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Blocking of tumor promoter-induced AP-1 activity inhibits induced transformation in JB6 mouse epidermal cells

(c-*jun*/phorbol esters/epidermal growth factor/cell transformation)

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ABSTRACT **AP-1 transcriptional activity is stimulated by the transformation promoters phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) and epidermal growth factor (EGF) in promotion-sensitive (P^+) but not in promotion-resistant (P^-) JB6 mouse epidermal cell lines. Although TPA stimulates expression of the *jun* and *fos* family genes, only c-*jun* expression shows higher elevation in P^+ cells than in P^- cells. The present study tests the hypothesis that induced AP-1 activity is required for tumor promoter-induced transformation in JB6 P^+ cells. Both retinoic acid and the glucocorticoid fluocinolone acetonide inhibited basal and TPA-induced AP-1 activities that were tested with a stromelysin promoter–chloramphenicol acetyltransferase reporter gene in P^+ cells. Since both retinoic acid and fluocinolone acetonide are active in inhibiting TPA-induced anchorage-independent transformation of P^+ cells in the dose range that blocks TPA-induced AP-1 activity, their antipromoting effects may occur through inhibition of AP-1 activity. To test the hypothesis with a more specific inhibitor, stable clonal transfectants of P^+ cells expressing dominant negative c-*jun* mutant encoding a transcriptionally inactive product were analyzed. All transfectants showed a block in TPA and EGF induction of AP-1 activity. All transfectants also showed inhibition of TPA-induced transformation, and most transfectants showed a block in EGF-induced transformation. These results indicate that AP-1 activity is required for TPA- or EGF-induced transformation. This work demonstrates that a specific block in induced AP-1 activity inhibits tumor promoter-induced transformation.**

Chemical carcinogenesis is a multistep process that includes initiation, promotion, and progression (1–4). While the initiation step is short-term and irreversible, tumor promotion is a long-term process that is partially reversible and requires chronic exposure to tumor promoter. The rate-limiting steps in multistage carcinogenesis occur during the promotion and progression phases. The JB6 mouse epidermal cell system of clonal genetic variants that are promotion sensitive (P^+) or promotion resistant (P^-) has enabled the study of genetic susceptibility to transformation promotion at the molecular level. In P^+ JB6 cells phorbol 12-myristate 13-acetate ("12-*O*-tetradecanoylphorbol 13-acetate," TPA) and epidermal growth factor (EGF) induce the irreversible formation of large, tumorigenic, anchorage-independent colonies in soft agar at a high frequency. In contrast, the P^- cells exhibit a response in soft agar that is 0.1–1% that of P^+ cells and the colonies are considerably smaller (5, 6).

A number of P^+/P^- differences in the response pathway for tumor promoter-induced transformation have been reported (7–10). A noteworthy difference involves the activator protein 1 (AP-1) complex, which activates gene expression in response to tumor promoters in P^+ but not in P^- JB6 cells (9). AP-1 is a heterodimeric complex containing products of the *jun* and *fos* oncogene families (11–13). Analysis of *jun* and *fos* family expression indicates that c-Jun, but not JunB, JunD, or c-Fos protein, may be a limiting constituent responsible for induction of AP-1 transcriptional activity in P^+ cells (14). The AP-1 complex transcriptionally activates genes that contain the sequence TGA(G or C)TCA, referred to as the AP-1 binding site or TPA-responsive element (TRE), in their promoters (11–13). There are several candidates for genes that respond to AP-1 that may be involved in tumor promotion or progression, including the genes for the metalloproteinases collagenase and stromelysin (transin) (15–17).

Brown *et al.* (18) have shown that a dominant negative c-*jun* mutant which specifically blocks AP-1 activity also blocks Ha-*ras* plus c-*jun*-induced cellular transformation. Retinoic acid (RA) and glucocorticoids have also been shown to block AP-1 activity (19–23). In this report, we have used both pharmacologic and molecular inhibitors to block induced AP-1 activity and to test the hypothesis that induced AP-1 activity is required for the tumor promoter-induced transformation response in P^+ cells.

MATERIALS AND METHODS

Materials. Fetal bovine serum was from BioWhittaker, TPA from Chemicals for Cancer Research, and EGF from Collaborative Research. [^{14}C]Acetyl coenzyme A and *in vivo* labeling grade [^{35}S]methionine were from Amersham. Lipofectin reagent was from BRL. Fluocinolone acetonide (FA) and RA were from Sigma. pHIV-CAT reporter plasmid was kindly provided by David Derse (Frederick Cancer Research and Development Center).

Cell Culture. Mouse epidermal JB6 P^+ cells (5, 6, 9) were grown at 36°C in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 25 μg of gentamicin per ml.

Immunoprecipitation of c-Jun and TAM67 Protein. TAM67 transfectants and vector-only transfectants were treated for 15 hr with TPA (10 ng/ml), EGF (10 ng/ml), or dimethyl sulfoxide (DMSO, 0.1%) prior to metabolic labeling with [^{35}S]methionine (0.2 mCi/ml; 1 mCi = 37 MBq). Cell lysates were collected (14) and immunoprecipitated with polyclonal

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Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; EGF, epidermal growth factor; P^+, promotion sensitive; P^-, promotion resistant; TRE, TPA-responsive element; CAT, chloramphenicol acetyltransferase; RA, retinoic acid; FA, fluocinolone acetonide; TNFα, tumor necrosis factor α; DMSO, dimethyl sulfoxide; HIV-1, human immunodeficiency virus type 1.

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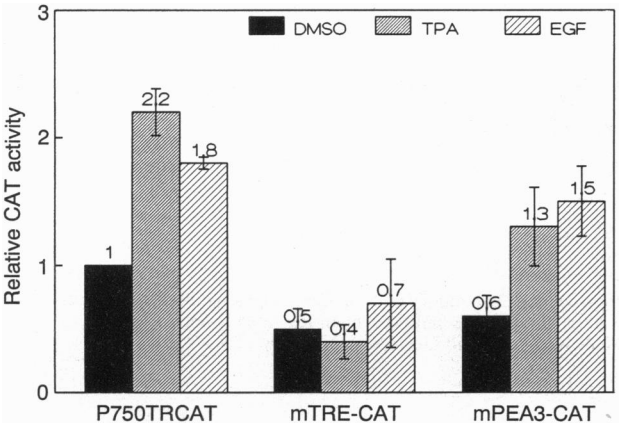

FIG. 1. AP-1 binding site is necessary for TPA- or EGF-induced stromelysin promoter-controlled transcription. JB6 P+ Cl 41 cells were transfected by a calcium phosphate method with 10 μg of P750TRCAT or mTRE-CAT or mPEA3-CAT plasmid DNA and 10 μg of sheared genomic DNA isolated from Cl 41. Results are expressed as the mean ± SEM of three experiments. Both TPA- and EGF-induced AP-1 dependent activities are statistically significantly different from that in the control group (Student *t* test: TPA vs. DMSO, $P < 0.05$; EGF vs. DMSO, $P < 0.01$).

rabbit c-Jun antibody (Ab-1, Oncogene Science). Immunoprecipitates were analyzed by SDS/12.5% PAGE.

Assay for Promotion and Antipromotion of Anchorage Independence. JB6 P+ clone (Cl) 41 and Cl 41.5a cell lines were exposed to TPA in 0.33% agar medium. For antipromotion assay, antitumor promoters were added with TPA and simultaneously diluted into the soft-agar cell medium. TPA-dependent colony induction was determined at 14 days.

Transfection and Chloramphenicol Acetyltransferase (CAT) Assay of AP-1 Activity and NF-κB Activity. Recombinant DNA containing 750 bp of the rat stromelysin promoter (P750TRCAT) driving the heterologous CAT reporter gene contained an AP-1 binding sequence at position −70 (15, 24, 25). The mTRE-CAT reporter, a site-directed mutant with two point mutations in the AP-1 site (15), and mPEA3-CAT, a site-directed mutant with a point mutation in each of the PEA3 sites at positions −208 to −200 and −191 to −199 of the stromelysin promoter, were produced by using the oligodeoxynucleotide 5′-GCAAGAAGCATTTCTTGG-3′ and a site-directed mutagenesis kit (Amersham, version 2). JB6 Cl 41 cells were transfected for 4–5 hr by calcium phosphate procedures with 10 μg of the CAT reporter plasmid DNA and 10 μg of sheared genomic DNA isolated from Cl 41; cells were then changed to medium containing 2% fetal bovine serum. Additions were 24–42 hr later and total cell extracts were prepared after 8 hr of treatment. CAT enzyme activity was measured by a diffusion-based assay (New England Nuclear). The results are expressed as the relative rate of accumulation of [^{14}C]acetylated product. Relative AP-1-dependent activity was calculated as the value with P750TRCAT minus the value with mTRE-CAT. NF-κB-dependent CAT activity was tested in JB6 cells just as described for the stromelysin–CAT reporter gene except that different times of TPA treatment and amounts (10 or 30 μg) of pHIV-CAT plasmid reporter DNA were used. The pHIV-CAT reporter (26) contains a 196-bp *Taq* I–*Hin*dIII fragment of the long terminal repeat of human immunodeficiency virus type 1 (HIV-1) with two NF-κB binding sites linked to the CAT gene. In HeLa cells, transfection was for 12 hr and treatment with TPA or tumor necrosis factor α (TNFα) was for 3 or 4 hr.

Stable Transfections and G418 Selection. Stable transfection of JB6 Cl 41 cells was performed with the pMexMTH-neoTAM67 (TAM67) or the pMexMTH-neo plasmid. pMex MTH-neo is a mammalian expression vector in which the gene of interest is under the transcriptional control of the mouse metallothionein promoter. TAM67 is a truncated transcriptionally inactive form of c-*jun* (18). Two micrograms of plasmid DNA was transfected with Lipofectin reagent (BRL) into mouse JB6 P+ Cl 41 cells at 50–70% confluence in 60-mm dishes, according to the manufacturer's recommendation, and the cells were selected in medium containing the neomycin analogue Geneticin (G418, GIBCO) at 400 μg/ml. Individual clones were ring-isolated and expanded in the presence of G418 and analyzed for introduced TAM67 expression by Northern blotting and immunoprecipitation.

RESULTS

The AP-1 but Not the PEA3 Binding Site Is Necessary for TPA- or EGF-Induced Expression of the Stromelysin–CAT Reporter. The gene for the metalloproteinase stromelysin is induced by both TPA and EGF in a cell type-specific manner and contains an AP-1 and several PEA3 (Ets binding) elements in its promoter (15, 24, 27). To assess the role of the AP-1 binding site in the induction of stromelysin promoter-driven transcription in JB6 cells, we used three reporter constructs: 750 bp of the rat stromelysin-promoter containing one AP-1 site and two PEA3 sites linked to the CAT gene (P750TRCAT); P750TRCAT, with two point mutations in the AP-1 site (mTRE-CAT) (15); and a mutant with two point mutations of P750TRCAT in the PEA3 sequences (mPEA3-

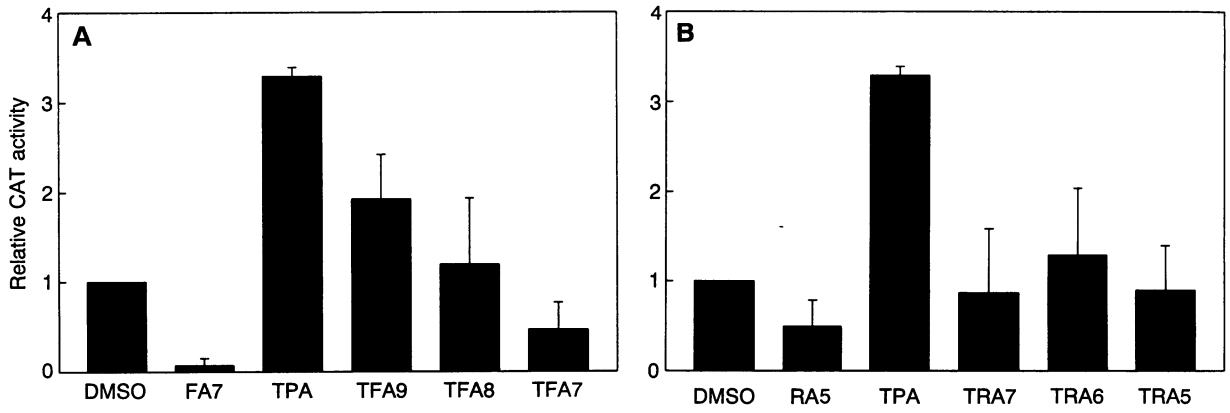

FIG. 2. FA and RA block TPA-induced AP-1 activity. JB6 P+ Cl 41 cells were transfected with P750TRCAT as described in Fig. 1. Results are expressed as the mean ± SEM of three experiments. (*A*) Inhibitor was FA tested at 10^{-7} M in the absence of TPA (FA7) and at 10^{-9}, 10^{-8}, and 10^{-7} M in the presence of TPA (TFA9, TFA8, and TFA7, respectively). (*B*) Inhibitor was RA tested at 10^{-5} M in the absence of TPA (RA5) and at 15^{-7}, 10^{-6}, and 10^{-5} M in the presence of TPA (TRA7, TRA6, and TRA5).

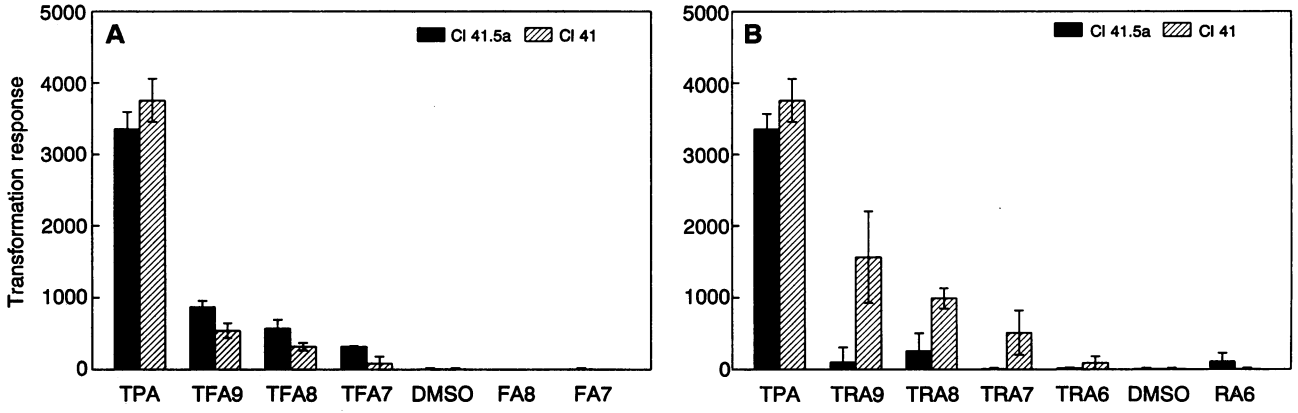

FIG. 3. Inhibition of TPA-induced anchorage-independent transformation by RA or FA. Ten thousand JB6 P^+ cells were exposed simultaneously to DMSO (0.01%) or TPA (3 ng/ml; 5 nM) and inhibitor in 0.33% agar and scored for colonies at 14 days. Transformation response is expressed as the number of soft-agar colonies per 10^4 suspended cells. (*A*) Inhibitor was FA (10^{-9} M–10^{-7} M, FA9–FA7). (*B*) Inhibitor was RA (10^{-9} M–10^{-6} M; RA9–RA6).

CAT). Eight hours of TPA or EGF treatment induced about 2-fold higher CAT activity than that found in uninduced cells (Fig. 1). AP-1-dependent activity was calculated by subtracting the AP-1-independent value seen with mTRE-CAT. After this subtraction, the fold induction of AP-1 activity was calculated to be 3.8 and 2.4, respectively, for TPA and EGF in JB6 P^+ cells (Fig. 1 legend) (9). Both values are significantly higher than that of the control group ($P < 0.05$ and $P < 0.01$, respectively). The induction of P750TRCAT requires the AP-1 binding sequence, since mutation of the AP-1 binding site caused loss of inducibility. In contrast, the PEA3 sequence, to which the Ets oncoprotein binds (28), appears not to be required, as PEA3 mutation had no effect on inducibility (Fig. 1). The basal activity of these promoters is influenced by both the AP-1 and PEA3 elements, however, since mutation in either of these sites resulted in some decrease in CAT activity in the unstimulated control cultures (DMSO). Thus, while basal CAT activity appears to be regulated by both PEA3 and AP-1, TPA- or EGF-induced activity of the stromelysin–CAT gene is regulated only by AP-1 in JB6 cells.

RA and Glucocorticoid Repress TPA-Induced AP-1 Activities in P^+ Cells. Since RA and glucocorticoids have been shown to inhibit AP-1 activity in several model systems, we tested these agents against TPA-induced activity in JB6 cells. The TPA-induced CAT activity was abolished when transfected cells were exposed to TPA plus RA or fluocinolone acetonide (FA) (Fig. 2). FA produced dose-dependent inhibition of the TPA-induced CAT activities at 1–100 nM, whereas RA showed 90–100% inhibition of induced CAT activity at 0.1–10 μM. Both FA and RA also blocked basal AP-1 activity.

RA and FA Block TPA-Induced Transformation in P^+ Cells. To test whether the same concentration range of inhibitor needed to block AP-1 activity also blocks TPA-induced transformation, a soft-agar transformation assay was carried out. RA and FA blocked TPA-induced transformation in a concentration-dependent manner in two P^+ cell lines, Cl 41 and Cl 41.5a (Fig. 3). In agreement with an earlier observation, the sensitivity of Cl 41.5a to antipromotion by RA appeared to be greater than that with Cl 41 (29). The concentration-dependent range for blocking TPA-induced transformation appears identical to that active in blocking TPA-induced AP-1 activity by FA (1–100 nM). For RA, although the concentrations that inhibited induced AP-1 and transformation showed overlap, AP-1 activity was more sensitive to inhibition at 0.1 μM RA.

Overexpression of Dominant Negative c-*jun* Mutant Blocks TPA- or EGF-Induced AP-1 Activity. Since significant inhibition of TPA-inducible AP-1 transactivation activity was achieved in P^+ cells at antipromoting doses of RA and FA, the AP-1 complex (Jun/Fos) may play a critical role in executing the P^+ transformation response. FA and RA, however, exert pleiotropic effects. The dominant negative mutant of c-*jun*, TAM67, has been shown to be a specific inhibitor of AP-1 activity in rat embryo cells (18, 30). The mechanism appears to involve TAM67 protein forming reduced-activity complexes with endogenous proteins of the Jun and Fos families. To specifically block AP-1 activity in JB6 cells, the TAM67 c-*jun* mutant in the pMexMTH vector (under the transcriptional control of the metallothionein promoter) was transfected into P^+ Cl 41 cells. After G418 (*neo*) selection, eight clonal TAM67 transfectants (M1–M8) and three *neo*-only transfectants (N1–N3) were obtained. All of the TAM67 transfectants showed expression of the introduced 1.3-kb TAM67 mRNA and 29-kDa TAM67 protein (Fig. 4 and data not shown). Densitometric analysis of the c-Jun immunoprecipitated protein bands indicated that there were no significant differences in c-Jun protein levels after EGF or TPA treatment in the TAM67 or *neo*-only transfectants (Fig. 4 and data not shown). TAM67 transfectants M1 and M4 expressed higher levels of TAM67 protein than did M2, M3, M5, M6, M7, and M8 transfectants regardless of TPA or EGF treatment. With the exception of M3 after EGF, no consistent changes in TAM67 protein levels were observed in the TAM67 transfectants after TPA or EGF treatment. Previous results in our laboratory have shown that treatment with Zn^{2+} does not increase the expression of mRNA or protein from a pMexMTH-p53 expression construct (31). In agreement with these results, 25 μm Zn^{2+}, the

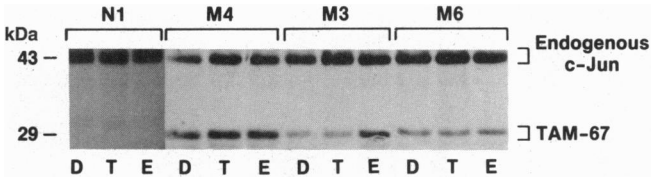

FIG. 4. Expression of introduced dominant negative c-*jun* mutant TAM67. c-Jun and TAM67 proteins were immunoprecipitated from TAM67 transfectants. Before metabolic labeling, transfectant cells were treated for 15 hr with TPA at 10 ng/ml (lanes T) or EGF at 10 ng/ml (lanes E) and compared with control DMSO-treated cells (lanes D). Endogenous c-Jun was precipitated in both TAM67 and *neo*-only transfectants (upper bracket). The introduced TAM67, seen as multiple bands at ≈29 kDa, was detected only in the TAM67 transfectants (lower bracket). The figure represents a composite of two different gels.

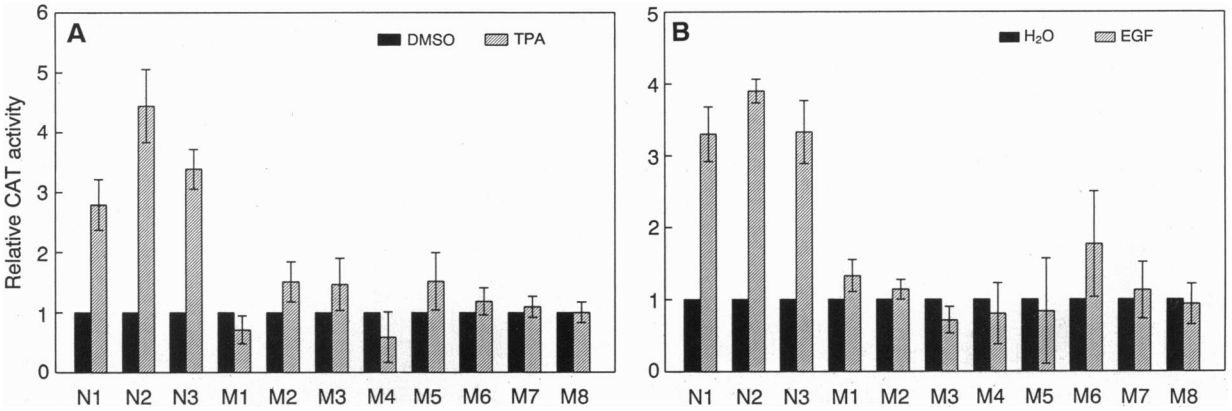

FIG. 5. Expression of TAM67 blocks TPA- or EGF-induced AP-1 activity. TAM67 transfectants and *neo*-only transfectants were transfected with P750TRCAT reporter plasmid and CAT assay was performed as described in Fig. 1, except that 42 hr after transfection the cells were exposed to 0.01% DMSO, TPA at 10 ng/ml (*A*), or EGF at 10 ng/ml (*B*). AP-1 activity was measured as described in Fig. 2. The DMSO-treated groups are control groups with values designated as basal level of CAT and normalized as 1, and thus have no error bars. As calculated from one experiment, the absolute basal levels of AP-1 activity in three *neo*-only transfectants and eight TAM67 transfectants were 45.6 ± 15.0 and 48.0 ± 20.3 cpm/hr per 0.25 ml, respectively. Results are expressed as the mean ± SEM of three experiments.

highest tolerated dose, did not increase the expression of introduced TAM67 mRNA (data not shown).

TPA induced 3- to 4.5-fold increases in AP-1 activities in the *neo*-only transfectants (Fig. 5). All eight of the TAM67 transfectants showed little or no TPA-induced AP-1 activity. To test the role of AP-1 activity in transformation promotion more generally, the response to the transformation promoter EGF was also examined. About 3- to 4-fold induction of AP-1-driven CAT activity by EGF was observed for the three *neo*-only transfectants. The EGF-induction of CAT activity was blocked in seven of the eight TAM67 transfectants. Transfectant M6 showed only partial inhibition of the EGF-induced AP-1 activity. The basal levels of AP-1 activity were not inhibited by introduced TAM67 (see Fig. 5 legend). In addition, compared with *neo*-only transfectants, overexpression of TAM67 did not affect monolayer growth rate in medium containing 1%, 3%, or 5% fetal bovine serum (data not shown).

To check the possibility that TAM67 might block other TPA- or EGF-induced transcription factors, we tested a CAT reporter controlled by an HIV-1 promoter sequence containing two NF-κB and no AP-1 sites (26). Both basal and induced CAT activities following exposure to TPA or TNFα were readily detected in HeLa cells transfected with this construct (data not shown). While induced 5-fold TRE–CAT activity was blocked by cotransfection of HeLa cells with 10 μg of TAM67 plasmid DNA, the induced NF-κB-dependent CAT activity was not affected by cotransfection of TAM67 DNA. However, neither basal nor TPA- or TNFα-induced NF-κB CAT activity was seen following transfection into JB6 cells whether or not they were expressing TAM67 (data not shown). Thus, it appears unlikely that TAM67 acts in JB6 cells to nonspecifically or indirectly block induced NF-κB or induced PEA3 (Fig. 1)-dependent transcriptional activity, but it is likely that TAM67 acts specifically to block induced AP-1 activity.

TPA- or EGF-Induced Transformation Is Blocked by Introduced TAM67. We further explored whether the transfectants expressing the dominant negative c-*jun* mutant could repress tumor promoter-induced transformation. Fig. 6 summarizes the results of these experiments. While the three *neo*-only transfectants showed a high frequency of transformation with exposure to TPA, all eight TAM67 c-*jun* transfectants were blocked for TPA-induced transformation. Six of the eight TAM67 transfectants also lost the EGF-induced transformation response (Fig. 6*A*). The other two, M3 and M6, showed a transformation response (Fig. 6*B*). In M6 the partially repressed AP-1 levels following EGF exposure may be sufficient to support a transformation response to EGF (Figs. 5 and 6). In M3, however, EGF-induced AP-1 activity appears not to be necessary for EGF-induced transformation. In summary, knockout of the AP-1 response by TAM67 appears in most cases to produce a loss of the transformation response induced by tumor promoters. Transfectants M1 and M4, the highest TAM67 protein expressors, were also the most inhibited for TPA-induced AP-1 activity and transformation.

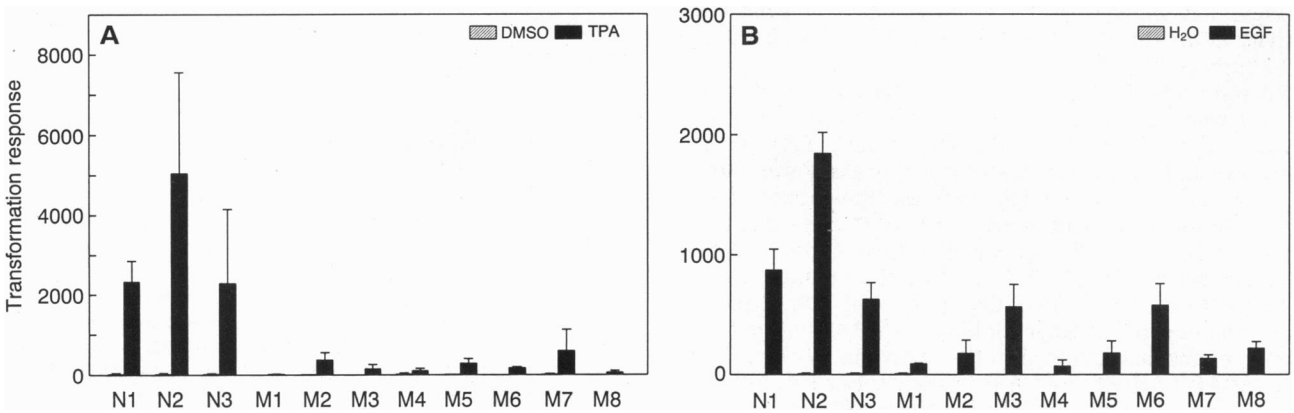

FIG. 6. TAM67 blocks TPA- or EGF-induced transformation response. Independent clonal TAM67 (M1-M8) or neo (N1-N3) transfectant cells (10^4) were exposed to 0.01% DMSO (control group), TPA at 10 ng/ml (*A*) or EGF at 10 ng/ml (*B*) in 0.33% agar and scored for colonies at 14 days. Transformation response is expressed as TPA- or EGF-induced soft-agar colonies per 10^4 suspended cells. Values for solvent control ranged from 0 to 120 colonies per 10^4 suspended cells. Results are expressed as the mean ± SEM of three experiments.

DISCUSSION

The results demonstrate that AP-1 activation is required for tumor promotion as modeled by JB6 cells, in which progression from the preneoplastic to a neoplastic stage can be assessed. Our first approach to inhibiting induced AP-1 activity utilized two pharmacological agents that have been shown to repress AP-1 activity in several other cell systems. AP-1 repression involves protein–protein interaction between active receptors (RA receptors α, β, and γ; glucocorticoid receptor) and c-Jun protein (19, 22, 32). The interaction requires the DNA-binding domain of the active receptors and the leucine zipper of Jun. These cross-couplings of AP-1 and intracellular hormone receptors are cell-type specific (33). In JB6 cells, RA and FA repressed TPA-induced AP-1 activities and transformation in the same dose range, suggesting that the AP-1 complex (Jun/Fos) may play a critical role in executing the P^+ response and that inhibition of its activity may explain the antipromoting activity of RA or FA. The other approach to testing the hypothesis that AP-1 activity is required for induced transformation utilized stable introduction of a dominant negative mutant of c-*jun*, TAM67, into JB6 cells. The TAM67 mutant protein has been shown to form homodimers or heterodimers (with other Jun or Fos family members) which bind to AP-1 sequences that subsequently demonstrate no or diminished AP-1 transactivation (18). Expression of TAM67 in JB6 cells blocked TPA- or EGF-induced AP-1 activities and also blocked induced anchorage-independent transformation. The question of whether TAM67 blocks the induction of tumorigenic phenotype awaits the development of an *in vivo* assay that will distinguish P^+ from P^- cells. AP-1 activity has been implicated in Ha-*ras*- and c-*jun*-induced cotransformation of rat embryo cells (18). The present report demonstrates that knockout of tumor promoter-induced AP-1 activity also knocks out the tumor promoter-induced transformation response. Although the above results suggest the conclusion that induced AP-1 activity is necessary for the induction of transformation, they do not address the issue of sufficiency. The loss of induced transformation response following loss of induced transactivation in this system suggests that the tumor promoter-induced transformation is accomplished in part by activation of AP-1-responsive effector genes.

Matrisian and coworkers (15, 24) have reported that the rat stromelysin promoter–CAT gene construct contains an AP-1 binding sequence at position −70 which is necessary for TPA-growth factor- and oncogene-induced stromelysin–CAT gene expression in several cell lines. By using mutants of this promoter–CAT construct in JB6 cells, we have also demonstrated that this AP-1 binding sequence is necessary for TPA- or EGF-induced activity, whereas an intact Ets oncoprotein-controlled PEA3 sequence is not. This suggests that induced P750TRCAT activity specifically measures AP-1-dependent induced transcriptional activation. With an HIV-1 promoter–CAT reporter that contains two NF-κB binding sequences, neither basal nor TPA- or TNFα-induced transcriptional activity was detected in JB6 P^+ cells. These results suggest that neither Ets nor NF-κB transcription factor is involved in tumor promoter-induced transformation in JB6 P^+ cells and that neither can be an alternative target to explain the promotion-blocking activity of TAM67.

Blocking of preneoplastic progression may be more feasible than reversing cancer. In view of the importance of AP-1 activity in tumor promoter-induced transformation as suggested in this report, AP-1 might be used as a molecular target for prevention of carcinogenesis. The studies presented here suggest that derivatives of RA or FA might function in chemoprevention against targeted AP-1. Also suggested is the possibility that "gene prevention" involving direct introduction of a gene such as TAM67 into preneoplastic cells may protect them from progressing to cancer in response to tumor promoters. Blocking of AP-1 activity, or events downstream of protein kinase C or growth factor receptors (34), might be more specific for inhibiting the process of carcinogenesis, with fewer side effects on normal growth and differentiation, than direct receptor blocking would be. In fact, inhibition of induced AP-1 by a dominant negative c-*jun* mutant seems not to be accompanied by effects on growth rate or by knockout of basal AP-1 activity in JB6 cells.

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