Dietary energy restriction, in part through glucocorticoid hormones, mediates the impact of 12-*O*-tetradecanoylphorbol-13-acetate on jun D and fra-1 in Sencar mouse epidermis

Joseph Przybyszewski[1], Weiqun Wang[1,3], Angela Au[1], Courtney Perry[1], Megan Guetzko[1], Ken Koehler[2] and Diane F. Birt[1,4]

[1] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011-1061
[2] Department of Statistics, Iowa State University, Ames, IA 50011-1061
[3] Currently with the Department of Human Nutrition, Kansas State University, Manhattan, KS 66506-1407
[4] Corresponding Author; Email: dbirt@iastate.edu.

Supported by the National Institute of Health Grant R01 CA77451-01, American Institute for Cancer Research Grants 97B039, 95B126-REV, United States Department of Agriculture, Nutrition and Wellness Research Center and by the Iowa Agriculture and Home Economics Experiment Station Hatch Act and State of Iowa funds.

This is the accepted manuscript of an article published in *Molecular Carcinogenesis* 49, no. 6 (2010): 592–602, http://dx.doi.org/10.1002/mc.20625.

Keywords: cancer prevention, tumor promotion, activator protein-1, mouse epidermis

Abbreviations: adx, adrenalectomized; AL, *ad libitum*; ANOVA, analysis of variance; AP-1, activator protein-1; CCS, corticosterone; DER, dietary energy restriction; ERK, extracellular response kinase; EMSA, electrophoretic mobility shift assay, PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl-13-phorbol acetate.

Abstract: Dietary energy restriction (DER, 40% calorie reduction from fat and carbohydrate) inhibited mouse skin carcinogenesis and decreased 12-*O*-tetradecanoyl-13-phorbol acetate (TPA)-induced activator protein-1 (AP-1):DNA binding previously. This study measured protein levels of c-jun, jun B, jun D, c-fos, fra-1 and fra-2 and examined their contribution to AP-1:DNA binding by electrophoretic mobility shift assay (EMSA) with supershift analysis in the epidermis of control and DER Sencar mice exposed to TPA. TPA significantly increased c-jun, jun B, c-fos, fra-1 and fra-2 and decreased jun D within 3-6 hours after treatment. AP-1:DNA binding reached a maximum 2.5-fold induction over controls 4 hours after TPA treatment and antibodies to jun B, jun D and fra-2 in the EMSA binding reaction resulted in supershifts in both acetone- and TPA-treated mice 1-6 hours after treatment.
The effect of corticosterone (CCS) and DER on the AP-1 proteins and on the composition of the AP-1:DNA complex was measured in adrenalectomized (adx) mice. DER reduced the TPA impact on jun D and enhanced the induction of fra-1. In addition, CCS-supplemented groups had significantly lower jun D and higher fra-2 than adx groups and sham groups. While sham animals treated with either acetone or TPA contained jun B, jun D and fra-2 proteins in the AP-1:DNA complex by supershift analysis, fra-2 was no longer seen in adx DER animals. In summary, our study supports potential roles for jun D, jun B and fra-1 in the DER regulation of AP-1 function in the Sencar mouse skin carcinogenesis model.

INTRODUCTION

DER is the reduction of calories from dietary fat or carbohydrate compared to a freely fed diet. When animals are treated with a skin carcinogenesis regimen, those given a DER diet with a 40% energy reduction had 50-85% fewer skin tumors than freely fed animals [1]. Inhibition of breast [2], liver [3], colon [4] and pancreas cancers [5] have also been reported in energy-restricted animals. Although the molecular mechanisms for this significant cancer preventive dietary effect are not well understood, advances in understanding the cellular pathways contributing to the DER effect have taken two overlapping directions.

First, previous work in our laboratory has demonstrated that DER's cancer preventive effect occurred during the tumor promotion stage of skin carcinogenesis [6] and that DER caused decreases in the overall activity and the levels of specific isoforms of protein kinase C (PKC) [7] and extracellular response kinase (ERK) [8;9]. Reductions in c-jun protein, c-jun mRNA and AP-1:DNA binding in mouse epidermis [10] by DER are further evidence that DER is inhibiting the mitogen activated protein kinase/AP-1 pathway. AP-1 activation has been shown to be important in keratinocytes in tumor promotion [11] and progression [12;13]. Since we demonstrated that DER reduces TPA-induced c-jun protein levels [10], we hypothesized that DER may impact tumor promotion by decreasing the TPA effects on AP-1 proteins including effects on concentration and/or DNA binding of AP-1 proteins. To test this hypothesis, we examined the effects of TPA and DER on c-jun, jun B, jun D, c-fos, fra-1 and fra-2 proteins by western blot and electrophoretic mobility shift analyses in SENCAR mouse epidermis treated *in vivo* with TPA. As previously reported [14], Fos B was not detectable in mouse epidermis by western blot analysis.

Second, animal studies have shown that the adrenal glands are important for the inhibition of TPA-induced papilloma formation by food restriction in Sencar [15] and CD-1 mice [16]. Since it is well-known that glucocorticoid hormones are potent inhibitors of skin carcinogenesis [17] and tumorigenesis [18], it is not surprising that DER was associated with increased levels of glucocorticoid hormones [19] including CCS [20]. However, because DER decreased signaling down the AP-1 pathway through PKC, ERK and AP-1:DNA binding [7;9;10] without apparent effects on glucocorticoid receptor (GR) levels or activities [20], this study investigated the effect of glucocorticoid hormones on AP-1 constituent proteins in adrenalectomized (adx) and corticosterone (CCS)-supplemented adx mice fed a DER or ad libitum (AL) diet. We thus further hypothesized that adrenalectomy should reverse the attenuating effects of DER on TPA-induced alterations in concentration, and/or DNA binding of AP-1 constituent proteins and that supplementation of CCS to adx mice restores the attenuating effect of DER on the TPA-induced increase in concentrations and DNA binding of AP-1 proteins.

In summary, we hypothesized that 1) dietary energy restriction would inhibit TPA-induced changes in the concentration and/or DNA binding of AP-1 proteins, 2) adrenalectomy would reverse the effect of dietary energy restriction on the TPA-induced changes in concentration and/or DNA binding of AP-1 proteins and 3) corticosterone supplementation of

adrenalectomized mice would restore the effect of dietary energy restriction on the TPA-induced changes in concentrations and/or DNA binding of AP-1 proteins.

Using *in vivo* TPA treatment to stimulate potential early events in cancer promotion in epidermal cells and DER and glucocorticoid hormone manipulation to prevent, inhibit or interfere with these effects, this is the first report using DER to identify the potential positive and negative transactivator functions of AP-1 constituents in the Sencar mouse skin carcinogenesis model. Our data suggests a potential role for jun D as a negative transactivator and that jun B and fra-1 are important in the regulation of AP-1 function in the Sencar mouse skin carcinogenesis model. As hypothesized, DER reversed the TPA effects on jun D expression but, unexpectedly, enhanced the TPA effects on fra-1.

MATERIALS AND METHODS

Animals and diet. Female SENCAR mice, aged 6-8 weeks, were purchased from NIH facilities in Frederick, MD. The mice were housed in a humidity- and temperature-controlled room on a 12 hour light/dark cycle and allowed 1 week to adjust to their environment before surgery. Modified AIN-93 and 40% energy restricted diet pellets were obtained from Harlan Teklad Premier Laboratory Diets (Madison, WI, #TD99433 and #TD99467, respectively), stored at -20° C and were used within 6 months. Diet composition was as previously reported [20]. For the time course study, the shaved dorsal skins of the mice were treated with 3.2 nmol TPA in 200 µL acetone or with 200 µL acetone alone at 1, 3, 4, 6, 12, 18 and 24 hours prior to killing by cervical dislocation. For the dietary energy restriction study, mice were given control or 40% DER diets for 10-12 weeks and then treated with 3.2 nmol TPA in 200 µL acetone or with 200 µL acetone alone 4 hour prior to killing. All experiments were approved by the Institutional Animal Care and Use Committee at Iowa State University.

Adrenalectomy. Surgeries on shaved mice were performed between 7:00 A.M. and 9:00 A.M. The mice were intraperitoneally injected with pentobarbital, 75 mg/kg, approximately 20 min prior to surgery. The anesthetized mice were swabbed with 70% ethanol and the dorsal skin was cut and fascia opened to reveal the kidney. The adrenal glands and surrounding fatty tissue were removed and the fascia was sutured with gut pliabilized nylon and collagen sutures (Ethicon Inc., Somerville, NJ) and the wound closed with wound clips. In sham animals, these same procedures were followed except that the adrenal glands were exposed and disrupted but left intact. Immediately after surgeries, adrenalectomized mice were given saline (0.9%) in an ethanol vehicle (0.6%) or 60 µg/ml CCS in drinking water to mimic the increase in CCS reported in DER mice [7]. One week after surgeries, animals were given 20% energy restricted diets for one week and then 40% DER diets for the remainder of the study (10-12 weeks).

Western blot analysis. Whole cell lysates were prepared from mouse epidermis as previously described [20] except that the depilation step with Nair® was omitted and the protein content of each lysate was determined by the bicinchoninic acid (BCA) protein assay kit (Sigma, St. Louis, MO). Equal amounts of 25 or 100 µg of whole cell mouse epidermal proteins were combined with equal volumes of 2x sample buffer (20 mM sodium monophosphate, 4% SDS, 20% glycerol, 10% B-mercaptoethanol, 200 mM dithiothreitol, 0.02% bromophenol blue), boiled for

5 min and electrophoresed on a 10% polyacrylamide SDS minigel. Proteins were transferred to a PVDF membrane (Biorad, Hercules, CA) in 25 mM Tris, 192 mM Glycine, 15% methanol for 2-3 hours. The membrane was blocked with 5-7% non-fat dry milk in TBST (20 mM Tris, 137 mM sodium chloride, 0.05% tween-20) for 1-3 hours at room temperature (RT) followed by a 1 hour incubation in 1:200 dilution of the primary goat polyclonal antibody obtained from Santa Cruz Biotechnology (Santa Cruz, CA) at RT. After 4 x 5 min TBST washes, the membrane was incubated in a 1:1000 dilution of anti-goat HRP secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 hour at RT, washed 4 x 5 min with TBST and detected with ECL plus detection kit (Amersham Biosciences Corp, Piscataway, NJ). Banding patterns were imaged on a Molecular Dynamics PhosphorImager® (Sunnyvale, CA) in chemiluminescence mode and quantitated using ImageQuant® software. Band intensities were in the linear range of detection by the phosphoimager. Band identification for each antibody was according to the positive controls from the manufacturer.

Electrophoretic mobility supershift analysis. Epidermal cell nuclear proteins were extracted from mouse dorsal skins as previously described [10] and quantitated using a Coomassie® Plus protein assay reagent kit (Pierce Biotechnology, Rockford, IL). The AP-1 consensus sequence:
5'-CGC TTG ATG AGT CAG CCG GAA-3'
3'-GCG AAC TAC TCA GTC GGC CTT-5'

(Promega, Madison, WI) was endlabeled with ^{32}P-ATP (7000 Ci/mmol, MP Biomedicals, Costa Mesa, CA) using T4 polynucleotide kinase (Promega, Madison, WI) according to the manufacturer's instructions including the removal of ^{32}P-ATP by Sephadex G25 spin columns (Roche, Indianapolis, IN). Procedures for the electrophoretic mobility shift analysis of the timing of TPA-induced AP-1:DNA binding were as previously reported [10]. Supershift EMSA of the composition of AP-1 constituent proteins in the AP-1:DNA complex in nuclear proteins of TPA-treated and DER mice used the same procedure with a minor addition. Briefly, for the binding reaction, approximately 100,000-200,000 cpm of ^{32}P-radiolabeled AP-1 consensus sequence and 0.5 µg poly(dI-dC) (Amersham Biosciences, Piscataway, NJ) were incubated with 15 µg nuclear protein at room temperature for 10 min followed by incubation with 400 ng antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to each of the AP-1 constituent proteins for an additional 30 min. This binding reaction was loaded onto a pre-run 5% polyacrylamide gel, electrophoresed and the resulting gel was dried at 85°C for 45 min using a gel dryer (Biorad Laboratories Hercules, CA). The dried gel was exposed to a phosphorscreen (Molecular Dynamics, IL) overnight prior to imaging the banding pattern with a Molecular Dynamics Phosphorimager® (Sunnyvale, CA) in phosphor mode and quantitating the radioactivity using ImageQuant® software.

Statistics. For EMSA results, the logarithm of the radioactivity of the shifted band was analyzed for differences between groups using analysis of variance (ANOVA), as the logarithm of the values were more nearly normally distributed, and differences between means were tested by t-test of the least squares means. For Western results of the time course experiment, the logarithm of the normalized relative chemiluminescence of the protein band for the time points

were analyzed for difference from the acetone-treated control group using ANOVA followed by Dunnett's Multiple Comparison Test on GraphPad Prism Software (San Diego, CA). For Western results of the DER experiment, the logarithm (base 10) of the normalized relative chemiluminescence of the protein band for the time points were analyzed by 3-way ANOVA using the GLM procedure with treatment, diet and surgery as factors and Tukey-Kramer t tests on SAS software. Significant changes in body weight over time were determined by single factor ANOVA followed by Dunnett's post test using GraphPad Prism software comparing body weights between experimental groups at each age during DER feeding.

RESULTS

Timing of TPA effects on AP-1:DNA binding. To determine the time after treatment that resulted in the highest induction of AP-1:DNA binding by TPA and select an appropriate time for the DER study, we first studied the binding of AP-1 to its consensus DNA sequence as measured by EMSA 1, 3, 4 and 6 hours after treatment of SENCAR mouse dorsal skin with a tumor promoting dose of TPA. The image of a representative EMSA experiment with one animal used for each treatment in each lane containing the AP-1:DNA binding reaction is shown in Figure 1A. A summary of the EMSA results for AP-1:DNA binding representing n = 8 observations per treatment group per time point is shown in Figure 1B. We observed that the induction of AP-1:DNA binding reached a maximum 4 hours after TPA treatment (2.5-fold increase over acetone control, $P < 0.05$) and was still significantly increased at 6 hours. Surprisingly, we demonstrated that AP-1:DNA binding was significantly decreased to one-half that of the controls at 1 hour after TPA treatment ($P < 0.05$). In a parallel study, supershift EMSA analysis showed that antibodies to jun D and jun B resulted in a supershifted AP-1:DNA band in all of the six acetone-treated and five TPA-treated animals (3hour post TPA: n = 3; 1hour post TPA: n = 2) examined (see Figure 2 for results from one mouse). The presence of jun D and jun B proteins in the AP-1:DNA complex was seen in both acetone- and TPA-treated mouse samples at all time points measured. Fra-2 shifts occurred in two of two acetone-treated and one of the three TPA-treated samples while Fra-1 was not detected by supershift in acetone- or TPA-treated samples.

Based on the results of the AP-1:DNA binding time course study, we selected 4 hours after TPA treatment for collection of skin samples from the dietary energy restriction study in order to best characterize potential inhibitory effects of DER on TPA-induced AP-1:DNA binding.

Timing of TPA effects on AP-1 constituent protein levels. To study potential early and late responses of TPA on AP-1 protein levels, we harvested epidermal samples 1, 3, 4, 6, 12, and 24 hours after TPA treatment and compared the Western analysis results with acetone-treated controls. The images of individual representative Western blot experiments with one animal used for each treatment in each lane for Jun and Fos family proteins are shown in Figures 3A and 3C, respectively. Summaries of the results of western blots for Jun and Fos family proteins representing 4-6 observations of individual animals per treatment group per time point are shown in Figures 3B and 3D. We found that the maximum increase in jun B protein level occurred at 6

hours (2.4-fold, $P < 0.05$) and stayed significantly above controls for at least 24 hours after TPA treatment; while the TPA induction of c-jun spiked at 4 hours, the only time point showing a significant increase (1.7-fold, $P < 0.05$) and jun D protein was not significantly altered (Figure 3B). For Fos family proteins, significant increases in c-fos protein occurred at 3, 4, 6 and 24 hours after TPA treatment with the maximum induction at 6 hours (1.9-fold, $P < 0.05$) while fra-2 protein gradually increased to a significant level at 24 hours (2.2-fold, $P < 0.05$) after TPA treatment (Figure 3D). The most dramatic induction was in fra-1 which, like jun B, reached a maximum level at 6 hours, was increased 8.1-fold ($P < 0.05$) and stayed significantly above controls for at least 24 hours after TPA treatment. Though detected in positive controls (data not shown), levels of fos B protein in mouse epidermis were below the limits of Western analysis detection as reported previously by others [14].

Effects of DER on body weights. Body weights for freely fed and DER mice over 15 weeks of feeding are shown in Figure 4. At the end of the 10 weeks on their respective diets, freely fed mice in either sham or adx groups gained an average of 3-4% of their body weight while freely fed adx/CCS-supplemented mice lost 8% of their body weight compared to their starting weight at 11 weeks of age. Diets with a 40% reduction in calories from fat and carbohydrate relative to freely fed animals resulted in 9%, 0% and 20% losses of body weight in sham, adx and adx/CCS groups, respectively, compared to their 11-week old starting weight. Following the start of the DER diet, weight loss in DER animals continued for 4 weeks, then their body weights remained constant for the remainder of the experiment showing at most 11%, 14% and 20% weight losses over the course of the experiment for sham, adx and adx/CCS groups, respectively. Single factor ANOVA comparisons of body weight between the experimental groups showed a significant weight loss beginning at age 13 weeks for all DER groups compared to freely fed animals ($P < 0.05$). In addition, DER/adx/CCS mice weighed significantly less than DER/adx and DER/sham animals ($P < 0.05$). There were no significant differences in body weight among freely fed groups or between DER/sham vs DER/adx animals.

Effects of DER and CCS on AP-1 protein levels. The effects of TPA, DER, adrenalectomy and CCS on the protein levels of individual AP-1 constituent proteins are summarized in Figure 5 and the results of ANOVA analysis are provided in Table I. Using 3-way ANOVA on log transformed data, treatment effects (acetone vs. TPA) were significant for c-jun ($P < 0.0001$), jun B ($P = 0.0006$), jun D ($P = 0.0009$), c-fos ($P = 0.0007$) and fra-1 ($P < 0.0001$). For each of these proteins except jun D, the mean value of all TPA-treated groups was higher than the acetone mean. ANOVA for jun D revealed a significant three way interaction ($P = 0.0204$) and individual Tukey-Kramer posthoc t test analyses showed that the mean jun D value of the AL/sham/TPA group was significantly lower than that of AL/sham/acetone ($P = 0.047$) accounting for the noted three way interaction. This significant TPA reduction was lost in DER/sham animals. Individual Tukey-Kramer posthoc t tests showed that the mean fra-1 value in the TPA-treated group was significantly higher than that observed in the corresponding acetone-treated control group ($P < 0.05$, except AL/adx/CCS: $P = 0.219$, AL/adx/NaCl: $P = 0.069$). In addition, DER not only enhanced the effect of TPA on fra-1 but also increased the significance of the acetone vs TPA comparison for all DER groups as shown in Table II.

Notably, much of this enhancement was due to the reduced fra-1 concentration in the acetone treated DER mouse skin. An interaction between TPA treatment and diet was observed in fra-1 ($P = 0.064$, Table I) and is accounted for by this differential TPA effect in the AL and DER groups. Differences by ANOVA due to glucocorticoid status (sham, adx, CCS) were found in jun D ($P = 0.008$) and fra-2 ($P = 0.026$) (Table I). While mean jun D values of CCS-supplemented groups were significantly lower than adx groups ($P = 0.02$) and sham groups ($P = 0.009$) by 3-way ANOVA, mean fra-2 values of CCS-supplemented groups were significantly higher than adx groups ($P = 0.04$) and sham groups ($P = 0.03$). An interaction between treatment and hormone was noted for c-jun ($P = 0.002$, Table I) and individual t tests showed that the mean c-jun value for DER/CCS/TPA was significantly higher than the mean of DER/CCS/acetone ($P = 0.043$) accounting for this interaction.

Effects of DER on constituents of the AP-1:DNA complex. Supporting the results of the time course experiments, supershift EMSA analysis showed that antibodies to jun D, jun B and fra-2 reduced the AP-1:DNA shifted band and resulted in a supershifted AP-1:DNA band in both acetone- and TPA-treated mouse samples as shown in a representative image (Figure 6). Because supershift EMSA analysis showed that the composition of the AP-1:DNA complex was identical in both acetone- and TPA-treated animals, the supershift EMSA results are pooled across acetone and TPA treatment groups and summarized in Table III. Because of the complexity of the changes in band patterns with supershift and the limited amount of nuclear protein available for this analysis, this data was not amenable to statistical analysis and we report it only to show that it was consistent with our time course results. However, we observed that while sham animals treated with either acetone or TPA contained jun B, jun D and fra-2 proteins in the AP-1:DNA complex by supershift analysis, fra-2 was no longer seen in adx DER animals ($n = 7$). When nuclear protein extracts from DER/adx/CCS animals were pooled to obtain enough for one supershift EMSA analysis no shifted bands were observed.

DISCUSSION

We hypothesized that DER would inhibit TPA-induced changes in the concentration and/or DNA binding of AP-1 proteins, that adrenalectomy would reverse the effect of DER on the TPA-induced changes and that corticosterone supplementation of adrenalectomized mice would restore the effect of DER on the TPA-induced changes. While treatment with TPA, as a main class tested using 3-way ANOVA, caused significant overall changes in all but fra-2 of the AP-1 constituent proteins, we found that only jun D and fra-1 levels were significantly altered by TPA in the AL/sham group. This TPA effect was reversed by DER for the jun D protein but enhanced for the fra-1 protein. For jun D, adrenalectomy did not completely reverse the effect of DER to the significant TPA effect seen in the AL/sham group, nor did corticosterone supplementation completely restore the DER effect on TPA-induced changes to that of the DER/sham group (see Figure 5). For fra-1, adrenalectomy did not significantly alter the TPA-induction in the DER group, however, corticosterone supplementation enhanced the TPA induction over the DER sham group (see Table II).

TPA-induced alterations in AP-1:DNA binding and AP-1 constituent protein levels have been reported in a number of cell culture transformation model systems and in cells from knock-in and knock-out mouse models (see references [21-24] for reviews). Evidence from cultured cell models suggests that differences in the abundance and composition of AP-1:DNA complexes may play a role in transformation [25;26]. Using the Sencar mouse skin carcinogenesis animal model, our study expands the understanding of the transactivator functions of AP-1 proteins in response to a tumor promoter (TPA), a tumor inhibitor (DER) and adrenal gland hormones (as mediators of DER effects) by measuring protein levels and DNA binding of AP-1. Our rationale for omitting DMBA treatment is based on previous findings in our lab [8] that activities of ERK-1 and ERK-2 (upstream kinases in the AP-1 pathway) were unaffected by a variety of regimens of treatment with DMBA followed by TPA. Limited by the inability to measure phosphorylated AP-1 proteins, we attempted to immunoprecipitate phosphorylated AP-1 proteins but were unsuccessful due to the small size of the mouse skin samples. However, our EMSA findings are similar to those in cell culture systems. While c-jun and c-fos are typically considered strong AP-1 transactivators [22], our Western immunoblot results on the timing of TPA effects on AP-1 proteins showed a relatively weak, early and transient activation of c-jun and c-fos in mouse skin. Supershift EMSA analysis provided no evidence that these proteins were found in the AP-1:DNA complex of either control- or TPA-treated mice. Our observations are consistent with an earlier report where c-jun and c-fos supershifts were not observed with a TPA induced AP-1:DNA complex from ICR mouse skin [27]. These authors, however observed a diminished AP-1:DNA complex as evidence of the presence of these constituent proteins in the complex. In the present study, jun B and fra-1 showed a strong, long-lasting activation by TPA. In fact, 6 hours after TPA treatment, while the levels of c-jun and c-fos proteins were starting to decrease, jun B and fra-1 were reaching their peak induction. Thus, jun B and fra-1 may be considered potential strong transactivators based on the relative magnitude of their protein induction (> 2 fold increase) by TPA in the time course experiments and c-jun and c-fos as potential modest transactivators (> 50% increase). Of these, only c-jun, jun B and fra-1 showed TPA-induced increases of at least 50% over controls 4 hours after TPA treatment in the time course experiment. While we hypothesized that TPA induction of these proteins would be significantly decreased by DER, we found no significant diet effect for any protein by 3-way ANOVA. As predicted from the TPA time course experiment, TPA significantly increased fra-1 protein levels compared to acetone controls. Surprisingly, this TPA induction was at least doubled by DER. Interestingly, the effect of DER on fra-1 was to enhance, not reduce, the TPA induction. In light of our observations and recent literature findings, the following discussion supports a role of fra-1 in replacing c-fos and acting as a repressor; while, jun B may act as an activator and substitute for, or add to, the activity of c-jun and jun D may be a negative activator.

Fra-1

Fleischmann, et al [28] showed that fra-1 replaces c-fos dependent functions in mice. Using a knock-in approach, they deleted the c-fos gene and replaced it with fra-1 and found that the slower growth and developmental traits of c-fos null mice were reversed by overexpression of fra-1 without altering the expression of c-fos target genes in mouse fibroblasts. As Milde-

Langosch [29] concluded, instead of acting as AP-1 transactivators, fra-1 may replace c-fos and act as an adaptor for other transcriptional factors or as an AP-1 repressor. Contrary to this, Young et al [30] suggest that fra-1 is required to activate AP-1 in an ERK-dependent manner in JB6 cells but that this dependency may be both tissue and gene specific. Thus, while the results from our time course experiment suggest a transactivator function for fra-1, the fact that DER enhanced the TPA stimulation of fra-1 suggested that fra-1 may be acting as a transcriptional repressor in the presence of c-fos in mouse skin. It should be noted that, while c-fos was not detected by supershift analysis, assessment of protein levels in the DER experiment was conducted 4 hours after TPA treatment, at the junction in time when c-fos was starting to diminish while fra-1 was beginning to increase.

Jun B

In vitro studies have shown that jun B had one-tenth the DNA binding activity of c-jun [31] and, in certain cell types, jun B was shown to act as a negative regulator of cell proliferation [21-24;32]. While DER had no impact on jun B protein expression, the concomitant decrease in c-jun and increase in jun B in the time course experiment and the frequent presence of jun B and the apparent absence of c-jun in the AP-1:DNA complex by supershift analysis of control- and TPA-treated mice is consistent with reports that jun B may substitute for c-jun in the mouse epidermal model. For example, Passegue etal [33] replaced c-jun with jun B by homologous recombination and expressed jun B on a c-jun-null background and found that jun B can regulate genes targeted by c-jun and substitute for c-jun in mouse embryonic development and cell proliferation. As Passegue noted, to maintain AP-1 transactivation, higher jun B levels are probably required in the absence (or reduced levels) of c-jun.

Jun D

Although the marginal 17% reduction in jun D, 6 hours after TPA, in the time course experiment was not significant; the DER experiment showed a significant 34% decrease in jun D protein levels by TPA in AL/sham animals. As we hypothesized, DER reversed this reduction in jun D by TPA in sham animals from a 34% reduction by TPA in AL animals (individual Tukey Kramer t test, $P = 0.047$) to a 15% reduction in DER animals ($P = 0.97$) relative to acetone controls. These results suggest that glucocorticoid hormones may be important in the DER effect on jun D. While the diet experiment showed a significant TPA-induced reduction of jun D protein levels, Zhoa et al [34] showed that jun D protein levels were increased 24 hours after TPA treatment in skin of DMBA-initiated MnSOD transgenic and non-transgenic mice. However, as in our finding, Zhoa showed that jun D demonstrated a clear supershift in the AP-1 complex. Like Zhoa, our results suggest a major role for jun D in early stages of TPA-induced AP-1 activation. Also, as we hypothesized, DER reversed the TPA effects on jun D through a mechanism that was partially mediated by corticosterone.

In conclusion, our results support roles for fra-1 in the presence of c-fos; and junD in suppressing downstream TPA induced events and jun B as a transactivator adding to the activity of c-jun in DER modulation of mouse skin carcinongenesis. We also showed that DER reversed the impact of TPA on jun D and enhanced the effect of TPA on fra-1 and that these DER effects were partially mediated through glucocorticoid hormones in Sencar mouse epidermis. These

observations may relate to our prior finding that DER abolished TPA-induced AP-1 luciferase mRNA expression in an AP-1 reporter transgenic luciferase mouse model [35] and further support the importance of DER in inhibiting AP-1 transactivation of AP-1 targets. Our results highlight the complexity of regulation of AP-1 activity through processes involving the amount of AP-1 constituent proteins and composition of AP-1:DNA complexes. It is notable that the DER mice in these experiments had reduced body weights compared to controls, but they were not strikingly low weights, they were in a healthy underfed state.

REFERENCES

1. Birt DF, Pinch HJ, Barnett T, Phan A, Dimitroff K. Inhibition of skin tumor promotion by restriction of fat and carbohydrate calories in SENCAR mice. *Cancer Res.* 1993; 53, 27-31.

2. Thompson HJ, Zhu Z, Jiang W. Dietary energy restriction in breast cancer prevention. *J.Mammary.Gland.Biol.Neoplasia.* 2003; 8, 133-142.

3. Kritchevsky D. Caloric restriction and experimental carcinogenesis. *Adv.Exp.Med.Biol.* 1992; 322, 131-141.

4. Kritchevsky D. Colorectal cancer: the role of dietary fat and caloric restriction. *Mutat.Res.* 1993; 290, 63-70.

5. Nair SC, Toshkov IA, Yaktine AL, Barnett TD, Chaney WG, Birt DF. Dietary energy restriction-induced modulation of protein kinase C zeta isozyme in the hamster pancreas. *Mol.Carcinog.* 1995; 14, 10-15.

6. Birt DF, Pelling JC, White LT, Dimitroff K, Barnett T. Influence of diet and calorie restriction on the initiation and promotion of skin carcinogenesis in the SENCAR mouse model. *Cancer Res.* 1991; 51, 1851-1854.

7. Birt DF, Duysen E, Wang W, Yaktine A. Corticosterone supplementation reduced selective protein kinase C isoform expression in the epidermis of adrenalectomized mice. *Cancer Epidemiol.Biomarkers Prev.* 2001; 10, 679-685.

8. Liu Y, Wang W, Hawley J, Birt DF Adrenalectomy abrogates reduction of 12-O-tetradecanoylphorbol-13-acetate-induced extracellular signal-regulated protein kinase activity in the epidermis of dietary energy-restricted SENCAR mice: implications of glucocorticoid hormone. *Cancer Epidemiol.Biomarkers Prev.* 2002; 11, 299-304.

9. Liu Y, Duysen E, Yaktine AL, Au A, Wang W, Birt DF. Dietary energy restriction inhibits ERK but not JNK or p38 activity in the epidermis of SENCAR mice. *Carcinogenesis* 2001; 22, 607-612.

10. Przybyszewski J, Yaktine AL, Duysen E, Blackwood D, Wang W, Au A, Birt DF Inhibition of phorbol ester-induced AP-1-DNA binding, c-Jun protein and c-jun mRNA by dietary energy restriction is reversed by adrenalectomy in SENCAR mouse epidermis. *Carcinogenesis* 2001; 22, 1421-1427.

11. Dong Z, Cmarik JL, Wendel EJ, Colburn NH. Differential transformation efficiency but not AP-1 induction under anchorage-dependent and -independent conditions. *Carcinogenesis* 1994; 15, 1001-1004.

12. Li JJ, Rhim JS, Schlegel R, Vousden KH, Colburn NH. Expression of dominant negative Jun inhibits elevated AP-1 and NF-kappaB transactivation and suppresses anchorage independent growth of HPV immortalized human keratinocytes. *Oncogene* 1998; 16, 2711-2721.

13. Dong Z, Crawford HC, Lavrovsky V, Taub D, Watts R, Matrisian LM, Colburn NH. A dominant negative mutant of jun blocking 12-O-tetradecanoylphorbol-13-acetate-induced invasion in mouse keratinocytes. *Mol.Carcinog.* 1997; 19, 204-212.

14. Angel P, Szabowski A, Schorpp-Kistner M. Function and regulation of AP-1 subunits in skin physiology and pathology. *Oncogene* 2001; 20, 2413-2423.

15. Stewart JW, Koehler K, Jackson W, Hawley J, Wang W, Au A, Myers R, Birt DF. Prevention of mouse skin tumor promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition. *Carcinogenesis* 2005; 26, 1077-1084.

16. Pashko LL, Schwartz AG. Reversal of food restriction-induced inhibition of mouse skin tumor promotion by adrenalectomy. *Carcinogenesis* 1992*;* 13, 1925-1928.

17. Belman S, Troll W. The inhibition of croton oil-promoted mouse skin tumorigenesis by steroid hormones. *Cancer Res.* 1972; 32, 450-454.

18. Slaga TJ, Lichti U, Hennings H, Elgjo K, Yuspa SH. Effects of tumor promoters and steroidal anti-inflammatory agents on skin of newborn mice in vivo and in vitro. *J.Natl.Cancer Inst.* 1978; 60, 425-431.

19. Armario A, Montero JL, Jolin T. Chronic food restriction and the circadian rhythms of pituitary-adrenal hormones, growth hormone and thyroid-stimulating hormone. *Ann.Nutr.Metab* 1987; 31, 81-87.

20. Yaktine AL, Vaughn R, Blackwood D, Duysen E, Birt DF. Dietary energy restriction in the SENCAR mouse: elevation of glucocorticoid hormone levels but no change in distribution of glucocorticoid receptor in epidermal cells. *Mol.Carcinog.* 1998; 21, 62-69.

21. Shaulian E, Karin M. AP-1 as a regulator of cell life and death. *Nat Cell Biol* 2002; 4, E131-E136.

22. Hess J, Angel P, Schorpp-Kistner M. AP-1 subunits: quarrel and harmony among siblings. *J Cell Sci* 2004; 117, 5965-5973.

23. Mechta-Grigoriou F, Gerald D, Yaniv M. The mammalian Jun proteins: redundancy and specificity. *Oncogene* 2001; 20, 2378-2389.

24. Jochum W, Passegue E, Wagner EF. AP-1 in mouse development and tumorigenesis. *Oncogene* 2001; 20, 2401-2412.

25. Bernstein LR, Colburn NH. AP1/jun function is differentially induced in promotion-sensitive and resistant JB6 cells. *Science* 1989; 244, 566-569.

26. Bernstein LR., Walker,S.E. Tumor promotion resistant cells are deficient in AP-1 DNA binding, JunD DNA binding and JunD expression and form different AP-1-DNA complexes than promotion sensitive cells. *Biochim.Biophys.Acta* 1999; 1489, 263-280.

27. Chun K-S, Kim S-H,Yong-Sang Song Y-S,SurhY-J. Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* 2004; 25,713-722.

28. Fleischmann A, Hafezi F, Elliott C, Reme CE, Ruther U, Wagner EF. Fra-1 replaces c-Fos-dependent functions in mice. *Genes Dev.* 2000; 14, 2695-2700.

29. Milde-Langosch K. The Fos family of transcription factors and their role in tumourigenesis. *Eur.J Cancer* 2005; 41, 2449-2461.

30. Young MR, Nair R, Bucheimer N, Tulsian P, Brown N, Chapp C, Hsu TC, Colburn NH. Transactivation of Fra-1 and Consequent Activation of AP-1 Occur Extracellular Signal-Regulated Kinase Dependently. *Mol.Cell.Biol.* 2002; 22, 587-598.

31. Deng T, Karin M. JunB differs from c-Jun in its DNA-binding and dimerization domains, and represses c-Jun by formation of inactive heterodimers. *Genes Dev.* 1993; 7, 479-490.

32. Shaulian E, Karin,M. AP-1 in cell proliferation and survival. *Oncogene* 2001; 20, 2390-2400.

33. Passegue E, Jochum W, Behrens A, Ricci R, Wagner EF. JunB can substitute for Jun in mouse development and cell proliferation. *Nat Genet* 2002; 30, 158-166.

34. Zhao Y, Xue Y, Oberley TD, Kiningham KK, Lin SM, Yen HC, Majima H, Hines J, Clair D. Overexpression of Manganese Superoxide Dismutase Suppresses Tumor Formation by

Modulation of Activator Protein-1 Signaling in a Multistage Skin Carcinogenesis Model. *Cancer Res* 2001; 61, 6082-6088.

35. Birt DF, Przybyszewski J, Wang W, Stewart J, Liu Y. Identification of molecular targets for dietary energy restriction prevention of skin carcinogenesis: an idea cultivated by Edward Bresnick. *J. Cell. Biochem.* 2004; 91,258–264.

Figure Legends

Figure 1. Timing of TPA Effects on AP-1:DNA Binding in SENCAR Mouse Epidermis. Panel A, Image of a typical EMSA gel with one mouse represented in each lane showing AP-1:DNA binding in SENCAR mouse dorsal skins treated in vivo with acetone or 3.2nmol TPA for the indicated times. In addition to about 70,000 cpm radiolabeled probe and 0.5 µg poly(dI-dC), the lanes contents are as follows: lane 1 60 µg epidermal nuclear extract (eNE) from a 4 hour TPA-treated mouse + 100-fold excess of unlabeled AP-1 oligo; lane 2 same as lane 1 except 100-fold excess of unlabeled AP-2 oligo; lanes 3-9 each contain 60 µg eNE from a mouse treated with acetone or TPA for the times as indicated. Panel B, AP-1:DNA binding was determined by EMSA of nuclear protein extracts from epidermal cells of mouse dorsal skin. The symbol (with standard error bars) for the 0 hour time point is the average of 29 acetone-treated mice. All other symbols (with standard error bars) represent the averages of 8 mice per time point. Statistical significance compared to 0 hour acetone control by the student two sample tTest ($P < 0.05$) is indicated by *.

Figure 2. EMSA supershift analysis of the timing of TPA effects on the composition of the AP-1:DNA complex. Image of a typical EMSA supershift gel showing the shifted AP-1:DNA band and the supershifted band in the same SENCAR dorsal skin nuclear extract from a mouse 4 hours after treatment with acetone control. The binding reaction of each lane contains about 100,000 cpm of labeled probe, 0.5 µg poly(dI-dC), 15 µg epidermal nuclear extract from a single mouse and 400ng of the antibody indicated and incubated for 30min before separation by electrophoresis. In this image, all lanes of the gel were loaded with the nuclear extract from the same mouse. This blot was repeated for 4-10 mice per treatment/diet group.

Figure 3. The Timing of TPA Effects on Jun (Panels A,B) and Fos Proteins (Panels C,D). Examples of Western immunoblot images for c-jun, jun B and jun D proteins (Panel A) and c-fos, fra-1 and fra-2 proteins (Panel C) where the first lane (PC) contains the positive control provided by the manufacturer and other lanes each contain 100 µg SENCAR epidermal whole cell lysate from one mouse exposed to TPA or acetone for the times as indicated. Panels B,D, Relative amounts of each protein are plotted as chemiluminescent response against time where the symbols represent the means (± SE) for $n = 6$ mice per group and the zero time point is the mean of the 0, 4, 24 hour acetone treatments ($n = 12$). Time points that are significantly different from acetone-treated controls using ANOVA followed by Dunnett's Multiple Comparison Test on log transformed data ($P < 0.05$) are as indicated in the legend using different symbols (*, $, #) for each protein. The arrow identifies the jun B band used for quantitation.

Figure 4. Effects of dietary energy restriction, adrenalectomy and corticosterone supplementation on Sencar mouse body weights. Values are mean (± standard error) of the body weight for $n = 6$-21 mice per group. Analysis by one-way ANOVA showed significant differences between DER groups compared to freely fed animals starting 2 weeks after beginning the DER diet ($P < 0.05$). In addition, Dunnett's post test performed on DER mouse groups at 21 weeks of age showed that

DER/adx/CCS mice had a significantly different mean body weight from DER/adx and DER/sham animals ($P < 0.05$). Abbreviations, adx, adrenalectomized; AL, *ad libitum* fed; CCS, corticosterone supplemented; DER, 40% dietary energy restricted; sham, sham operated control.

Figure 5. Effects of DER, TPA and corticosterone on Jun (Panels A, B, C) and Fos (Panels D, E, F) Proteins. Relative levels of each protein by Western analysis are plotted as chemiluminescence output expressed as the means within 95% confidence intervals for n = 3-11 per group (number of mice for each observation provided at the base of the bar) in whole cell lysates from dorsal skins of Sencar mice treated with acetone or TPA for 4 hours and fed the indicated diet with or without the adrenal gland as indicated. Abbreviations are as in Fig 4. Significant effects for each protein by 3-way ANOVA are shown for main effects of TPA treatment as *, glucocorticoid status as #, diet as $ and a bracket encloses acetone and TPA groups that are significantly different from each other by Tukey-Kramer posthoc analysis ($P < 0.05$) except for the Fra-1 AL/ADX groups where $P < 0.07$.

Figure 6. EMSA supershift analysis of the DER effects on the composition of the AP-1:DNA complex. Image of a typical EMSA supershift gel showing the shifted AP-1:DNA band and the supershifted band as indicated in SENCAR mouse dorsal skins of freely fed sham-operated treated with acetone for 4 hours. The binding reaction is as in Fig 2.