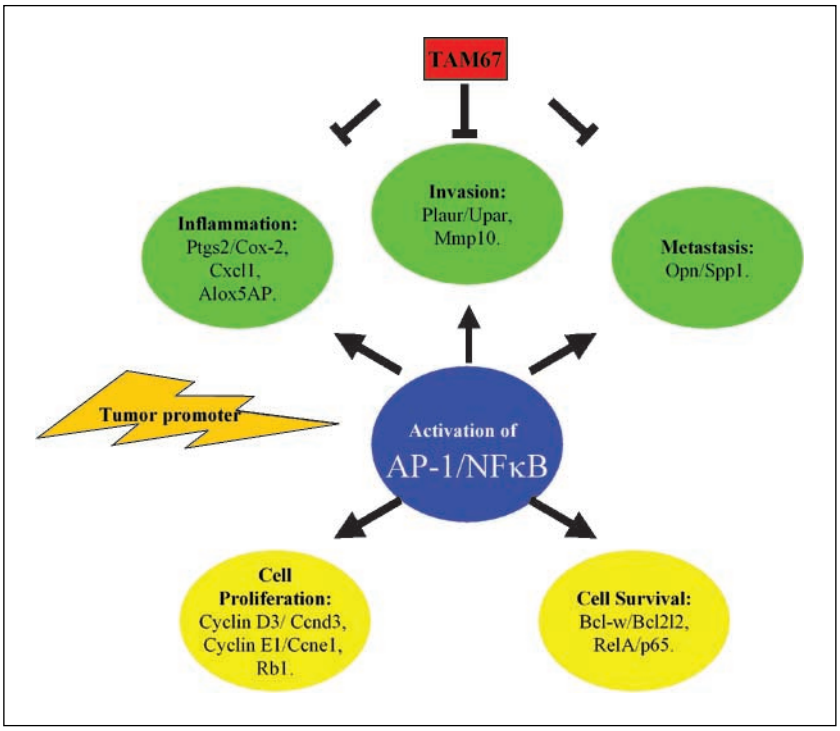

Figure 2. TAM67 inhibits tumor promotion without targeting cell proliferation or cell survival. *Green,* those genes and functions down-regulated by TAM67; *yellow,* those genes and functions unchanged by TAM67 expression (tumor-promoted TAM67 transgenic/tumor-promoted nontransgenic = 1.0 ± 0.1). All genes depicted as TAM67 targets showed statistically significant inhibition of tumor promoter–induced expression by TAM67 or were confirmed as TAM67 targets by quantitative PCR.

shown in Supplementary Fig. S1. All experimental values presented represent an average of three independent RNA samples. The lack of TAM67 effects on HPV16-E7- or TPA-induced hyperproliferation or cell survival (7, 9), coupled with the requirement for opposite regulation by tumor promoter and inhibitor TAM67, provides a powerful tool to enrich for genes functionally significant in tumor promotion.

Dominant-negative c-Jun (TAM67) inhibits TPA-induced gene expression. In DMBA/TPA-treated epidermis, of the 10,440 informative genes (i.e., expressed at a level 2 SD above background in at least 50% of the experiments), 3,970 genes changed expression level more than 1.5-fold in response to a single 6-h dose of TPA. Of these 3,970 genes, 904 were oppositely regulated by TPA treatment and TAM67 expression (Fig. 1*A*). Of the 904 TPA-regulated genes, 461 genes were induced by TPA treatment and inhibited by TAM67 expression (Fig. 1*B*). Because AP-1 most often activates, rather than inhibits transcription, these 461 genes are likely to be enriched for direct targets of TAM67. Of the 16 TPA-induced genes inhibited more than 4-fold by TAM67, induction of 9 was completely blocked (Supplementary Table S1*A*). Seven of the nine showed significant inhibition by a pooled *t* test. Among these nine completely blocked genes were arachidonate 5-lipoxygenase–activating protein (Alox5ap), urokinase plasminogen activator (Plaur) and osteopontin (Spp1/Opn), already shown to be involved in driving tumor promotion and progression (29–32). Despite the *P* value of 0.062, the inhibition of TPA-induced Opn mRNA expression by TAM67 was confirmed by quantitative PCR (Table 1), showing about 15-fold inhibition. As shown in Supplementary Fig. S2, epidermal osteopontin protein levels did not change at 6 h in response to TPA or to TAM67, possibly reflecting a need to measure osteopontin in basement membrane or other ECM where it functions. A cutoff of 2-fold instead of 4-fold with complete block identified a total of 65 genes. Expression profiling thus identified a limited set of TPA-induced genes in which induction was completely blocked by TAM67 expression under conditions previously shown to specifically inhibit tumor promotion.

Dominant-negative c-Jun (TAM67) inhibits E7-enhanced gene expression. Epithelial expression of HPV16-E7 induces epidermal hyperplasia, hyperkeratosis, and spontaneous skin tumors (24). HPV16-E7 synergizes with chemical tumor promoters during the promotion stage of carcinogenesis to produce papillomas. Expression of TAM67 protects mice from E7-enhanced tumor promotion (9). Of the 940 genes oppositely regulated by E7 and TAM67, 549 genes were down-regulated by TAM67 (Fig. 1*B*). Supplementary Table S1*B* shows that of the 18 E7-induced genes inhibited more than 4-fold by TAM67, induction of 9 was completely blocked. Five of these nine genes showed significant inhibition by a pooled *t* test. Expression of the oncogene growth-related gene 1 (Gro1/Cxcl1) was inhibited more than 7-fold by TAM67 (Supplementary Table S1*B*). Cxcl1 positively regulates angiogenesis, a process in which tumors depend for survival, growth, invasion, and metastasis (33). When the cutoff was 2-fold instead of 4-fold, the number of genes completely blocked was 43. Thus, dominant-negative c-Jun (TAM67) down-regulates and completely blocks induction by E7 of a limited set of genes potentially important for HPV16-E7–enhanced carcinogenesis.

Dominant-negative c-Jun (TAM67) inhibits E7-enhanced TPA-induced gene expression. E7 expression greatly enhances TPA-induced papilloma formation. As previously shown, TAM67 expression blocks both TPA-induced and E7-enhanced tumor promotion (7, 9). Although TAM67 expression inhibits E7/TPA-induced papilloma formation by as much as 5-fold, the magnitude of TAM67-induced change in gene expression seen in the combined E7/TPA background is considerably muted compared with the TAM67 effects with E7 or TPA alone. Instead of the more than 4-fold inhibition by TAM67 seen in the E7 or TPA background, maximal inhibition in the combined E7/TPA background was just over 2-fold. This muting of TAM67-induced

change may be explained, in part, by an increased number and magnitude of expression of induced genes seen with the combination of tumor promoters. Treatment with TPA induced 45 of the 9,708 informative genes (0.46%) >10-fold. E7 expression up-regulated 15 of the 9,011 informative genes (0.17%) >10-fold. The combination of TPA and E7 induced 61 of the 9,762 informative genes (0.62%) more than 10-fold. Of the 625 genes oppositely regulated by TAM67 and the combination of E7 and TPA, 242 genes were down-regulated by TAM67 expression. Three of these were completely blocked for induction. One of these, Tradd, was significantly inhibited. Tradd, a death domain–containing adaptor molecule that interacts with tumor necrosis factor receptor superfamily, member 1a (Tnfrsf1a/TNFR1) mediates programmed cell death signaling and NFκB activation (Supplementary Table S1*C*). Thus, TAM67 eliminated the combined tumor promoter induction of gene expression for a limited set of genes.

Reported AP-1/c-Jun target genes are oppositely regulated by tumor promoter and dominant-negative c-Jun (TAM67). A review of the literature reveals 17 published AP-1/c-Jun target genes to be oppositely regulated by at least one tumor promotion regimen and TAM67 *in vivo* (Supplementary Table S2; refs. 34–38). Among the known c-Jun targets are two tumor suppressors, caveolin 1 (Cav1) and sprouty homologue 1 (Spry1), which are up-regulated in response to TAM67 expression. Both Cav1 and Spry1 are reported to inhibit Ras/Map kinase signaling (39, 40). Mediators of invasion and metastasis that are known targets of AP-1, including MMP-10, Plaur/uPAR, CD44, and Spp1/Opn, are down-regulated by TAM67 expression in mouse skin. Transcription of cell proliferation (e.g., cyclin D3/Ccnd3 and Rb1) and cell survival genes (e.g., Bcl2l2/Bcl-w and RelA/p65), however, does not seem to be targeted by TAM67 because these genes showed expression ratios for tumor promoter plus TAM67; tumor promoter minus TAM67 that were 1.0 ± 0.1 (Fig. 2). As predicted by the phenotype of the mice, TAM67 expression specifically inhibits tumorigenesis and tumor progression without targeting transcription of genes that mediate cell proliferation or cell survival.

Tumor promoter–induced Cox-2 expression is inhibited by dominant-negative c-Jun (TAM67). To be relevant to the range and combinations of exposures that cause human cancer, we were interested in TAM67 down-regulated genes that were common to all three epidermal tumor promotion protocols (TPA alone, E7 alone, or the combination of TPA and E7). Genes belonging to this triple

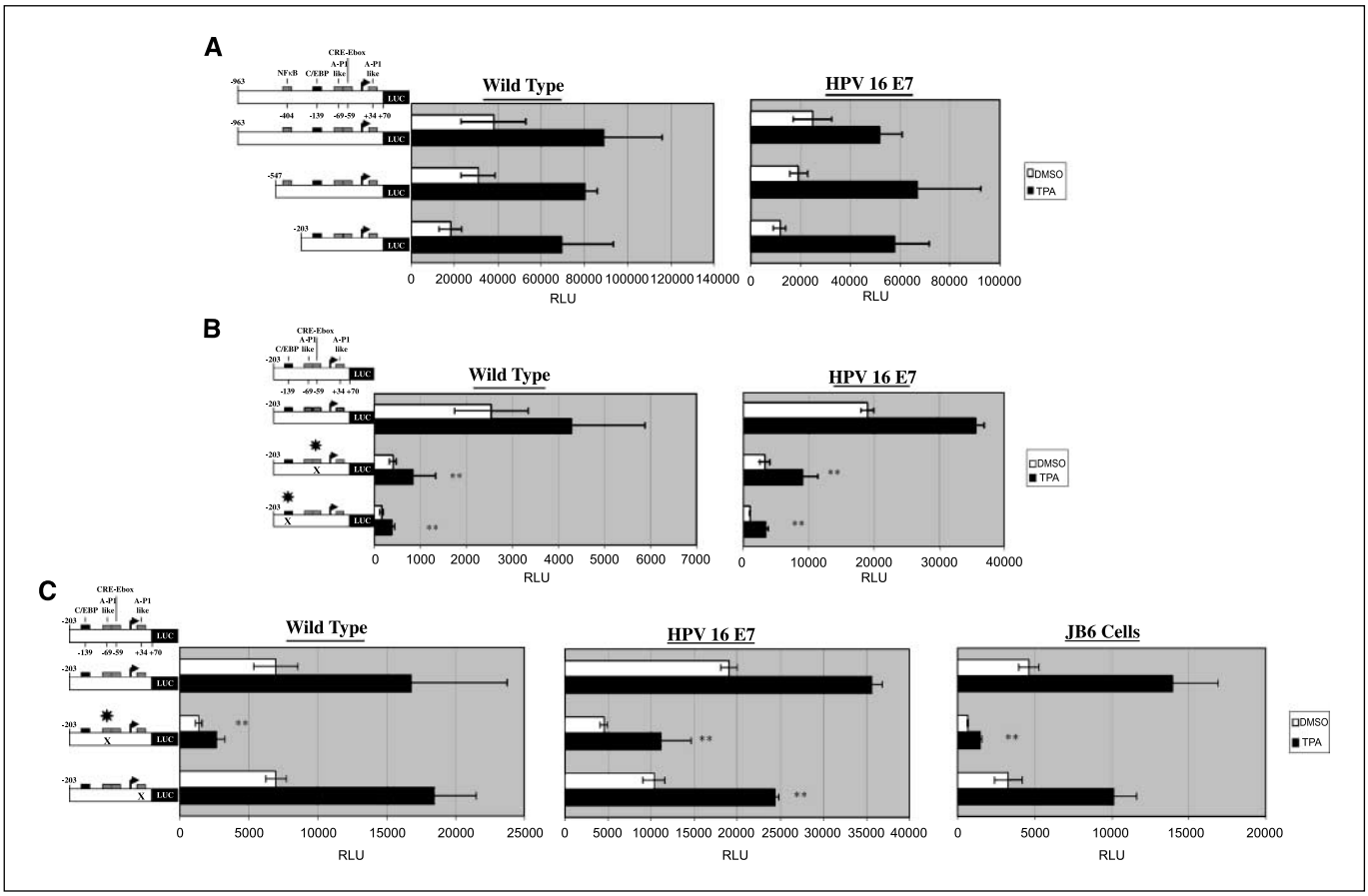

Figure 3. CRE/Ebox, C/EBP, and AP-1–like sites, but not NFκB sites, are required for Cox-2 promoter activation. Wild-type and E7 transgenic primary keratinocytes were cultured in fully supplemented Epilife medium containing 0.05 mmol/L $CaCl_2$ and treated with TPA (10 ng/mL) or DMSO in Epilife medium containing 50% supplements and 0.05 mmol/L $CaCl_2$ for 22 h. Transformation-sensitive JB6 cells were cultured in Eagle's MEM (EMEM) containing 4% FBS and treated with TPA (10 ng/mL) or DMSO in EMEM containing 0.2% FBS for 22 h. The relative luciferase activity of a series of mouse Cox-2 promoter reporter deletion and point mutant constructs was measured in the presence or absence of TPA. *A,* all sequences upstream of −203, including the highly conserved NFκB site, are not required for full TPA-induced promoter reporter activity. The CRE and C/EBP sites are, however, required for basal and TPA-induced activity (*B*). An AP-1–like site at position −69 is also required for both basal and TPA-induced promoter reporter activity in primary keratinocytes and transformation-sensitive JB6 cells (*C*). ×, point mutations. *Columns,* averages of six wells and representative of three experiments. Statistical significance was determined using a two-tailed paired *t* test. **, $P < 0.01$.

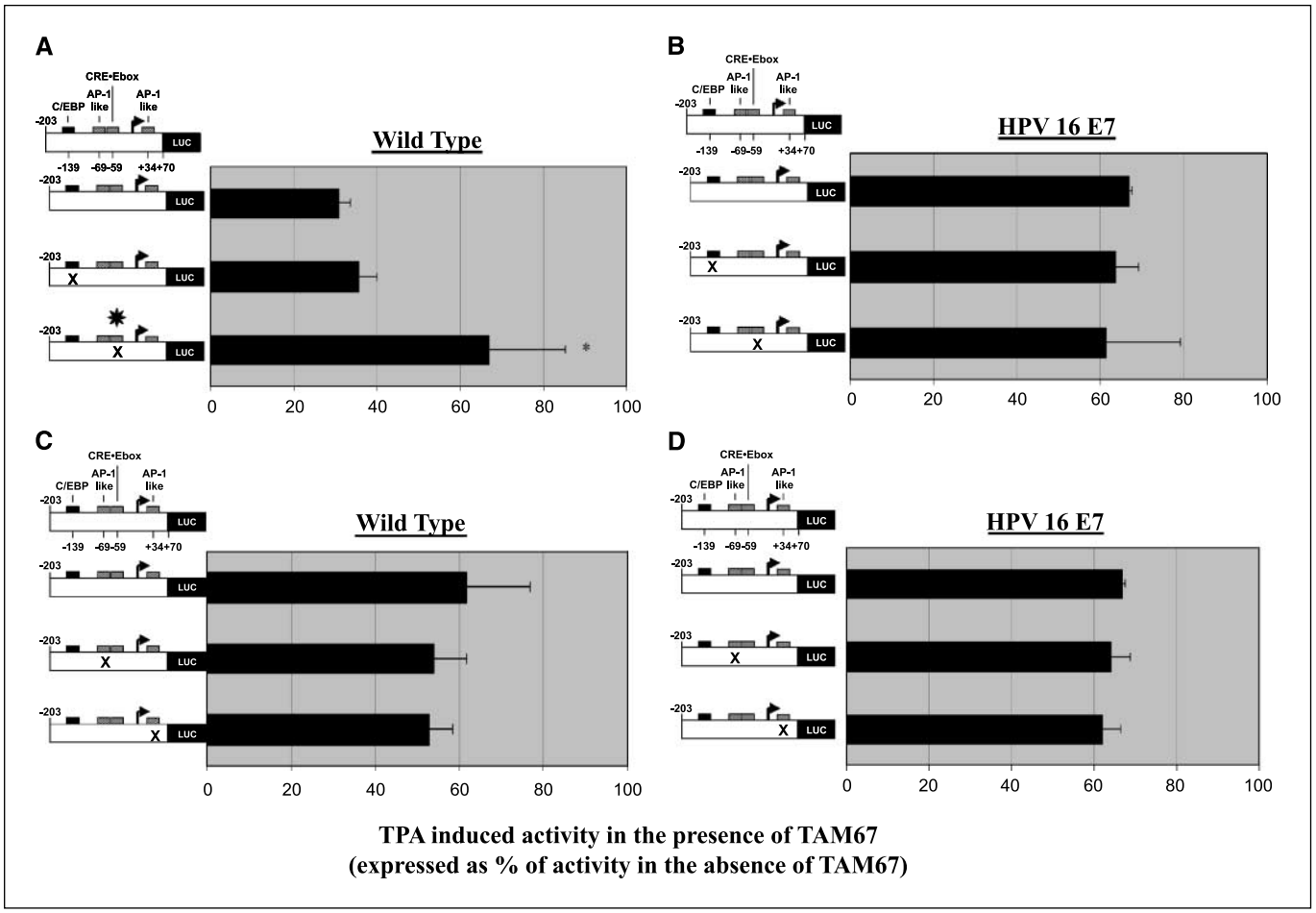

Figure 4. CRE/Ebox is required for suppression of Cox-2 promoter activation by dominant-negative c-Jun (TAM67) in wild-type primary keratinocytes. The relative luciferase activity of mouse Cox-2 promoter reporter deletion and point mutant constructs was measured in the presence of TPA and the presence or absence of transgenic TAM67. Wild-type (*A* and *C*) and E7 transgenic (*B* and *D*) primary keratinocytes were cultured, and luciferase activity was measured after 22 h of TPA treatment. ×, point mutations. *Columns,* averages of six wells and representative of three experiments. *, $P < 0.05$. The 100% value is assigned to the TPA-induced activity of each construct in the absence of TAM67. This 100% value represents a different average relative luciferase unit (RLU) value for each construct (see Fig. 3).

overlap group may have a more global role in tumor promotion. Cox-2, a major enzyme-mediating inflammatory responses and well recognized as a molecular target for chemoprevention, was one of only six genes down-regulated by TAM67 expression under all three tumor-promoting conditions tested (Supplementary Table S1*D*). Despite the lack of a *P* value in the significant range, quantitative PCR analysis confirmed the inhibition of Cox-2 transcription by TAM67 (Table 1), showing more than 4-fold inhibition of TPA-induced expression. As shown in Supplementary Fig. S2, Cox-2 protein levels were not significantly changed at 6 h in response to TPA or to TAM67. Whether transient changes in Cox-2 protein occurred before 6 h or delayed changes occurred subsequent to 6 h is presently unknown. This is the first report of *in vivo* down-regulation of Cox-2 mRNA expression levels by TAM67 under conditions in which TAM67 is known to specifically inhibit tumor promotion.

CRE/Ebox, CAAT/enhancer binding protein, and AP-1–like sites but not NFκB sites are required for Cox-2 promoter activation. To determine the regions of the mouse Cox-2 promoter required for basal and TPA-induced activity in primary keratinocytes, we examined the response of a series of Cox-2 promoter reporter constructs transiently transfected into wild-type and HPV16-E7 transgenic primary keratinocytes to TPA or solvent control. As shown in Fig. 3*A*, the promoter region upstream of −203, containing the highly conserved NFκB site at position −404, is not required for full TPA-induced activity of the Cox-2 promoter in either wild-type or HPV16-E7–expressing keratinocytes. As reported previously for other model systems (26, 41), the conserved CRE/Ebox and CAAT/enhancer binding protein (C/EBP) sites at positions −59 and −139, respectively, are necessary for both basal and TPA-induced activation of the Cox-2 promoter reporter in primary wild-type and E7 transgenic keratinocytes (Fig. 3*B*). Unexpectedly, the AP-1–like site at position −69 is also required for basal and TPA-induced Cox-2 promoter reporter activation in primary keratinocytes (Fig. 3*C*). The AP-1–like site at position −69 was also required for Cox-2 promoter activation in transformation-sensitive (P+) JB6 cells (Fig. 3*C*). To our knowledge, this is the first report of the requirement of this AP-1–like site for Cox-2 promoter activation.

CRE/Ebox is required for suppression of Cox-2 promoter activation by dominant-negative c-Jun (TAM67). We next addressed the question of which transcription factor binding sites are required for suppression of induced Cox-2 promoter activation by dominant-negative c-Jun (TAM67). To identify the

sites through which TAM67 acts to inhibit tumor promoter–induced Cox-2 expression in the mouse skin, we did Cox-2 promoter reporter assays in the presence of tumor promoter and TAM67 in primary keratinocytes. As shown in Fig. 4*C* and *D*, the highly conserved NFκB site (−404) and AP-1–like sites (−69 and +34) are not necessary for TAM67 inhibition of TPA-induced activation of the mouse Cox-2 promoter. Although the CRE/Ebox (−59) is required for the TAM67 inhibition of the TPA-induced Cox-2 promoter activation in wild-type primary keratinocytes (Fig. 4*A*), this site does not seem to be required in HPV16-E7 transgenic keratinocytes (Fig. 4*B*). Thus, TAM67 acts through the CRE/Ebox (−59) to inhibit Cox-2 promoter activation by TPA in wild-type keratinocytes. The sites responsible for TAM67 inhibition of Cox-2 in E7 transgenic keratinocytes were not discernible, possibly owing to low sensitivity associated with the relatively small magnitude of inhibition of transcription from this promoter by TAM67 in the E7 background.

AP-1 site is required for Opn promoter activation by TPA and for suppression of Opn promoter activation by dominant-negative c-Jun (TAM67). To determine the regions of the mouse Opn promoter required for basal and TPA-induced activity in transformation-sensitive JB6 cells, we examined the response of a series of Opn promoter reporter constructs transiently transfected into transformation-sensitive JB6 cells to TPA or solvent control. As shown in Fig. 5*A*, the promoter region upstream of −50, containing the conserved AP-1 site at position −76, is required for full TPA-induced activity of the Opn promoter.

We next addressed the question of whether an AP-1 transcription factor binding site is required for suppression of Opn promoter activation by dominant-negative c-Jun (TAM67). To identify the sites through which TAM67 acts to inhibit tumor promoter–induced Opn expression, we did Opn promoter reporter assays in the presence of tumor promoter and TAM67 in the transformation-sensitive JB6 cells. As shown in Fig. 5*B*, the deletion of the promoter region upstream of −50 shifts the inhibition by TAM67 from 50% to 30% (P = 0.02), suggesting that the −76 AP-1 site is required for the suppression by TAM67 of TPA-induced activity of the Opn promoter.

In summary, induction by TPA of the prometastatic protransformation gene Opn is completely blocked by TAM67 in mouse epidermis. Cox-2, a proinflammatory gene, is down-regulated by TAM67 expression in the presence of TPA, E7, or both. Quantitative PCR confirmed the microarray results for both. Both genes are transcriptional targets of TAM67.

Discussion

This analysis has identified TAM67 target genes that seem to be functionally significant in the mechanism by which the TAM67 inhibitor selectively inhibits tumor promotion and tumor progression. Spp1/Opn, Plaur, MMP-10, and Alox5AP seem to be important during TPA-induced tumor promotion, whereas Cxcl1/Gro1 may be important during E7-enhanced tumor promotion. Cox-2 has emerged as a transcriptional target of TAM67 that is regulated through the CRE site. In addition, a previously uncharacterized AP-1–like site (−69) has been implicated in basal and TPA-induced Cox-2 expression. OPN is a transcriptional target of TAM67 that is regulated in part through an AP-1 site. The implicated TAM67 target genes seem to be regulators of

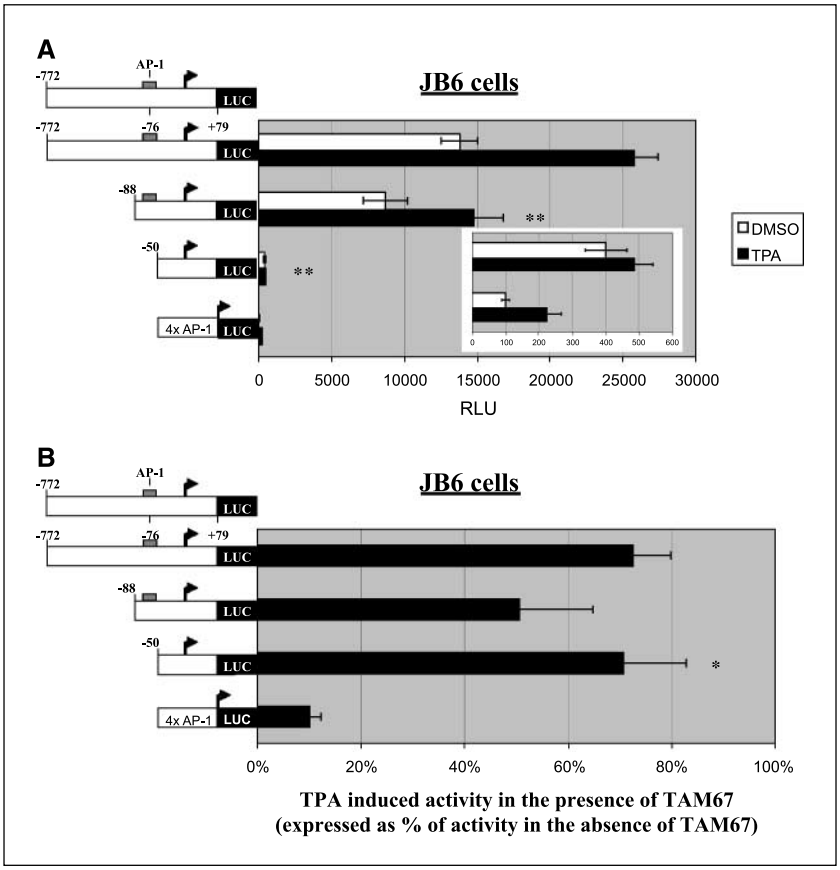

Figure 5. AP-1 site is required for the suppression of Opn promoter activation by dominant-negative c-Jun (TAM67) in transformation-sensitive JB6 cells. JB6 cells were cultured in EMEM containing 4% FBS and treated with TPA (10 ng/mL) or DMSO in EMEM containing 0.2% FBS for 22 h. The relative luciferase activity of a series of mouse Opn promoter reporter deletion constructs was measured in the presence or absence of TPA. *A,* sequences upstream of −88 are required for full TPA-induced promoter reporter activity. Sequences between −88 and −50, including an AP-1 site at position −76, are also required for basal and TPA-induced activity (*A*). *B,* sequences between −88 and −50, including an AP-1 site at position −76, are also required for suppression of Opn promoter activation by dominant-negative c-Jun (TAM67) in transformation-sensitive JB6 cells. The 100% value is assigned to the TPA-induced activity of each construct in the absence of TAM67. This 100% value represents a different average RLU value for each construct (see *A*). *Columns,* averages of six wells and representative of three experiments. Statistical significance was determined using a two-tailed paired *t* test. *, $P < 0.05$; **, $P < 0.01$.

inflammation, invasion, and metastasis rather than regulators of cell proliferation or cell survival, an observation predicted by the phenotypes of the TAM67 mice.

Dominant-negative c-Jun (TAM67) targets invasion/metastasis-associated but not tumor promotion-specific genes during tumor promotion. Interestingly, many of the TAM67 targets implicated after a single 6-h dose of the tumor promoter TPA are genes known to be involved in invasion and metastasis (Fig. 2). Plaur/uPAR, inhibited more than 4-fold by TAM67, is a major driver of tumor cell invasion and metastasis. Many of these genes had not previously been established as important for earlier stages of carcinogenesis. This is a significant observation because these genes now emerge as promising treatment targets during early-, middle-, and late-stage carcinogenesis. Genes important for maintaining tumor phenotype and stimulating aggressive cancer development now emerge as important in tumor promotion and early tumorigenesis. What did not emerge were genes unique to tumor promotion. Examining gene expression at later times during tumor promotion following single or multiple doses of TPA may reveal target genes unique to tumor induction. Because the rate-limiting steps in tumorigenesis occur during tumor promotion and progression, the functionally significant TAM67 targets implicated in tumorigenesis are important targets for cancer prevention. These observations raise the possibility that all of the epigenetic makings of cancer are present early in carcinogenesis during tumor promotion.

Dominant-negative c-Jun (TAM67) targets proinflammatory genes. Targeting proinflammatory genes seems to be important in the mechanism by which the AP-1/NFκB inhibitor TAM67 specifically inhibits tumorigenesis. TAM67–down-regulated genes Plaur/uPAR, Alox5AP, Spp1/Opn, and Ptgs2/Cox-2 are important in urokinase plasminogen activator (PLAU) signaling, leukotriene, and prostaglandin biosynthesis. Alox5ap, critical for the cellular synthesis of proinflammatory leukotrienes, is also one of the genes most inhibited by TAM67. Spp1/Opn, an extracellular matrix molecule overexpressed in many pathologic processes ranging from inflammation to metastasis, was also among the genes in which expression was most inhibited by TAM67. Moreover, Cox-2, although less inhibited, is a well-established mediator of inflammation. It is also interesting to note that markers of inflammatory cell infiltration and angiogenesis, including Cxcl1/Gro1, were down-regulated in tumor promoter–treated TAM67 transgenic mice. Cxcl1 also functions downstream of Cox-2 (42). TAM67 expression seems to short-circuit signaling to the inflammatory response. The inflammatory response is important not only for invasion and metastasis, but also for angiogenesis, a process that is needed for tumorigenesis as well as for tumor progression (43–45).

Dominant-negative c-Jun (TAM67) does not target genes that regulate cell proliferation and survival. As predicted by the selective effects of TAM67 in the mouse model, the transcription of cell proliferation genes (cyclin D3/Ccnd3, Rb1) and cell survival genes (Bcl2l2/Bcl-w, Rela/p65) is not inhibited by TAM67 (Fig. 2). It is important to note that the specificity of TAM67 targeting is dose dependent. Low-dose TAM67 expression, as is seen in the TAM67 transgenic mice, specifically inhibits induction of transcription from 4× AP-1 and NFκB promoter reporters without inhibiting induced transcription from a cell proliferation–associated SRE promoter reporter or cFos (ref. 46 and Supplementary Fig. S3).

Cox-2 and Opn are transcriptional targets of TAM67. Designation of AP-1 target genes is commonly based on overexpression of AP-1–dependent genes such as c-Jun or inspection of promoters to identify recognizable AP-1 sites. Demonstration of transcriptional targeting is often not pursued. TAM67-inhibited RNA expression does not establish a gene as a transcriptional target, but rather calls for analysis of the response to TAM67 of transcription from the gene promoter. Here, the transfection analysis of the Cox-2 and Opn promoters was conducted in cells that also showed TAM67 inhibition of endogenous gene expression. The Cox-2 promoter analysis implicates the CRE, but not NFκB, site as a target of TAM67. The Opn promoter analysis implicates an AP-1 site as a target of TAM67. The lack of observed regulation of TAM67 target genes through NFκB sites is consistent with the apparent lack of NFκB requirement for driving skin carcinogenesis (13). Chromatin immunoprecipitation (ChIP) analysis will be important for further characterization of the regulation by TAM67 of the endogenous Cox-2 and Opn promoters, as well as other endogenous target gene promoters. However, ChIP is restricted by the lack of effective antibodies and the low level of TAM67 expression needed to inhibit transcription. In summary, dominant-negative c-Jun (TAM67) targets genes important in inflammation, invasion, and metastasis, but not cell proliferation or survival when it specifically prevents tumor promotion.

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Dominant-Negative Activator Protein 1 (TAM67) Targets Cyclooxygenase-2 and Osteopontin under Conditions in which It Specifically Inhibits Tumorigenesis

Connie P. Matthews, Alysia M. Birkholz, Alyson R. Baker, et al.

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