Multistage carcinogenesis consists of initiation, promotion, and progression. Tumor promotion is a stepwise process: It occurs with comparatively low frequency, requires the chronic action of tumor promoters, and does not necessarily involve genotoxic damage. The mouse skin model is an excellent example of multistage carcinogenesis. The concentration of initiator (typically dimethylbenz[a]anthracene; DMBA) can be decreased to produce genotoxic damage in the absence of tumor formation. Sequential exposure of initiated cells to various tumor promoters (e.g., 12-O-tetradecanoylphorbol-13-acetate; TPA) at an optimal concentration results in a robust tumor response. A concentration of > 0.2 nM TPA or 0.02 nM EGF produced a significant increase in transformation response as well as in extracellular signal-regulated protein kinase (ERK), SRE, or AP-1 activation. Treatment with > 0.4 U/mL (2.35 pM) TNFα increased NFκB activity and transformation response in a dose-dependent manner. However, transformation response decreased at > 33 U/mL TNFα due to a cytotoxic response. These findings suggest that the signaling pathway leading to the activation of ERK, TCF, and AP-1 proteins constitutes a major factor determining the risk of tumor promotion by TPA or EGF. Cell toxicity in addition to NFκB activation should be considered in predicting TNFα-induced transformation response. Key words: activator protein-1, epidermal growth factor, nuclear factor κB, serum-response element, 12-O-tetradecanoylphorbol-13-acetate, transformation, tumor necrosis factor-α. *Environ Health Perspect* 110:865–870 (2002). [Online 18 July 2002] http://ehpnet1.niehs.nih.gov/docs/2002/110p865-870/suzukawa/abstract.html

Multistage carcinogenesis is consistent with putative thresholds for promotion of neoplastic transformation in the mouse epidermal JB6 model.
tumor-promoting agents in vivo (3). To determine if there is an activation threshold for promoter-induced transformation and to determine how much activation of transformation-relevant transcription factors is required, we established stable reporter clones. Dose–response relationships of transformation, AP-1, SRE, and NFκB activation by TPA, EGF, or TNFα revealed activation levels above which there was risk of neoplastic transformation.

Materials and Methods

Cell culture and reagents. Promotion-sensitive mouse epidermal JB6 cells, clone 41, were as previously described and were maintained accordingly (23,24). In brief, JB6 cells were cultured in Eagle’s minimal essential medium (EMEM; BioWhittaker, Walkersville, MD) supplemented with 4% fetal bovine serum (FBS), 2 mM l-glutamine and 25 mg/mL gentamicin (Life Technologies/Gibco, Gaithersburg, MD). TPA was purchased from LKT Laboratories, Inc. (St. Paul, MN). EGF (receptor grade) was purchased from Upstate Biotechnology (Lake Placid, NY, lot 19319). All other cell culture reagents were purchased from BioWhittaker or Life Technologies/Gibco. TNFα was purchased from PeproTech Inc. (Rocky Hill, NJ). Specific activity is ≥ 1 × 10^5 U/mg.

Plasmids and stable transfection. SRE-luciferase reporter construct containing five tandem SRE sequences (AGGATGTC-CATATTAGGAGCATCT) was purchased from Stratagene (La Jolla, CA). AP-1 or NFκB luciferase reporter plasmids consisting of luciferase reporter gene driven by the promoter harboring the appropriate element were described previously (13,14). The AP-1 reporter plasmids consist of firefly luciferase genes driven by an AP-1-responsive promoter containing four copies of flanked AP-1 consensus sequence (TGCACTATGAGTGCATG) from GCN4 and a minimal albumin promoter region with TATA box: AAGGTTAAGCTATGATATATTAGGGAGATCTTCTGCGACACAGATCATCCTTCTATGCACCCCTACATCAACCATGACCTCTTCTATCATACCCACCCCTACATCCTTCTTCTATCATACCCACCCCTACATGACCTCTTCT.

The NFκB reporter plasmids consisted of firefly luciferase reporter genes driven by a minimal NFκB-responsive region from an interleukin-6 (IL-6) promoter containing two copies of NFκB-responsive elements in a sense orientation: GACTCTAGAGATTACAATGTGGGATTCTTCCCAT - GTCGACAGTTTTATCACTGATCTGGGA AAATCCCCACATGAAAAATCATTCTCCGCCC. Because there are no other known responsive cis-elements identified in the above sequences, any cross-family activation should occur at the level of protein–protein, not at the level of protein–DNA interaction.

We performed transfections according to the Fugene6 protocol from Roche molecular biologicals (Indianapolis, IN). In brief, 1 × 10^5 cells were seeded in 10-cm dishes. The next day, 15 µL of Fugene6, 4 µg of reporter DNA, and 1-0.5 µg of pCDNA empty vector were added to 0.5 mL of medium of complete medium. After 10 min of incubation, transfection mixture was added to cell culture dishes. Cells were incubated for 48 hr. G418 selection was started on the transfected population with 500 µg/mL.

Luciferase assay of reporters. We seeded 1 × 10^5 cells/well of reporter cells in 24-well plates. On the next day the cells were starved in EMEM with 0.2% FBS for more than 24 hr to lower the basal transcription factor activation. We treated the resulting cells with various concentrations of TPA, EGF, or TNFα in EMEM with 0.2% FBS for 3 hr. We observed little or no cell detachment. The stimulated cells were collected and lysed at 3 hr of treatment. The cells were lysed directly on the plate after a single wash with phosphate-buffered saline. We assayed the resulting cell lysates for luciferase activity using the Luciferase Assay Kit (Promega, Madison, WI) and DYNEX Luminometer (DYNEX Technologies, Chantilly, VA). Three independent wells were used for each condition in each experiment. We calculated percent activation of each reporter by the following equation: % activation = [sample relative luciferase units (RLU) – basal RLU]/(maximum RLU – basal RLU) × 100. We used the average RLU of three wells as sample RLU.

Western blots. We seeded 1 × 10^5 cells/well of cells in six-well plates. Cells were starved as described under luciferase assay. Cells were treated with TPA or EGF for 30 min, washed with ice-cold phosphate-buffered saline once, then lysed with lysis buffer (2% SDS, 50 mM Tris-HCl pH 7.5). Western immunoblotting was performed according to the ECL protocol from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Anti-ERK-1/2 (p44/42 MAP Kinase) and anti-phosphoERK-1/2 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). In brief, 20–40 µg of whole-cell lysates were boiled and denatured in sample buffer containing SDS and dithiothreitol (NOVEX, San Diego, CA) followed by gel electrophoresis using NuPAGE 10% Bis-Tris prepacked gel (NOVEX) in 4–morpholine-propanesulfonic acid buffer. The proteins were electro-transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, NH) using a semi-dry transfer blotting system from EnproTech Co. (Hyde Park, MA). The resulting protein-bound membrane was blotted with selected antibodies and visualized using ECL reagents (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) and X-OMAT AR film (Kodak, Rochester, NY). The band intensities were monitored by Kodak digital camera (DC120) and analyzed by its image-analyzing program (Kodak 1D). We determined the lowest and highest intensity as 0% and 100% activation, respectively. Although the peak activation was observed at 30 min, ERK activation was sustained for at least 6 hr (data not shown).

Anchorage-independent transformation assay. We performed promotions of neoplastic transformation assays as described previously (25). In a 60-mm tissue culture dish, 10,000 JB6 cells were resuspended in 1.5 mL of 0.33% agar in EMEM with 10% FBS and layered over 7 mL of 0.5% agar in EMEM with 10% FBS. Both layers of agar were supplemented with DMSO, phosphate-buffered saline, or various concentrations of tumor promoter TPA, EGF, or TNFα. The cells were cultured at 36°C for 14 days, and the resulting colonies were counted by an automated image analysis system supported by Image Pro-Plus (version 3.0.1) software (Media Cybernetics, Silver Spring, MD). We scored colonies > 8 cells. The transformation responses are presented as number of colonies per 10,000 cells per 60-mm tissue culture dish.

Results

To assess activation response to small-molecule inducers (tumor promoters), we generated stably transfected reporter cell lines. Such stable reporter cell lines offer the advantage of eliminating the variability that arises with repeated transient transfections. Nine, six, and three clonal transfectants harboring AP-1-, SRE-, and NFκB-luciferase reporter constructs were isolated, respectively. All of them were sensitive to tumor promoter-induced transformation and exhibited basal and tumor promoter-induced luciferase activity. Among them, we selected two clones of each harboring a luciferase reporter for further analysis.

Activation of MAP kinase ERK-1, -2, and SRE-dependent transcription by TPA or EGF. Using anti-phospho-ERK-1/2 antibody, we measured the amount of phospho-ERK-1/2, an activated form of ERK, in SRE-luciferase reporter (S13) cells treated with varying concentrations of TPA or EGF. Treatment of cells with 0.023–16 nM TPA and 0.030–20 ng/mL (5.0 pM–3.3 nM) EGF produced a dose-dependent increase of ERK activity (Figure 1A, C). Parallel measurements of SRE activation by TPA or EGF are shown in Figure 1B and D. To facilitate the comparison, the optical densities of phospho-ERK shown in Figure 1A and C are plotted with the SRE reporter activation shown in Figure 1B and D. TPA treatment yielded similar
dose–response curves for SRE and ERK activation, suggesting that SRE activation is a legitimate, alternative indicator of ERK activation under defined conditions. We observed a significant increase of SRE activation at the EGF concentration needed to produce detectable activation of ERK-2. The dose–response curve of ERK activity by EGF did not completely coincide with the one of SRE-Luciferase activation (Figure 1D). This suggests that other MAP kinases such as Jun N-terminal kinase and/or p38 kinase might be involved in the activation of SRE at progressively higher concentrations of EGF (26).

Two SRE reporter clones (S12 and S13) showed different basal luciferase activity and produced 3.3- and 2.9-fold increase of luciferase activity by 5.3 nM TPA, respectively (data not shown). When the data are plotted as percent of maximum activation, the two clones show similar dose response to TPA or EGF treatment (Figure 2A, B).

**Activation of AP-1-dependent gene expression and transformation response by TPA or EGF.** Two independent AP-1 reporter clones (A3 and A9) also showed concentration-dependent activation of AP-1-dependent transcription in response to TPA or EGF treatment (Figure 3A, B). Apparent threshold concentrations for producing significant activation of AP-1 are 0.2 nM TPA and 0.12 ng/mL (19.8 pm) EGF. We determined anchorage-independent transformation response to TPA or EGF at varying concentrations. More than 0.2 nM TPA or 0.12 ng/mL (0.02 nM) EGF—concentrations that produced significant activation of SRE or AP-1—also produced a significant increase in transformation response (Figure 4A, B).

**Activation of NFκB-dependent gene expression and transformation response by TNF.** NFκB reporter clones N3 and N5 showed concentration-dependent NFκB activation by TNFα (Figure 5). Significant NFκB activation occurred at 0.4 U/mL, and maximal activation occurred at 33 U/mL. Determination of the transformation response to TNFα revealed that although the number of colonies increased up to 11 U/mL TNFα, it sharply decreased with higher doses (Figure 6A). This dose–response curve is consistent with our previous report (5). The number of total objects (colonies plus single cells) at the time of soft agar assay decreased at more than 11 U/mL TNFα (Figure 6B, lower panel). This suggests that the decreased transformation response at high dose is caused by TNFα induced cell toxicity.
A comparison of TNFα-induced NFκB activation and transformation response for clone N3 is shown in Figure 6B (top panel). The most noteworthy feature of this comparison is the dissociation of TNFα-mediated NFκB activation from the transformation response at progressively higher doses. The slight shift in the TNFα dose response for transformation of N3 clonal cells, relative to the parental clone 41 cell line, indicates that we selected clones with greater sensitivity to the cytotoxicity response.

We chose to focus on the correlation between second messenger activation and inducible (≥ 2-fold) transcriptional activities. Less than a 2-fold induction does not provide sufficient separation from background signal standard deviation to provide meaningful comparisons. Because TPA or EGF produced < 2-fold maximal induction of NFκB activation, and because TNFα produced less than 2-fold maximal AP-1 activation, dose–response analyses for the respective ligands were not pursued using the respective cloned reporter cell lines.

**Discussion**

These results establish that there are thresholds of activation of ERK-1, ERK-2, AP-1, or NFκB above which there is risk of transformation by TPA, EGF, or TNFα. Concentrations > 0.2 nM (0.12 ng/mL) TPA (Figure 4A), 0.12 ng/mL (0.02 nM) EGF (Figure 4B), or 1 U/mL TNFα (Figure 6A) produced significant increases in transformation response by mouse epidermal JB6 cells, while responses to lower concentrations were comparable to background. Fifty percent of maximal response to TPA was seen at 0.69 ± 0.19 nM for SRE activation, AP-1 activation, and transformation response (Figure 7). Fifty percent of maximal response to EGF was seen at 1.4 ± 0.6 ng/mL (229 pM) for SRE activation, AP-1 activation, and transformation response (Figure 8). A concentration of 10 ng/mL TPA and 10 ng/mL EGF are equal to 1.6 nM and 0.17 nM, respectively. These relatively high concentrations, which produce maximal responses, are typical concentrations used in previous reports (11,29). The magnitude of AP-1 activation thus predicts transformation response by TPA or EGF. Because AP-1 activation is required for TPA- or EGF-induced transformation of JB6 cells (13) and for tumor promotion in mouse skin (7), AP-1 activation is a good predictor of transformation response by TPA or EGF. This finding allows one to do 3-hr assays instead of time-consuming 14-day assays to assess promotion of transformation response to TPA or EGF.

Regression analysis shows the close relationship between SRE activation, AP-1 activation, and transformation responses to TPA or EGF. The magnitude of SRE activation with a slope of 45° (Figure 9A). Moreover, the magnitude of transformation response is closely related to the magnitude of AP-1 activation by TPA or EGF, again with a slope close to 45° (Figure 9B). This indicates that the magnitude of either SRE activation or AP-1 activation constitutes a reliable predictor of the magnitude of transformation risk in response to TPA or EGF. The amount of SRE activation indicates the amount of ERK activation by TPA or EGF (Figure 1B). Consistent with previous findings (11,22), ERK activation not only is essential but is also a major determinant of AP-1 activation, which in turn is a major determinant of transformation response to TPA.

SRE activation leading to c-fos transcription, although a good risk indicator, is not sufficient for transformation because promotion-resistant cells can induce c-fos expression in response to TPA or EGF (24). Thus, the tight correlation between ERK and SRE activity in the initial 3 hr with the transformation response suggests that acute molecular readouts are also good alternative indicators of subsequent transformation response to TPA and EGF.

EGF and TNFα are biologically more significant tumor promoters than TPA because these are endogenous growth factors/cytokines produced in response to many stimuli. In vivo studies with TNFα knockout mice showed TNFα is required for TPA-induced tumor promotion (30,31). The present study indicates that TNFα has a concentration range for producing transformation response. At TNFα concentrations associated with maximal transformation response (11 U/mL), NFκB activation is approximately 70% of maximum. Moreover, NFκB activation continues to increase at progressively higher TNFα concentrations, despite the sharp switch from transforming activity to cytotoxicity. This dissociation...
suggests that, although activation of NFκB plays a role in producing risk, other factors must also be important in determining risk of TNFα-induced transformation. Low levels spanning the concentration range that precedes the cytotoxic response are predictive for transformation risk due to TNFα exposure, whereas high levels of NFκB activation associated with cytotoxicity have no predictive value.

In practical situations, multiple tumor promoters might be applied sequentially or simultaneously. Assaying an unknown agent in parallel with tumor promoter TPA, EGF, or TNFα at their threshold concentrations for promotion of transformation will allow one to determine whether exposure to the unknown agent alone activates transcription factor at levels that exceed transformation-relevant thresholds. The unknown agent can also be used in combination with one of the known tumor promoters, and the incremental activation due to the unknown can be compared with transformation-relevant levels of activation. Because the JB6 model has proved to be predictive for revealing molecular events that drive or prevent tumor promotion or maintain tumor phenotype in vivo (7) or in a human keratinocyte model (8,9), this model can be used as a sensitive and rapid initial screen to identify agents likely to present risk of tumor promotion.

The 3-hr transcription factor activation assays are reliable predictors of 14-day transformation outcomes in the JB6 model and of mouse skin tumor promotion. Activation of AP-1 and NFκB at 3 and 18 hr is required for transformation in the JB6 model (13,15). This is true for multiple classes of agents (6, 32–39). An agent that does not activate AP-1 or NFκB is unlikely to act as a tumor promoter. Although activation of AP-1 or NFκB is not sufficient for promotion of transformation, stimulated activation of either transcription factor increases the risk of tumor promotion. It is also known that the elevated AP-1 activation seen at 6 hr is sustained in mouse epidermis after 2 weeks of twice-weekly tumor promotion, and that, when dominant negative jun expression inhibits tumor promotion, it also inhibits the 2-week induction as well as the 6-hr induction of AP-1 (7).

Previous studies revealed that magnetic field exposure does not affect TPA-induced transformation response of JB6 cells (40,41). Defining molecular events that are required for cell transformation and thresholds in signaling pathways associated with the transformation response will allow for flexibility in the application of in vitro model systems directed at defining relative cancer risks at low-dose exposures. Molecular responses in cells cotreated with known tumor promoters

Figure 6. Low-dose similarity of TNFα-dependent dose–response curves for transformation response and NFκB activation. (A) Transformation response of Cl 41 and N3 cells to TNFα. Colonies > 8 cells were scored. Results represent the combined average of three independent experiments using duplicate samples (n = 6). Error bars indicate SD. (B) A comparison of TNFα-mediated NFκB activation (NFκB-Luc) and transformation response (Tx) for N3 cells (top panel). Percent transformation response = sample/maximum number of colonies × 100. The number of total objects (colonies plus single cells) per dish is plotted in the lower panel.

Figure 7. Similar thresholds for TPA-mediated SRE activation, AP-1 activation, and transformation. TPA-mediated SRE activation of S13 cells (SRE-Luc) from Figure 2A, AP-1 activation of A3 cells (AP-1-Luc) from Figure 3A, and transformation response (Tx) of A3 cells from Figure 4A were plotted as percent maximum activation for direct comparison. Percent transformation response = (sample – number of colonies in control dish)/(maximum number of colonies – number of colonies in control dish) × 100. Error bars indicate SD.

Figure 8. EGF-mediated transformation threshold is similar to AP-1 activation threshold. EGF-mediated SRE activation (SRE-Luc) of S12 cells from Figure 2B, AP-1 activation (AP-1-Luc) of A3 cells from Figure 3B, and transformation response (Tx) of A3 cells from Figure 4B were plotted as percent maximum activation to facilitate the direct comparison. Percent transformation response = (sample number of colonies in control dish)/maximum number of colonies – number of colonies in control dish) × 100. Error bars indicate SD.

Figure 9. AP-1 response is predictive for risk of transformation by TPA or EGF at both low and high doses. (A) Linear regression of AP-1 activation and SRE activation by TPA (R² = 0.96 and 0.94 for TPA and EGF, respectively). (B) Linear regression of transformation response and AP-1 activation by TPA and EGF (R² = 0.94 and 0.81 for TPA and EGF, respectively).
and unknown environmental contaminants (physical or chemical) can rapidly be quantitated to determine whether transformation-related signal transduction is induced by the unknown contaminant over a rationally selected range of TPA, EGF, or TNFα concentrations. Because the molecular response to TPA, EGF, and TNFα can be directly correlated with transformation response in the agar assay, the results can provide rapid analysis of relative risks. Within this context, we are particularly interested in the cancer risk associated with low-dose radiation exposures. It is difficult to unambiguously define the biological consequences of low-dose radiation exposures due primarily to low signal-to-noise ratio. We are optimistic that a cotreatment strategy using low-dose radiation and known tumor promoters will allow a robust measure of the effects of radiation on transformation-related signal transduction. If successful, this work will have broader applications in environmental health concerns, concerns that invariably involve low-dose exposures.

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