Stable Expression of a c-*JUN* Deletion Mutant in Two Malignant Mouse Epidermal Cell Lines Blocks Tumor Formation in Nude Mice[1]

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Abstract

We have stably expressed a *trans*-activation suppressing deletion mutant of the human c-*jun* gene (*TAM-67*) in the malignant mouse epidermal cell lines 10Gy5 and PDV. Expression of the p26 mJUN protein blocked both constitutive and inducible transcriptional *trans*-activation of several AP-1 responsive reporter chloramphenicol acetyltransferase constructs. p26 mJUN was able to block both 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and okadaic acid induced expression of the mouse stromelysin gene in 10Gy5 cells and TPA induced expression of the urokinase-type plasminogen activator gene in PDV cells as determined by Northern analyses. Both genes contain TPA response elements in their promoter regions and are known to be AP-1 responsive. The presence of p26 mJUN in nuclear extracts, as determined by Western blotting, did not detectably alter the DNA binding activity of endogenous AP-1 as determined by gel shift analysis with an oligonucleotide containing a single high affinity AP-1 binding site. UV cross-linking studies coupled with Western analyses identified DNA bound cJUN but not mJUN in nuclear extracts of stably transfected cell lines, suggesting that the mutant JUN protein may exert some of its antioncogenic effects in malignant mouse epidermal cells by a mechanism(s) not involving DNA binding. Malignant mouse epidermal cells which stably expressed the mutant JUN protein were not only inhibited in their AP-1 *trans*-activation response, but also in their ability to form s.c. tumors in nude mice. These results indicate that inhibition of AP-1 mediated transcriptional *trans*-activation alone can be sufficient to suppress the tumorigenic phenotype in a subset of malignant mouse epidermal cells.

Introduction

Cellular responses to external stimuli involve complicated signal transduction pathways resulting in changes in gene expression and, ultimately, phenotypic adaptation. Perturbations in these signal transduction pathways can lead to abnormal cellular responses including cancer. It is becoming increasingly clear that aberrant transcriptional regulation plays an important role in deregulated gene expression and carcinogenesis. For example, repeated applications of the phorbol ester tumor promoter TPA[3] to the skin of mice pretreated with a subcarcinogenic dose of 7,12-dimethylbenz-[a]anthracene leads to the development of benign papillomas (1). Also, treatment with either TPA or another tumor promoter, OA, has been shown to affect the pattern of AP-1 regulated gene expression in both cultured mouse epidermal cells and mouse skin *in situ* (2). Furthermore, TPA or OA treatment of cultured cells leads to a rapid and transient increase in their AP-1 DNA binding and *trans*-activating activity (3, 4). The association between tumor promotion and increased AP-1 activity suggests a causal role for AP-1 during cancer promotion and progression. Conversion from a benign to a malignant phenotype in epidermal cells appears to be mediated at least in part by acquisition of constitutive cellular AP-1 activity. For example, progression from benign to malignant phenotypes was achieved by transfection of v-*fos* into the benign keratinocyte cell lines 308 and SP-1 (5). A radiation associated malignant variant of the benign 308 cell line (10Gy5) also exhibits constitutive AP-1 DNA binding and *trans*-activating ability (6). Finally, loss of AP-1 DNA binding activity in human keratinocytes is associated with differentiation and loss of proliferative potential (7). Taken together, these results indicate that acquisition of constitutive AP-1 activity may be of significant mechanistic importance in the promotion and progression of skin cancer. We hypothesized that inhibition of AP-1 mediated cellular events might result in the inhibition of the malignant phenotype. To test this hypothesis, we expressed a c-*jun* deletion mutant, TAM-67, shown to act as a dominant negative transcription factor (8, 9), in malignant mouse epidermal cell lines.

Two malignant mouse epidermal cell lines used extensively by our laboratory, 10Gy5 and PDV, exhibit constitutive DNA binding and *trans*-activating activity by the AP-1 complex (6). The malignant 10Gy5 cell line was derived by X-irradiation of the benign cell line 308, which does not exhibit constitutive AP-1 activity, but in which AP-1 activity can be induced by treatment with TPA or OA. Since acquisition of constitutive AP-1 activity appears to be important during tumor promotion and progression in mouse skin, we reasoned that blocking this activity might affect tumor formation *in vivo*. A JUN mutant lacking its transcriptional ac-

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[3] The abbreviations used are: TPA, 12-*O*-tetradecanoylphorbol-13-acetate; OA, okadaic acid; AP-1, activator protein 1; CMV, cytomegalovirus; CAT, chloramphenicol acetyltransferase; uPA, urokinase-type plasminogen activator; kD, kilodalton(s); TRE, TPA response element; $-^{R}$, resistant; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl ammonium-methylsulfate.

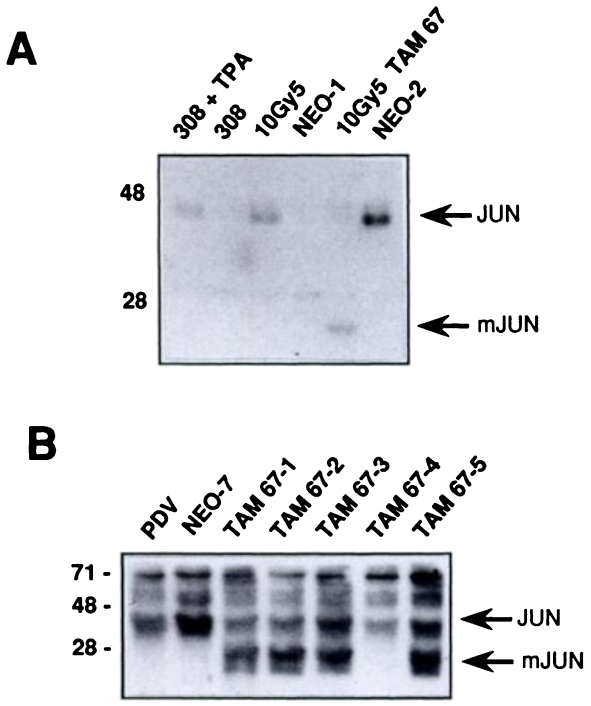


Fig. 1. Western analysis of TAM-67 stable transfectants from (*A*) the 308 lineage, and (*B*) the PDV lineage, showing expression of cJUN and mJUN. Nuclear proteins (*A*, 3 μg; *B*, 5 μg) were resolved by SDS-PAGE in 12.5% gels, transblotted to nitrocellulose, and incubated with 10 μg/ml of a rabbit polyclonal JUN antibody raised against a peptide from the DNA binding domain (see "Materials and Methods"). The 123-amino acid deletion mutant JUN protein is visible as a single band at the predicted molecular weight of 26,000 only in neo^R cell lines transfected with TAM-67.

tivation domain (TAM-67) had already been shown to inhibit AP-1 mediated transcription and oncogene induced cotransformation in rat embryo cells (9). We reasoned that this JUN mutant should also inhibit AP-1 mediated *trans*-activation in malignant mouse epidermal cells, thus suppressing, at least in part, their malignant phenotype. We now report that stable expression of the mutant c-JUN protein p26 mJUN is able to suppress the malignant phenotype of two carcinoma forming mouse epidermal cell lines, 10Gy5 and PDV.

Results

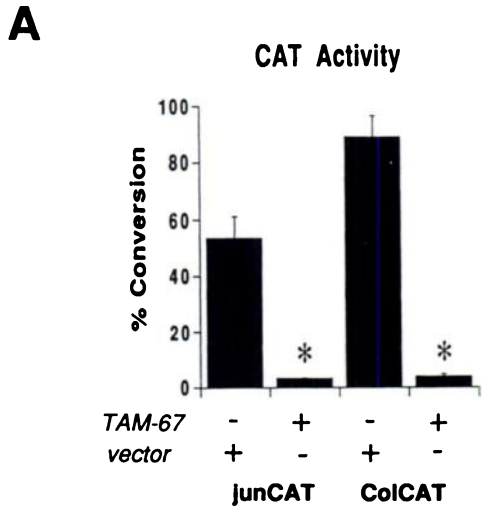
Detection of p26 mJUN by Western Analysis. Presence of the mJUN protein in stable neomycin resistant clones was determined by Western analysis. We anticipated the mass of the mJUN protein to be approximately 26 kD (8, 9). For detection by Western blotting, we chose an antibody that recognizes an epitope in the DNA binding domain which would be present in both the mutant and wild-type JUN proteins. Of two neomycin resistant cell lines derived from 10Gy5 cells transfected with pMexMth TAM-67, one (10Gy5 TAM-67) showed detectable steady-state levels of p26, indicating expression of the mJUN protein (Fig. 1*A*). The TAM-67 transfected clone which did not express the mutant protein was designated "NEO-2." Among neomycin resistant PDV clones transfected with pMexMth TAM-67, 4 of 5 clones expressed the mutant protein (Fig. 1*B*). In repeated experiments, expression of p26 mJUN did not consistently affect the expression of wild-type cJUN protein in stable transfectants derived from the PDV parental cell line (Fig. 1*B*), nor in the 10Gy5 TAM-67 cell line (data not shown).

p26 mJUN Expression Blocks *Trans*-activation of AP-1 Regulated Reporter Constructs. AP-1 mediated transcriptional *trans*-activation was determined by CAT assays on lysates of cells transfected with various AP-1 responsive reporter constructs as described in "Materials and Methods." Results from multiple independent transfections were quantified by phosphorimage analysis as described in "Materials and Methods." Fig. 2*A* illustrates that transient cotransfection of PDV cells with pCMV TAM-67 significantly inhibited transcriptional *trans*-activation of both the Col TRE/TK CAT and the −1.1-kb junCAT reporter constructs compared to cotransfection with reporter plus vector alone. This blocking activity was not due to a general decrease in cellular transcriptional activity, but rather was shown to be specific to TREs. Fig. 2*B* shows that *trans*-activation of a Gal-4 responsive reporter by a GAL-4/VP16 fusion protein was unaffected by TAM-67, whereas *trans*-activation of an AP-1 responsive reporter was inhibited by TAM-67.

Not only was constitutive AP-1 *trans*-activating ability blocked in these cells, but also inducible activity. Fig. 3 shows that OA inducible transcription of the −118 mouse stromelysin CAT (13E) reporter activity was abolished in 10Gy5 cells transfected with TAM-67 relative to cells transfected with vector only. OA inducible activity in the TRE minus reporter, 14E, was also abolished, but the magnitude of the inhibition was considerably less than that of the TRE containing 13E. These results indicate that expression of the mutant protein is sufficient to block both constitutive and inducible AP-1 mediated *trans*-activation even in the presence of wild-type JUN in 10Gy5 and PDV cells, thus confirming the dominant negative nature of the mutant protein.

mJUN Transfectants Exhibit Lower Transcript Levels of AP-1 Regulated Genes. Northern analyses revealed that tumor promoter induced transcription of genes containing TRE *cis*-regulatory elements was blocked or attenuated in both 10Gy5 and PDV cell lines stably expressing mJUN. Both TPA and OA induced transcription of the stromelysin gene was inhibited in the 10Gy5 mJUN cell line, whereas inducible transcript levels of the c-*jun* gene were modestly attenuated (Fig. 4*A*). In stable PDV cell lines expressing mJUN, TPA induced transcription of another AP-1 responsive gene, uPA, was also inhibited relative to the parental PDV cell line and vector-only transfectants (Fig. 4*B*). Fig. 4*B* also shows the 7S ribosomal RNA control for loading and transfer.

mJUN Stable Transfectants Retain Constitutive AP-1 DNA Binding. Electrophoretic gel mobility shift assays determined that constitutive AP-1 DNA binding was retained in all of the mJUN expressing cell lines to the same extent as the parental lines and vector only controls (Fig. 5*A*, and data not shown). The presence of only one shifted band in the mJUN transfected cell line at the same position as those in the parental and vector-only controls suggests that there is a single major form of AP-1 complex in these malignant mouse epidermal cells. The DNA binding complex must be predominantly composed of cJUN and cFOS, since preincubation of PDV TAM-67 nuclear extracts with antibodies specific to either JUN or FOS protein abolished AP-1 DNA binding (Fig. 5*B*). If p26 mJUN protein mediates its effects through DNA binding, one might expect a second bandshift from nuclear extracts of mJUN expressing cells. Although purified recombinant mJUN protein



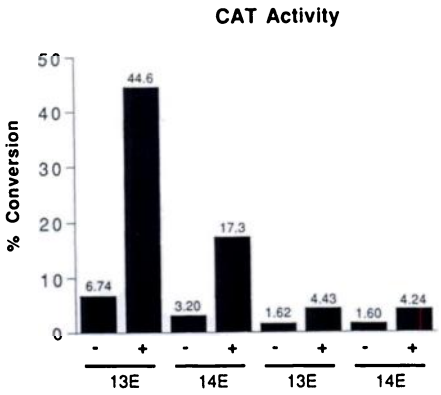


Fig. 3. OA induced transcription from the TRE containing mouse stromelysin promoter is blocked in 10Gy5 cells expressing the JUN mutant. Cells were transfected by DOTAP for 12 h with 3 μg 13E (−118 mouse stromelysin promoter) or 14E (−28 mouse stromelysin promoter) reporter construct along with 3 μg pCMV vector or TAM-67 as indicated. Twelve h after transfection, cells were treated with 50 ng/ml OA (+) or vehicle only (−). Cell lysates were obtained, and 10 μg protein were assayed for CAT activity at 37°C for 6 h. Data were quantified by phosphorimage analysis and are displayed as the mean of two independent transfections.

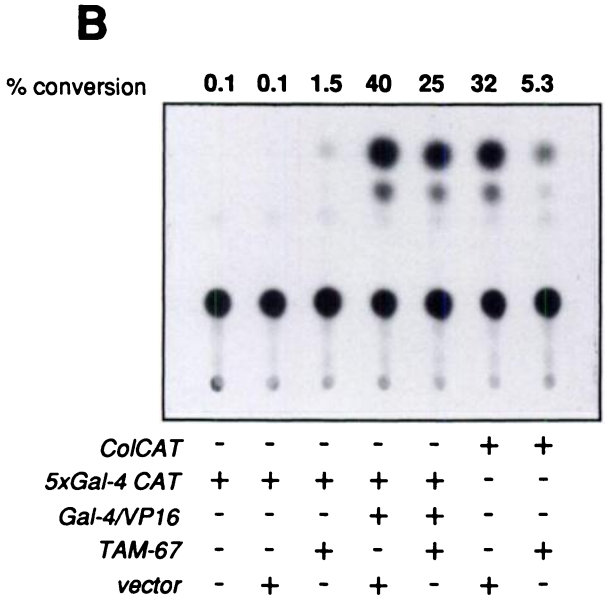


Fig. 2. *Trans*-activation of AP-1 responsive promoters is blocked by expression of TAM-67. *A*, results of CAT assays depicting inhibition of constitutive activation of both the Col TRE/TK CAT and the −1.1-kb junCAT by TAM-67 in PDV cells. Triplicate plates of cells at ~80% confluence were transfected with 3 μg of reporter and 3 μg of either pCMV TAM-67 or vector only by DOTAP for 12 h. After an additional 12 h in serum containing medium, cells were harvested, and lysates were prepared. CAT assays were performed with 20 μg of protein at 37°C for 4 h. Data were quantified by phosphorimage analysis and are displayed as the mean and SE of three independent transfections. *, statistically significant difference from vector-only control (*t* test, $P < 0.05$). *B*, TAM-67 specifically blocks AP-1 regulated transcription. PDV cells were DOTAP transfected for 12 h with 3 μg of each of the constructs indicated. Lysates were obtained, and CAT assays were performed with 20 μg of protein for 4 h. *Trans*-activation of the Gal-4 CAT reporter by the GAL-4/VP16 fusion protein was unaffected by vector or TAM-67, whereas *trans*-activation of the AP-1 responsive Col TRE/TK CAT reporter was specifically inhibited.

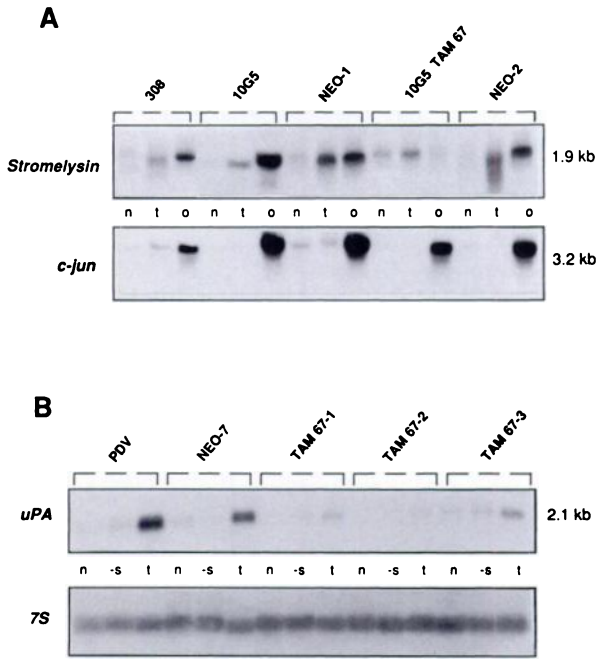


Fig. 4. Northern analyses demonstrating inhibition of tumor promoter induced transcript levels from several AP-1 responsive genes. Ten μg total RNA/lane were size fractionated, blotted, and probed as described in "Materials and Methods." *A*, tumor promoter induced mRNA levels of stromelysin and c-jun in 10Gy5 cells. Cells were serum deprived for 12 h and treated 12 h prior to RNA isolation as follows: *n*, no treatment; *t*, TPA (200 ng/ml); *o*, OA (50 ng/ml). *B*, TPA induced mRNA levels of urokinase-type plasminogen activator in PDV cells. Cells were untreated (*n*), serum deprived (−s), or TPA treated (200 ng/ml) (*t*) for 12 h prior to RNA isolation.

has been previously shown to bind DNA in gel mobility shift assays (9), we have observed no evidence for a second gel shifted complex in our nuclear protein extracts which would be indicative of mJUN bound to DNA.

p26 mJUN from Nuclear Extracts Fails to Bind DNA. To further examine the mechanism(s) by which mJUN mediates its inhibition of AP-1 regulated signal transduction, we performed UV cross-linking experiments to identify which protein(s) in our nuclear extracts were, in fact, capable of bind-

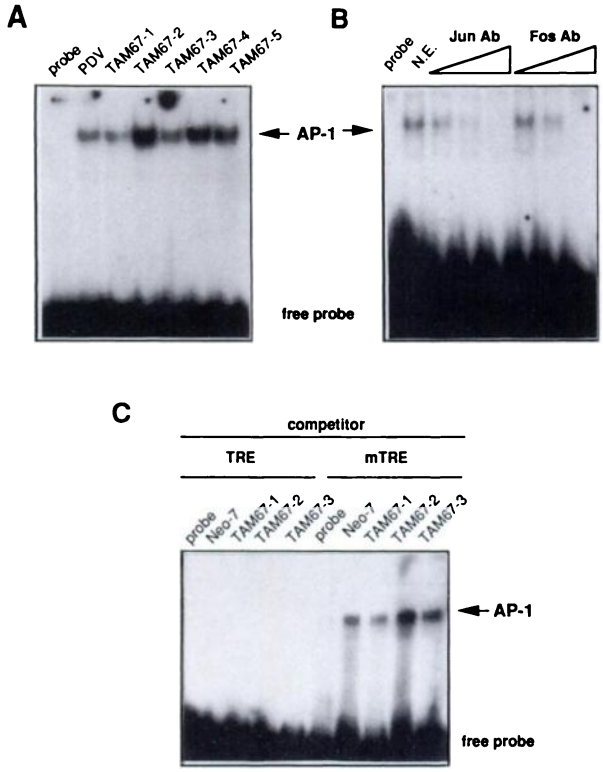


Fig. 5. Nuclear extracts (*N.E.*) of transfected cell lines retain normal AP-1 DNA binding. *A*, 1 μg of nuclear protein was incubated for 15 min with a ^{32}P labeled double stranded oligodeoxynucleotide probe containing the human collagenase TRE sequence. Samples were electrophoresed at 20 mA in a 5% polyacrylamide gel to resolve DNA bound complexes, wrapped in cellophane, and exposed to X-ray film. *B*, mJUN expressing cells exhibit a single major gel shifted complex consisting primarily of JUN and FOS. One μg nuclear protein was preincubated for 1 h at room temperature either alone or with 5, 10, or 15 μg antibody specific to either JUN or FOS, then incubated for 15 min with probe, electrophoresed, and exposed to X-ray film. *C*, the major gel shifted complex was not competed by a 15-min preincubation with unlabeled mutant TRE-like (*mTRE*) oligonucleotide, whereas preincubation with unlabeled wild-type TRE oligonucleotide completely abolished the shifted complex.

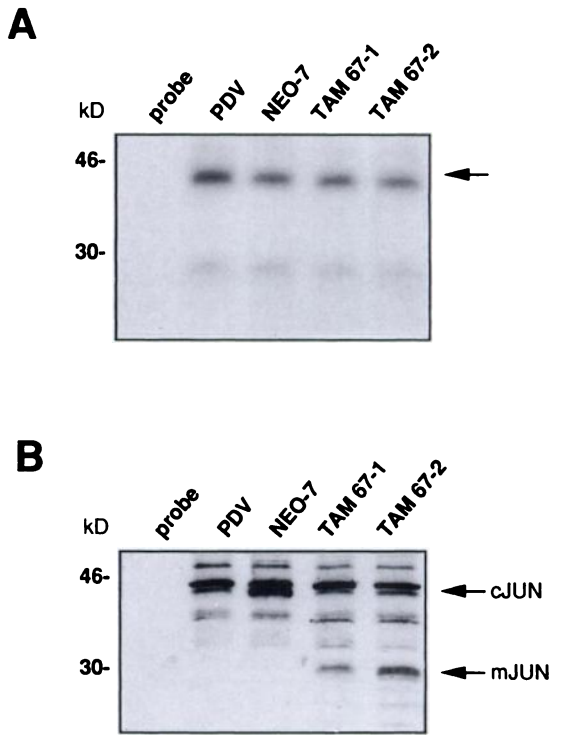


Fig. 6. *A*, UV cross-linking analysis showing a single prominent band (*arrow*) with an apparent molecular weight consistent with that of cJUN. Gel shift binding reactions were exposed to ~500 J/m² UV light from a UV transilluminator over 15 min at 4°C. Laemmli buffer was added, and the samples were boiled for 5 min and resolved by SDS-PAGE in a 12.5% gel. The free probe was cut off the bottom, and the gel was wrapped in cellophane and exposed to X-ray film for 48 h at −80°C. *B*, Western analysis showing both cJUN and mJUN present in the nuclear extract. Proteins in the UV cross-link gel (*A*) were transblotted to nitrocellulose, and JUN immunoreactivity was identified with 10 μg/ml of JUN specific antibody (Ab-1). Immunoreactivity was detected with horseradish peroxidase conjugated goat anti-rabbit IgG secondary antibody and the enhanced chemiluminescence reagent luminol (Amersham). The blot was wrapped in cellophane and exposed to X-ray film for 10 s.

ing to the radiolabeled TRE oligonucleotide. The results, shown in Fig. 6*A*, indicate a single major cross-linked protein (*arrow*) with an apparent molecular weight consistent with that of cJUN. A second, weaker activity was also detected in all of the samples, but there was no indication of differential binding activity between TAM-67 transfectants and vector-only transfectants or parental PDV cells. A longer exposure revealed a band with a molecular weight consistent with that of FOS, but still no difference in signal intensities between the TAM-67 transfectants and vector-only transfectants or parental cells in the region of 26 kD. The identity of the darker band as cJUN was confirmed by Western analysis of the gel, shown in Fig. 6*B*, as described in "Materials and Methods." The Western analysis also confirmed the presence of p26 mJUN in nuclear extracts of TAM-67 stably transfected cells (Fig. 6*B*).

mJUN Inhibits Tumor Formation in Nude Mice. Among cell lines which stably expressed the mJUN protein, *in vivo* tumor forming potential was markedly reduced compared to parental cell lines and vector-only transfectants. Table 1 illustrates that 10Gy5 mJUN was completely inhibited from tumor formation, whereas two different 10Gy5 NEO^R cell lines and parental 10Gy5 cells retained their tumor forming ability. Among PDV mJUN expressing cell lines, 4 of 8 cell lines were inhibited with respect to their ability to produce tumors in nude mice relative to the parental PDV cells and NEO^R vector-only transfectants. This inhibition of tumor growth could not be accounted for by decreased proliferation of the TAM-67 transfectants, since their growth rates *in vitro* were no different than those of vector-only transfectants or parental cells (data not shown). The finding that any PDV transfectant was nontumorigenic was striking. Among dozens of NEO^R PDV stable transfectants generated over several years in our laboratory and produced with a variety of different constructs, none has ever failed to form tumors in nude mice.[4]

Discussion

Deregulated expression of AP-1 regulated genes is a hallmark of tumor promotion in the mouse skin by TPA (2).

[4] F. E. Domann, A. Borchers, and G. T. Bowden, unpublished observations.

Table 1 Tumor development by mutant JUN transfectants in nude mice

Cell line	p26mJUN expression	No. of mice with tumors	Average time to tumor (weeks)
PDV	–	2/2, 2/2	4, 4
PDV-2	–	4/4, 4/4	4, 4
PDV-6	–	4/4, 4/4	4, 4
NEO-7	–	2/2, 2/2	4, 4
NEO-21	–	2/2, 2/2	4, 4
2/NEO-1	–	4/4, 4/4	4, 4
5/NEO-1	–	4/4, 4/4	4, 4
12/NEO-1	–	4/4, 4/4	4, 4
15/NEO-2	–	4/4, 4/4	4, 4
TAM-67-4	–	2/2, 2/2	4, 4
TAM-67-1	+	0/2, 1/2	>8, 8
TAM-67-2	+	2/2, 2/2	4, 4
TAM-67-3	+	0/2, 0/2	>8, >8
TAM-67-5	+	2/2, 2/2	4, 4
10/TAM-67-5	+	0/4, 0/4	>6, >6
11/TAM-67-1	+	0/4, 1/4	>6, 6
6/TAM-67-9	+	4/4, 4/4	4, 4
6/TAM-67-8	+	4/4, 4/4	4, 4
308	–	0/2, 0/2	>16, >16
10Gy5	–	2/2, 2/2	12, 12
NEO-1	–	2/2, 2/2	12, 12
NEO-2	–	2/2, 2/2	12, 12
TAM-67	+	0/2, 0/2	>16, >16

Based on our published data (6), we hypothesized that acquisition of constitutive AP-1 DNA binding and *trans*-activating ability was causally associated with malignant progression in mouse keratinocytes. To test this hypothesis, we stably expressed the dominant negative transfactor TAM-67 in two different malignant mouse epidermal cell lines. Expression of the p26 mJUN protein in these cell lines was associated with suppression of their tumor forming potential in nude mice. Although inhibition of oncogene mediated transformation by dominant negative transcription factors has been investigated previously (10–12), inhibition of tumor forming ability in malignant cell lines generated by physical or chemical carcinogens has not previously been reported. This is an important observation because tumor formation *in vivo* is arguably the most relevant endpoint from a carcinogenesis point of view. Thus, a single dominant acting antioncogene can mediate, at least in part, inhibition of a malignant phenotype in the mouse skin model of multistage carcinogenesis.

As mentioned, the idea of using dominant negative transcription factors to reverse oncogene mediated transformation is not new. For example, Okuno *et al.* (10) used a dominant negative c-FOS mutant to effectively inhibit JUN mediated transformation in chicken embryo fibroblasts. Similarly, Sawyers *et al.* (11) used a dominant negative c-MYC to block BCR-ABL mediated transformation of primary mouse pre-B marrow cells. Inhibition of c-*jun*/*ras* and c-*fos*/*ras* mediated cotransformation of rat embryo cells by the protein encoded by the TAM-67 construct has also been previously reported, as has its ability to inhibit AP-1 mediated transcriptional *trans*-activation (8, 9). Recently, inhibition of tumor development *in vivo* was reported for a LexA/v-JUN chimeric protein expressed in *ras* transformed NIH 3T3 cells (13). However, to our knowledge, we present the first evidence of a dominant negative c-JUN mutant capable of suppressing *in vivo* tumor formation by carcinogen initiated malignant tumor cells.

Interestingly, one-half of the PDV TAM-67 stably transfected clones in this study were not inhibited from tumor formation in the nude mouse assay. This observation indicates that inhibition of AP-1 mediated signal transduction is not always sufficient to inhibit tumorigenesis in this model system. These results could be explained by clonal heterogeneity with respect to stage of progression. Since carcinogenic progression is a multistage process, it is reasonable to suggest that different clones within a population could exist at various stages of progression, and that dependence on AP-1 activity for malignant progression may no longer be necessary after a certain stage. If this is the case, then we may actually have identified some of these AP-1 independent variants among our clones which stably express the dominant negative JUN protein but which are not inhibited from tumor formation *in vivo*.

It has been shown previously that the purified TAM-67 mutant JUN protein can bind a TRE containing oligonucleotide *in vitro* (9). Therefore, the anticipated mechanism of action of this *trans*-activation domain deletion mutant was to "quench" or "block" endogenous cJUN/AP-1 activity (9) and thereby suppress AP-1 mediated *trans*-activation of gene promoters regulated by TREs. Our findings indicate, however, that although cJUN immunoreactivity and sequence specific DNA binding activity were readily detectable under our assay conditions, only immunoreactivity and not DNA binding activity was observed for p26 mJUN. One possible explanation for this observation is that we measured sequence specific DNA binding activity with only one type of AP-1 binding site (the human collagenase gene promoter TRE). Since there is probably a great deal of sequence heterogeneity with respect to what constitutes a TRE *in vivo*, it is still possible that mJUN binds TREs *in vivo* or in other promoter contexts. Sequence heterogeneity of AP-1 *cis*-regulatory elements *in vivo* would provide a number of potential targets through which mJUN could mediate its inhibitory effects on tumor formation by malignant mouse epidermal cells. Alternatively, our findings suggest that mJUN could mediate some of its antioncogenic effects through a mechanism independent of DNA binding in malignant keratinocytes. This notion is somewhat reminiscent of previous studies in which transformation and *trans*-activation by another AP-1 component, FOS, were not found to be closely associated. For example, studies with the FBR murine sarcoma virus v-*fos* oncogene showed that DNA binding and TRE dependent *trans*-activation were not required for the transforming properties of this oncoprotein (14). Similar results demonstrating a lack of correlation between transformation and TRE dependent *trans*-activation were obtained using a temperature sensitive mutant, v-FOS (15).

Our results suggest that maintenance of a malignant phenotype can be disrupted by the expression of exogenous proteins that interfere with AP-1 mediated signaling. Several endogenous negative regulators of JUN function have also been identified. One such inhibitor of JUN, IP-1, is a protein which has been shown to negatively regulate JUN DNA binding (16). Another protein, Jif-1, a recently identified JUN binding protein and putative tumor suppressor, has been shown to interact with the leucine zipper region of JUN and to decrease DNA binding and *trans*-activation by JUN, suggesting that Jif-1 is an endogenous negative regulator of JUN (17). Other as yet unidentified cellular proteins also probably

bind to and regulate JUN function. Thus, in addition to regulation by phosphorylation (18) and redox control mechanisms (19), novel protein:protein interactions appear to provide another level of regulation of JUN and, therefore, of AP-1 activity. We speculate that mJUN could mediate its activity in mouse epidermal cells through a mechanism analogous to that of other JUN interacting factors. Such a mechanism would not necessarily require DNA binding, but rather, may involve protein:protein interactions which sequester or render inactive a necessary component(s) of the AP-1 signal transduction pathway or transcriptional initiation complex. We are currently engaged in studies to identify these potential interactions. In conclusion, the results that we have obtained here support the idea that constitutive AP-1 activity plays a functional role in the maintenance of the malignant phenotype of mouse squamous cell carcinoma cells.

Materials and Methods

Cell Lines and Culture Conditions. The benign epidermal cell line 308 (20) was generously provided by S. H. Yuspa. 10Gy5 cells were derived from a surviving clone of 10Gy X irradiated, logarithmically growing 308 cells. Early passage squamous cell carcinoma producing PDV cells (21) were a gift of N. E. Fusenig. All other cell lines were stably transfected clones of either one of the parental cell lines. Cell cultures were maintained in minimal essential medium (GIBCO) supplemented with 7.5% fetal bovine serum (GIBCO), 2.5% calf serum (GIBCO), and 100 units/ml penicillin/streptomycin (GIBCO). Cells were trypsinized and reseeded weekly at a 1:6 dilution and were fed fresh medium every 3–4 days.

Plasmids. The construction of mammalian expression vectors containing the human c-JUN transcriptional activation mutant (TAM-67) has been described previously (8). Briefly, in pCMV TAM-67, expression of the mutant complementary DNA is driven by the CMV promoter. In pMexMth, TAM-67 expression is driven by the mouse metallothionein I promoter. Only the latter construct expresses the bacterial neomycin resistence gene (NEO^R) and can therefore be adequately selected for stable expression. Transcriptional *trans*-activation assays were performed by transient transfection of the cells with CAT reporter constructs containing AP-1 responsive elements in several promoter contexts. These reporters included the human collagenase promoter Col TRE/TK Cat (22) (kindly provided by P. Angel), the jun promoter (−1.1-kb junCAT) (23) (kindly provided by M. Karin), and the −118 mouse stromelysin promoter (13E).[5] The −28 mouse stromelysin promoter (14E), which lacks the TRE, served as the control for AP-1 specific effects on this promoter. To determine specificity of TAM-67 mediated transcriptional inhibition, we used a reporter whose expression is not regulated by AP-1, but rather by the yeast specific *trans*-activator protein, GAL-4. When cotransfected with an expression construct encoding a chimeric *trans*-activator composed of the GAL-4 DNA binding domain fused to the VP16 *trans*-activation domain, reporter expression is activated (24). These constructs were kindly provided by P. Robbins.

[5] J. P. Levy, F. E. Domann, K. Holliday, H. Fujiki, and G. T. Bowden. Unique okadaic acid regulation of the mouse stromelysin gene, manuscript in preparation.

Transfections. For transient transfections, 2–5 μg DNA were delivered by liposome mediated transfer using DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's specifications. To generate stable lines, cells were transfected by electroporation. Briefly, cells were harvested by trypsinization and washed twice with HBS (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-70 mM NaCl-0.75 mM Na_2PO_4, pH 7.1). Then 2×10^6 cells were resuspended in 1 ml HBS, mixed with 10 μg of each DNA to be transfected, and chilled on ice for 5 min, after which they were subjected to a charge of 250 μFarads at a 250 V potential using a Bio-Rad electroporator. The cells were immediately plated in minimal essential medium containing dishes and incubated at 37°C for 48 h. Cells were then selected for stable plasmid integration using the neomycin analogue geneticin (G418; GIBCO) at a concentration of 250 μg/ml active G418 for the PDV cells and 400 μg/ml for the 10Gy5 cells. After the initial selection period of 3–4 days, the medium and dead cellular debris were removed from the plate, and the plates were fed fresh selection medium. Within 4–6 weeks after transfection, discrete colonies were ring cloned from the plates into 24-well plates and expanded under continued selection. Isolated clones were then screened by Western analysis for their ability to produce the mutant JUN protein.

Western Analysis. Nuclear protein extracts were prepared as described (25) and quantified with Bio-Rad reagent. These extracts were used for both Western and gel mobility shift assays. For Western analysis, 5 μg of nuclear protein were boiled for 5 min in Laemmli buffer and subjected to SDS-PAGE. Samples were electrophoresed through a 3% polyacrylamide stacking gel and resolved in a 12.5% gel. The size fractionated proteins were transferred to nitrocellulose by electroblotting at 100 V for 2 h. The nitrocellulose membrane was blocked in 2% nonfat dry milk in TBST (10 mM Tris, pH 8.0-150 mM NaCl-0.05% Tween-20) for 1 h. The blot was washed once in TBST and primary antibody (anti-c-Jun Ab-1; Oncogene Science) was added at a dilution of 1:10 in TBST milk. The blot was rocked at 4°C for 12 h and then washed three times with TBST. The blot was probed with ^{125}I-labeled goat anti-rabbit γ-globulin in TBST at a dilution of 1:3000 for 1 h and then washed three times in TBST. The blot was wrapped in Saran wrap and exposed to X-ray film for 12–24 h at −80°C.

Northern Analysis. Logarithmically growing cells were serum deprived for 12 h, and then treated with TPA (200 ng/ml) or OA (50 ng/ml) for 12 h. Total cellular RNA was isolated by guanidinium lysis as described (26). RNA was quantitated by spectrophotometry, and 10 μg total RNA were added to each lane. Samples were electrophoresed and blotted to GeneScreen (DuPont). Conditions for prehybridization, hybridization, and washouts were as described (2). Northern blots were probed with random primed ^{32}P labeled probes made from partial complementary DNAs of the mouse stromelysin, c-*jun*, *uPA*, and *7S* genes as described (2).

CAT Assays. Multiple 60-mm plates of cells were transfected by DOTAP as described above with 2–5 μg reporter construct either alone or with 2–5 μg vector or TAM-67 construct. Cells were transfected at 75–80% confluence, treated as indicated, and harvested for CAT assays. After treatment, cells were scraped in 250 mM Tris, pH 7.5, sonicated for 10 s, and incubated at 65°C for 10 min to inactivate endogenous acetylases. Samples were centrifuged for 5 min, and

the protein concentrations in the supernatants were determined with Bio-Rad reagent. An aliquot of this lysate (10–20 μg protein) was incubated with approximately 250,000 cpm [^{14}C]chloramphenicol (NEN) and 1 μg/ml butyryl-CoA (Sigma) at 37°C for 4–6 h. The incubation mixture was extracted with ethyl acetate, and the organic phase was harvested, dried, redissolved in 15 μl chloroform, and applied to the origin of a thin layer chromatography plate. The chromatograph was resolved in 95:5 chloroform:methanol solvent at room temperature. The thin layer chromatography plates were dried and exposed to film overnight, or exposed to a phosphorimaging plate for 2–4 h. Phosphorimaging plates were scanned and quantified with a Molecular Dynamics phosphorimaging system.

Gel Mobility Shift Assays. Oligonucleotide probes were labeled by incorporation of [^{32}P]dCTP (NEN) into the 5′ overhangs of the annealed, double stranded human collagenase TRE oligonucleotide 5′-agcttg**TGAGTCA**gccgctag-3′ (27) with Klenow DNA polymerase. The oligonucleotide binding assay was performed by mixing approximately 200,000 cpm probe with 1 μg of nuclear protein extract isolated as described above in the presence of 1 μg of polydeoxyinosinic-deoxycytidylic acid (Pharmacia) and 1× gel shift buffer (10 mM Tris, pH 7.5-50 mM NaCl-1 mM $MgCl_2$-0.5 mM EDTA-0.5 mM dithiothreitol-4% glycerol) at room temperature for 15 min. Competition experiments were performed with either the unlabeled TRE oligonucleotide or a mutant TRE-like oligonucleotide, 5′-agctTGAttacCTCAtccgctag-3′ (27). In antibody clearing experiments, various amounts of antibodies specific to JUN (Ab-2; Oncogene Science) or FOS (Ab-1; Oncogene Science) were preincubated with nuclear extracts for 1 h at room temperature prior to addition of probe. The binding reaction contents were resolved by gel electrophoresis on a 5% polyacrylamide gel. The gels were wrapped in cellophane and exposed to X-ray film for 4–24 h. Specific DNA-protein interactions were visualized as upwardly shifted bands in the gel.

UV Cross-Linking Analysis. For UV cross-linking studies, large scale (5×) gel shift binding reactions were set up and incubated for 10 min at room temperature followed by 10 min at 4°C. Binding reactions were exposed to broad spectrum UV light from a UV transilluminator at 4°C for 15 min (~500 J/m^2). Laemmli buffer was added, and the samples were boiled for 5 min and resolved by SDS-PAGE in a 12.5% gel. The gel was wrapped in cellophane and exposed to film for 48 h at −80°C to detect DNA cross-linked proteins. Subsequently, proteins present in the gel were electroblotted to nitrocellulose for 2 h at 100 V. The nitrocellulose membrane was then treated as a Western blot and incubated with the anti-cJUN antibody (Ab-1) as described above. Horseradish peroxidase conjugated goat anti-rabbit IgG was used as secondary antibody, and the signal was detected with the enhanced chemiluminescence reagent luminol (Amersham). The rapid signal detection (seconds) afforded by luminol allowed for the detection of immunoreactivity without interference from radioactively labeled proteins.

Tumorigenicity Assay. To assess the tumor forming potential of the parental and transfected cell lines, cells in log growth phase *in vitro* were trypsinized, washed in serum-free medium, and quantified by Coulter counter. Then 2 × 10^6 10Gy5 or 1 × 10^4 PDV cells were injected s.c. into athymic mice (Charles River) at each of two or four sites per mouse. Two mice were given injections of each cell line for a total of four sites per cell line. The mice were monitored twice weekly for the development of tumors. Sites were scored as positive for tumor growth when they reached 5 mm in diameter. Mice without tumors were observed for an additional 4 weeks after mice which developed tumors were removed from the study.

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