To the Graduate Council:

I am submitting herewith a dissertation written by Karen Gray Nelson entitled "A Study of Protein Modifications Induced by Phorbol Ester Tumor Promoters in Mouse Skin." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

Thomas J Slaga

T. J. Slaga, Major Professor

We have read this dissertation and recommend its acceptance:

Susan M Fischer

John S. Cook

Sankar Mitra

Accepted for the Council:

L Evans Roth

Vice Chancellor
Graduate Studies and Research

A STUDY OF PROTEIN MODIFICATIONS INDUCED BY PHORBOL ESTER TUMOR PROMOTERS IN MOUSE SKIN

A Dissertation

Presented for the

Doctor of Philosophy

Degree

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Karen Gray Nelson

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DEDICATION

This dissertation is dedicated to my husband Fred and my twin sons Christopher and Matthew.

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ABSTRACT

The purpose of this study was to determine if the phorbol ester tumor promoter 12-0-tetradecanoylphorbol-13-acetate (TPA) induced any specific changes in mouse epidermal proteins using the high resolution technique of two-dimensional electrophoresis. To accomplish this goal of determining the specificity and possibly the stage in promotion with which these protein changes were associated, epidermal proteins were analyzed (1) after treatment of adult mouse epidermis with several weakly promoting hyperplasiogenic agents, (2) following treatment with TPA in combination with various inhibitors of tumor promotion, (3) in basal kerotinocytes isolated from adult epidermis following treatment with TPA or several weakly promoting agents, and (4) during an initiation-promotion experiment.

The more soluble protein components not crosslinked by disulfide bonds were removed from the epidermis using 4M urea. Following the urea extraction, the remaining protein consisting primarily of the epidermal keratins were solubilized using 2% sodium dodecylsulfate and 10mM dithiothreitol (SDS-DTT). The pH range (pH 4-6) of the isoelectric focusing dimension was chosen such that maximal separation of individual keratin polypeptides was achieved. The proteins were resolved further by separation on the basis of their molecular weights in the second dimension which usually consisted of 10% SDS-polyacrylamide gels. The parameters that were used for the identification and characterization of some of the epidermal proteins were extractability, isoelectric points,

molecular weights, assembly into filaments in vitro, immunological crossreactivity, peptide maps, and amino acid composition.

Evidence was found which indicated that the potent tumor promoter TPA as well as the weakly promoting hyperplasiogenic agents, mezerein, ethylphenylpropiolate (EPP), and mechanical abrasion, induced similar modifications of epidermal proteins, particularly among the keratins. These keratin modifications progressed with time following treatment resulting in a keratin pattern which resembled that of newborn epidermis. The protein modifications induced by EPP, however, were quantitatively less than those observed with the other agents. This observation is supported by the findings of others which showed that many of the biochemical changes induced by EPP were quantitatively less when compared to those induced by TPA or mezerein. Significant keratin modifications were also observed in basal cells isolated from adult epidermis following treatment with TPA and the other weakly promoting hyperplasiogenic agents. Modification of the epidermal keratins, which represent the major differentiation product of the epidermis, could result in the alteration of the properties of the filaments formed from these proteins. Several other protein changes were observed among the more soluble proteins extracted with urea which were also prominent proteins in newborn epidermis. The induction of hyperplasia by strong or weak promoters appeared to result in shifts in the gene expression in adult epidermis to that of a newborn state, causing the reappearance of newborn proteins. These data indicated that many of the protein changes induced by TPA were associated with its hyperplasiogenic properties.

Further evidence which indicated that these protein changes were associated with hyperplasia and not necessarily related to complete promoting ability was obtained by studying the effects of various inhibitors of tumor promotion on the TPA induced protein modifications. The inhibitors which interfered with the induction of hyperplasia, fluocinolone acetonide (FA) and cycloheximide, were found to also interfere with the promoter-induced protein changes. Retinoic acid (RA) and tosylphenylalanylchloromethyl ketone (TPCK) which did not prevent the TPA induced hyperplasia did not significantly influence these protein changes.

The keratin polypeptide patterns of papillomas, carcinomas, and epidermis during an initiation-promotion experiment were studied to further understand the role that the keratin modifications play in two-stage carcinogenesis. Distinct keratin patterns were found to be associated with each of these tissues. The TPA-treated epidermis, initiated and noninitated, contained the same keratin modifications that were observed with one TPA treatment. These keratin changes were also features of all papillomas; however, papillomas could be distinguished from surrounding epidermis by the presence of considerable charge heterogeneity among the high molecular weight keratins. The most striking protein alterations observed in the carcinomas was a significant reduction in the high molecular weight keratins. In fact, the carcinoma keratin profiles were very similar to those patterns observed in the basal cells isolated from adult epidermis following a single treatment with a hyperplasiogenic agent. This suggests that the program of keratin synthesis

is fixed at the basal cell level of differentiation. Changes in keratin synthesis, therefore, may serve as a biochemical marker of malignant progression in mouse epidermis.

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LIST OF ABBREVIATIONS

TPA — 12-0-tetradecanoylphorbol-13-acetate

EPP — Ethylphenylpropiolate

SDS-DTT— 2% Sodium dodecylsulfate and 10mM dithiothreitol

RA — Retinoic Acid

FA — Fluocinolone acetonide

TPCK — Tosylphenylalanylchloromethyl ketone

DMBA — 7-12-dimethylbenz(a)anthracene

GGT — ɣ-Glutamyltranspeptidase

ODC — Ornithine Decarboxylase

CHAPTER I

INTRODUCTION

Mouse skin carcinogenesis has been divided into at least two stages; initiation which is brought about by a single application of a sub-threshold dose of a carcinogen, and promotion which is brought about by repetitive treatment with a noncarcinogenic tumor promoter. No apparent morphological modifications are induced in the epidermis by initiation, even though its effects are essentially irreversible. The mechanism of initiation probably involves a somatic cell mutation caused by covalent binding of electrophilic metabolites of carcinogens to cellular DNA (45, 54, 55).

The most potent and widely studied skin tumor promoters are the phorbol esters. The best phorbol ester tumor promoter, 12-0-tetra-decanoylphorbol-13-acetate (TPA), is a potent inflammatory and hyper-plasiogenic agent which induces numerous cellular and biochemical responses. These include stimulation of DNA, RNA and protein synthesis (3), increased phospholipid synthesis (31), altered cell morphology and differentiation (24, 26, 27), decreased responsiveness to β-adrenergic agonist (20) and G1 chalone (16), increased prostaglandin synthesis (43), induction of dark basal keratinocytes (15), increased histone synthesis and phosphorylation (29, 30), stimulation of the activities of the following enzymes: ornithine decarboxylase (ODC) (22), phosphodiesterase (21), phospholipase (47), and neutral proteases (42), decreased histidase activity (7) and induction of two proteins associated with keratohyalin

granule production (4, 5). It is difficult to distinguish which of these events are critical for tumor promotion. Those responses that appear to correlate best with promotion are the induction of epidermal hyperplasia, the stimulation of ornithine decarboxylase followed by elevation of polyamine levels, and the induction of dark basal keratinocytes (49, 54). Many of these cellular responses are probably normal events which occur upon stimulation of cell proliferation. The induction of epidermal hyperplasia appears to play an essential role in tumor promotion. There is a strong correlation between the promoting ability of a series of phorbol esters and their ability to induce hyperplasia (34). Further support comes from the observation that if hyperplasia is inhibited so is promotion (33). However, not all agents which induce inflammation and hyperplasia are promoters. For example, ethylphenylpropiolate (EPP) and acetic acid are very weak promoters at doses which induce a sequence of metabolic and morphologic changes similar to those caused by the promoter TPA (28, 56). Mezerein is a weakly promoting diterpene resembling TPA which induces similar cellular responses, especially with regard to the induction of hyperplasia and ODC activity. While epidermal hyperplasia produced by mechanical abrasion also acts as a weak promoter, the milder epidermal hyperplasia produced by hair plucking does not. It has been suggested that the intensity of hyperplasia is important for promoting activity (1, 2).

The use of inhibitors of tumor promotion such as the antiinflammatory steroids, retinoids, and protease inhibitors have contributed significantly to the search for critical events in tumor promotion (57, 58, 59). The

major mechanism by which antiinflammatory steroids such as fluocinolone acetonide (FA) inhibits promotion is thought to be due to their inhibition of promoter-induced hyperplasia and inflammation (57). Inhibition of promotion by the retinoids may be due to their inhibition of promoter-induced ODC activity and their influence on epithelial differentiation (58). Protease inhibitors such as tosylphenylalanylchloromethylketone (TPCK) may inhibit TPA induction of specific proteases that may be required for important events in promotion (59).

Recently, it has been demonstrated that the promotion is a multistage process consisting of at least two distinct stages (32). The first stage of promotion can be accomplished by only one TPA treatment. The second stage is brought about by repetitive treatments with mezerein, a weak promoter that brings about cellular responses similar to TPA. Although EPP is a potent hyperplastic agent, many of the cellular and biochemical changes induced by EPP are usually quantitatively less than those observed with TPA or mezerein. Ineffective as a first or second stage promoter, EPP may represent a third stage promoter, where the induction of cellular proliferation is the critical event. The effects of some inhibitors of promotion on the first and second stages have also been studied. The antiinflammatory steroid FA is an effective inhibitor of both stages of promotion. The protease inhibitor TPCK interferes with the first stage, while retinoic acid (RA), a retinoid, inhibits the second stage. Evidence obtained from these studies indicates that the induction of dark basal keratinocytes is critical for the first stage and that most of the other morphological and biochemical responses caused by TPA and mezerein are mostly likely associated with second stage or possibly even a third stage.

The major objective of this dissertation project was to determine if any specific changes in mouse epidermal proteins occurred in response to TPA using two-dimensional gel electrophoresis. In order to determine if any specific protein changes occurred in the promotion process, a comparison was made of the tumor promoter induced protein changes to those induced by weak or nonpromoting hyperplasiogenic agents. In addition, the effect of various inhibitors of tumor promotion on the protein modifications were studied in order to further determine their specificity and possibly the stage in promotion with which these protein changes were associated. To determine if TPA was inducing protein changes in a particular cell population of the epidermis, basal cells were isolated from TPA treated adult mouse epidermis and the protein alterations were investigated. A study of the protein changes that occurred in adult mouse epidermis, papillomas and carcinomas during an initiation-promotion experiment was also done to further characterize the role that these protein modifications played in two-stage carcinogenesis.

CHAPTER II

PROTEIN MODIFICATIONS INDUCED IN MOUSE EPIDERMIS BY POTENT AND WEAK TUMOR-PROMOTING HYPERPLASIOGENIC AGENTS

Introduction

The use of phorbol ester tumor promoters has led to increasing understanding of the cellular and biochemical mechanisms of two-stage skin carcinogenesis. Treatment of mouse skin with these promoters, especially the most potent phorbol ester, TPA, causes inflammation and hyperplasia. In addition, the phorbol esters induce many other morphological and biochemical alterations of normal skin function. They have been found to stimulate DNA, RNA, and protein synthesis (3); increase phospholipid synthesis (31); increase histone synthesis and phosphorylation (29, 30); alter cell morphology and differentiation (24, 26, 27); decrease responsiveness to β-adrenergic agonist (20) and G_1 chalone (16); increase ornithine decarboxylase activity (22); increase phosphodiesterase activity (21); increase protease activity (42); increase prostaglandin synthesis (43); decrease histidase activity (7); and induce dark basal keratinocytes (15). It is difficult to determine which of the many phorbol ester tumor promoter-related responses are essential events necessary for tumor promotion. For example, the role that epidermal hyperplasia plays in tumor promotion is a source of controversy (2, 24, 26-28, 33). Slaga and co-workers have demonstrated

that there is a strong correlation between the promoting ability of a series of phorbol esters and their ability to induce epidermal hyperplasia (34). Although most promoters are irritants (14, 33, 34), which induce inflammation and epidermal hyperplasia, not all irritants are promoters. For example, EPP is a very weak promoter (28) at doses which induce a sequence of metabolic and morphologic changes very similar, although usually quantitatively less than those caused by the potent promoter TPA. Mezerein is a diterpene similar to TPA which can induce most of the biochemical and morphological changes observed with TPA, but TPA is at least 50 times more active as a tumor promoter (19).

Epidermal hyperplasia produced by abrasion has also been shown to induce metabolic and morphologic changes similar to those induced by TPA (1). Recently, Argyris (2) demonstrated that epidermal hyperplasia produced by mechanical abrasion of mouse skin previously initiated with a carcinogen had weak promoting activity. He presented evidence indicating that the degree to which hyperplasia was induced was important for promoting activity. Hair plucking, which resulted in a milder epidermal hyperplasia than that induced by abrasion, did not act as a promoter.

Slaga and co-workers (32) have demonstrated that the promotion process can be divided into at least 2 distinct stages. Mezerein was found to be a potent 2nd-stage promoter, which indicates that the majority of the responses observed with TPA may be features of the 2nd-stage of promotion. Although it is ineffective as a 1st- or 2nd-stage promoter, EPP may be a promoter in the 3rd stage, in which the

induction of cellular proliferation is the critical event (Slaga, unpublished results). The stage of promotion at which abrasion may be effective is presently being investigated.

In this study, changes in mouse epidermal proteins that occurred in response to TPA were investigated by use of two-dimensional gel electrophoresis. I was particularly interested in possible modifications of the epidermal keratins, which are the major product of differentiation in the epidermis (11, 17, 40). To determine if the protein changes were essential for the promoting activity of TPA, I also compared the protein modifications caused by hyperplastic agents which have weak promoting activity but are active in the different stages of promotion. The results of this investigation indicate that many of the protein modifications induced by TPA are also induced by weakly promoting chemical and mechanical hyperplasia-inducing agents. Although these protein changes are not directly related to promoting ability, they may be essential to the 2nd stage of promotion.

Materials and Methods

Chemicals. TPA was obtained from Dr. P. Borchert (University of Minnesota, Minneapolis); mezerein was a gift from the late Dr. S. M. Kupchan (University of Virginia, Richmond) and Dr. J. Douros (National Cancer Institute, Bethesda, Md.); EPP was purchased from Aldrich Chemical Company (Milwaukee, Wis.). All the reagents for SDS-polyacrylamide electrophoresis were obtained from Bio-Rad Laboratories (Richmond, Calif.). Most of the isoelectric focusing ampholytes were from LKB (Durham, N. C.).

Nair was purchased from Carter Products (New York, N. Y.). The protease inhibitor phenylmethylsulfonylfluoride was from Sigma Chemical Company (St. Louis, Mo.). Staphylococcus aureus V8 enzyme used for peptide mapping was from Miles Laboratories (South Africa).

Treatment of animals and protein extraction. Female Sencar mice (7 to 8 weeks old) raised at Oak Ridge National Laboratory, were shaved at least 2 days before treatment. The 10 animals in each experimental group were treated topically with either TPA (5 μg), mezerein (2μg), EPP (13.9 mg) dissolved in 0.2 ml acetone, or acetone alone. The dosages were chosen so that comparable hyperplasia would be induced by each compound. Regenerative hyperplasia was also induced by mechanical abrasion. Shaved animals were anesthetized with Nembutal and their backs were abraded with a felt wheel mounted on a power-driven tool (2). The mice were killed by cervical dislocation at various times after treatment. Excess hair was removed from their backs by application of a chemical depilatory (Nair) for 5 minutes. After the depilatory cream was removed by thorough washing with cold water, the skins were removed, and the epidermis was scraped off with a razor blade. The epidermal proteins were isolated by a modification of the procedures of Fuchs and Green (12) and Dale and Ling (9). The epidermis was placed into a solution of 4 M urea and 1 mM phenylmethylsulfonylfluoride and homogenized with a Polytron PT-10 homogenizer for 45 seconds. The homogenate was centrifuged at 35,000 rpm for 30 minutes and the pellets were resuspended in 2% SDS-10 mM DTT, then incubated at 37° for 1 hour. The SDS-DTT extract was then centrifuged at 35,000 rpm for 30 minutes. The 4 M

urea supernatant containing the more readily soluble proteins of the epidermis and the SDS-DTT supernatant containing the more insoluble proteins (predominantly the keratins) were then dialyzed against distilled water, lyophilized, and stored at -20° until two-dimensional electrophoresis was performed. Figure 2.1 is a flow chart depicting the extraction procedure.

Two-dimensional electrophoresis. The method of O'Farrell (23) was employed. The isoelectric focusing dimension was run over a narrow acidic pH range (pH 4.0 to 6.0) in 5.5-mm glass electrophoresis tubes; this pH range was chosen because it would allow maximal separation of the individual keratins which have been shown to have isoelectric points within the range (8, 39). The 2nd dimension utilized 10% SDS-polyacrylamide slab gels. After electrophoresis, the proteins were fixed in 12% trichloroacetic acid, then stained in a solution of 0.1% Coomassie blue, 7% acetic acid, and 50% ethanol. Gels were destained in a solution of 7% acetic acid and 10% ethanol.

Immunological methods. Antiserum specific for keratins isolated from the two-dimensional gels was prepared in New Zealand white rabbits. The individual keratins were removed from stained two-dimensional gels and eluted electrophoretically into dialysis bags. This was followed by dialysis against distilled water and lyophilization. The keratins were suspended in complete Freund's adjuvant and injected at multiple sites including the footpads. This procedure was repeated twice. Double-diffusion experiments were performed by the method described by Yen and co-workers (44).

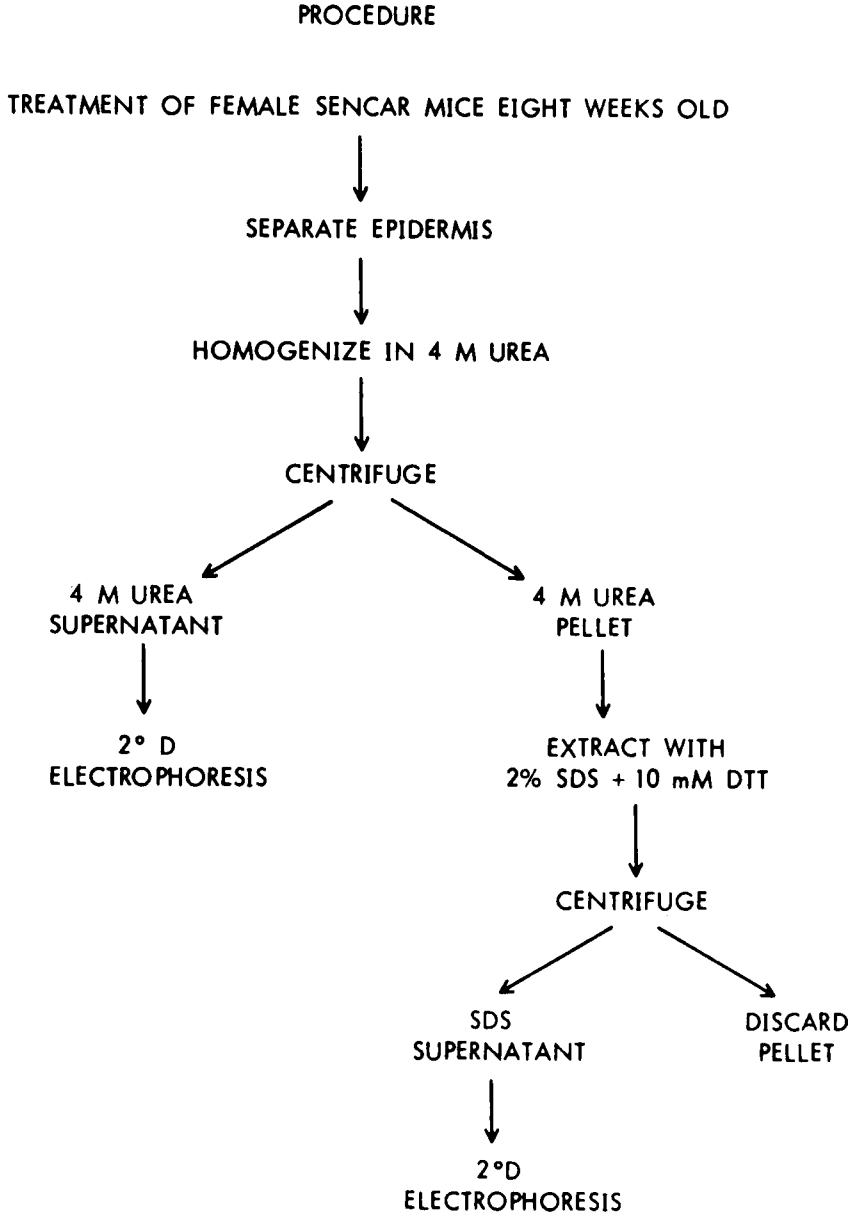

Figure 2.1. Flow chart depicting protein extraction procedure used with adult and newborn epidermis.

Peptide mapping. The peptide maps of the isolated keratins were examined by the procedure described by Cleveland et al. (6), which involves limited proteolysis followed by separation of the peptides by means of SDS-polyacrylamide electrophoresis. After separation of the keratin by two-dimensional electrophoresis, the individual proteins were cut from stained gels and equilibrated with Cleveland's SDS buffer overnight. To introduce enough protein to permit peptide maps to be detected with Coomassie Blue, I used 17.5-cm tubes. This allowed for a 3.5-cm gel-loading region, a 3.5-cm 4.5% SDS-polyacrylamide stacking gel, and a 10.5-cm 12% SDS-polyacrylamide running gel. The proteins were electrophoresed with 20 or 40 µg of Staphylococcus aureus V8 enzyme. Once the samples had stacked at the border of the 12% SDS-polyacrylamide running gel, the current was shut off for 1 hour. The tube gels were fixed and stained by the procedures described for the two-dimensional gels.

Amino acid analysis. After the keratins were separated by two-dimensional electrophoresis, the individual proteins were cut from stained gels and eluted from the gel pieces by electrophoresis into dialysis bags. The samples were then dialyzed overnight against 4 changes of distilled water. Proteins were precipitated with 25% trichloroacetic acid and washed with ethanol and ether. Approximately 200 µg of each purified keratin were hydrolyzed with 6 N HCl—1% 2-mercaptoethanol at 110° for 22 hours in sealed evacuated tubes. The amino acid composition was determined with a Beckman amino acid analyzer.

Keratin filament formation. Filaments were formed in vitro by the method of Steinert and Gullino (35). The proteins were extracted from the mouse epidermis in 8 M urea-0.05 M Tris-HCl (pH 8.8) and 0.025 M 2-mercaptoethanol for 3 hours. This was followed by centrifugation; first at 30,000 × g for 30 minutes to remove cellular debris and then at 250,000 × g for 2 hours to remove small particles. Filaments were produced by dialysis of the urea extracts against 5 mM Tris-HCl (pH 8.0) and 0.025 M 2-mercaptoethanol. These filaments could be pelleted by ultracentrifugation. For two-dimensional electrophoresis, the pelleted filaments were dissolved in the lysis buffer described by O'Farrell (23). For electron microscopy, filaments were negatively stained with 0.7% uranyl acetate.

Results

Two protein extracts (4 M urea and SDS-DTT) were analyzed by two-dimensional electrophoresis, as shown in Figure 2.1, page 10. The proteins were characterized at various times after exposure of mouse skin to either 5 µg TPA or the delivery vehicle, acetone. the 4 M urea extract removed the more soluble epidermal proteins. The remaining tissue was extracted with SDS-DTT for efficient solubilization of the keratins that were stabilized by disulfide bonds in the more terminally differentiated cells. The majority of the SDS-DTT extracted proteins that were resolved using my specified conditions of two-dimensional gel electrophoresis can be classified as epidermal keratins on the basis of their extractability, isoelectric points, and molecular weights.

Analysis of the SDS-DTT solubilized epidermal proteins by two-dimensional electrophoresis. As early as 12 hours after a single treatment with TPA (Figure 2.2A), the SDS-DTT extracted proteins were changed compared with the control (Figure 2.2F). TPA induced a new protein, designated 4, with an approximate molecular weight of 51,000. By 48 hours after treatment, the protein changes were much more distinct (Figure 2.2B). Not only was there a substantial increase in the amount of protein 4, but numerous other proteins began to appear on the gel profiles (proteins 40, 41, 42, and 43). These proteins had molecular weights ranging from 45,000 to 48,000. In addition, quantitative increases of protein 8 (M. W. 54,000) and protein 52 (M. W. 54,000) were observed. Several other proteins appeared in the lower-molecular-weight ranges which were absent from controls (Figure 2.2F). The protein patterns which emerged by 48 hours after a single treatment with TPA were also present at 72 hours after treatment (Figure 2.2C). Not only were there larger amounts of the new proteins at 72 hours than seen at 48 hours, but also several other proteins (proteins 26, 44, 46, 58, and 59) appeared, ranging in molecular weight between 30,000 and 50,000. At this time the lower-molecular-weight proteins (proteins 3, 4, 5, 8, 40, 41, 42, 43, and 46) were more prominent than the higher-molecular-weight protein (proteins 2a, 2b, 2c) seen in the controls.

At 1 week after TPA treatment, many of the protein changes appeared to be subsiding (proteins 4, 8, 40, 41, 42, 43, etc.) (Figure 2.2D). Note that proteins 2a and 2b consisted of at least 2 proteins each: a high-molecular-weight component (called $2a_1$ and $2b_1$), M. W. 62,000;

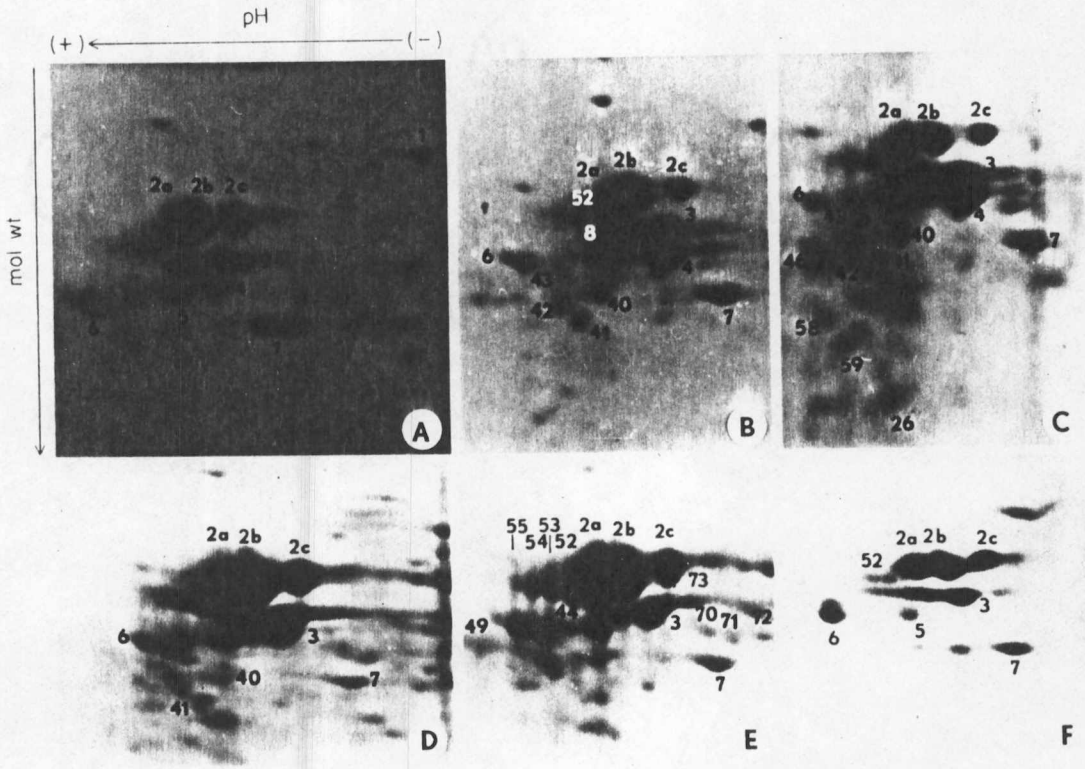

Figure 2.2. Two-dimensional gel profiles of the proteins extracted with SDS-DTT from adult Sencar mouse epidermis at various times after treatment with a single dose of TPA or acetone. The pH gradient of the isoelectric focusing gels ranges between pH 4.0 and 6.0. Numbers and letters were assigned to individual proteins for ease of comparison. These proteins were predominantly the epidermal keratins. The time points investigated were (A) 12 hours, (B) 48 hours, (C) 72 hours, (D) 1 week, and (E) 2 weeks after a single application of TPA. The protein profiles of Sencar mice treated with the delivery vehicle, acetone, did not change significantly at various times after treatment. Therefore, only the gel profiles of proteins extracted 48 hours after acetone treatment will be shown (F).

and a lower-molecular-weight component (called $2a_2$ and $2b_2$), M. W. 60,000. In general, only small amounts of the higher-molecular-weight components could be detected in the acetone control protein samples. However, 1 week after TPA treatment these high-molecular-weight components appear to increase.

The TPA-induced protein profile did not return to normal even after 2 weeks following a single TPA treatment (Figure 2.2E). Instead, the two-dimensional gel profile of the SDS-DTT extracted proteins from TPA-treated epidermis appeared remarkably similar to that of newborn SDS-extracted proteins (proteins 2a, 2b, 44, 49, 52, 53, 54, 55, 70, 71, and 72) (Figure 2.3A). Proteins 2a and 2b were predominant in both the TPA and newborn profiles, each consisting of at least 2 components. Furthermore, similarities were noted among the groups of proteins designated 52, 53, 54, and 55. These proteins were more acidic than keratin proteins 2a, 2b, and 2c and had very similar streaking patterns along the SDS dimension. This was indicative of both charge and size heterogeneity. Since keratins have been shown to be phosphorylated (39), it is possible that some of the proteins within the groups designated 52, 53, 54, and 55 were actually phosphorylated derivatives of the major keratins (proteins 2a, 2b, 2c, 3, and 5) having different numbers of phosphate groups. If this was the case, then TPA could induce in the adult epidermis posttranslational modification of keratin which resembled the newborn state.

In summary, TPA induced several changes in the SDS-DTT extractable proteins, which were probably epidermal keratins. There was a progressive

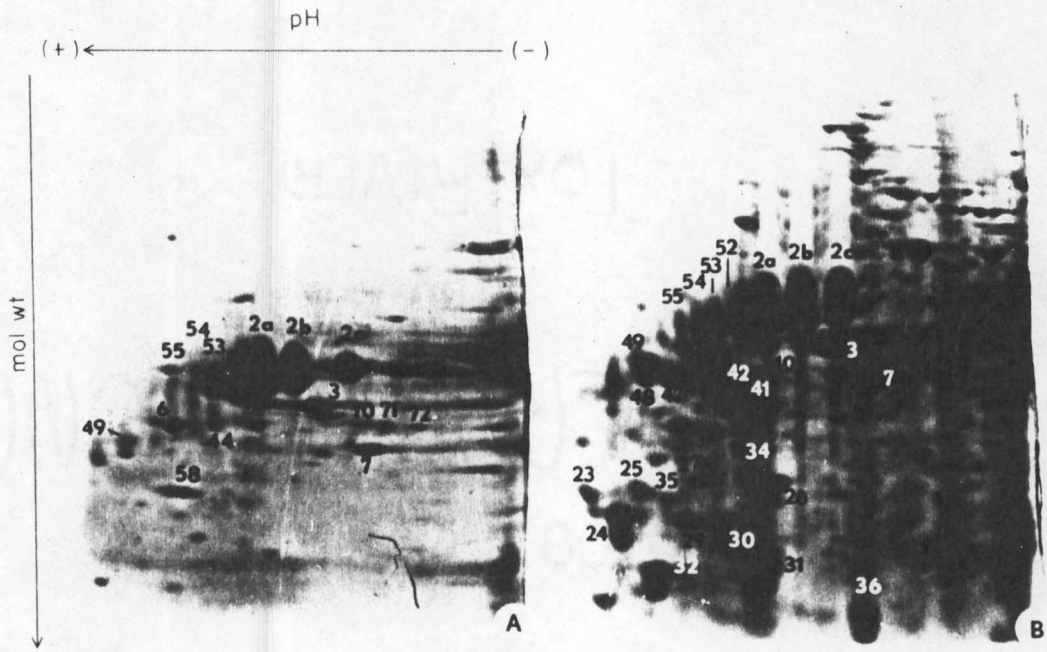

Figure 2.3. Two-dimensional gel protein patterns of Sencar newborn epidermis. (A) SDS-DTT extracted proteins; (B) 4 M urea solubilized proteins.

change of these proteins with time after TPA treatment, culminating in the appearance of newborn keratin patterns.

Identification and characterization of the epidermal keratins. The majority of SDS-DTT solubilized proteins (proteins 2a, 2b, 2c, 3, 4, 5, 6, 7, 8, 40, 41, 42, 43, and 44) that appear in the gels are probably the epidermal keratins. The keratins are a group of closely related, but distinct, fibrous proteins which associate in the form of 80-Å tonofilaments; they represent the major differentiation product of the epidermis (11-13, 17, 40). Gel electrophoresis can be used to separate the keratins into a family of polypeptides with molecular weights ranging from 40,000 to 68,000. The keratins of the living cell layers initially exist in a reduced form which probably favors polymerization into filaments. However, as terminal differentiation proceeds, the keratins become cross-linked by intermolecular disulfide bonds which are necessary for stabilization of the filaments in the fully differentiated, dead, stratum corneum. The keratins can be extracted from living cells with urea or SDS solutions, but a reducing agent is required for solubilization of these proteins from terminally differentiated cells. Keratins can reassemble into filaments *in vitro* if the denaturant is removed by dialysis (35, 39). Individual keratins have been shown to have similar amino acid compositions, peptide maps, and immunological reactivity (11).

The criteria used in this paper for the identification of the keratins are: (1) extractability, (2) isoelectric points, (3) molecular weights, (4) reassembly into filaments *in vitro*, (5) immunological cross-reactivity, (6) amino acid composition, and (7) peptide mapping.

Proteins 2a, 2b, 2c, 3, 4, 5, 6, 7, 8, 40, 41, 42, 43, and 44 meet the first three criteria, as they were the most abundant proteins in an SDS-DTT extract and they had molecular weights and isoelectric points similar to published values (8, 39).

Filament formation. When mouse epidermal proteins were extracted with a urea buffer and a reducing agent, then dialyzed for removal of the denaturant, the keratins reassembled spontaneously into filaments (Figure 2.4A). These filaments could be pelleted by centrifugation, and the polypeptide composition could be analyzed by two-dimensional gel electrophoresis (Figure 2.4, B and C). The gel protein profiles showed that the filaments derived from the acetone-treated epidermis (Figure 2.4B) contain all the major proteins (proteins 2a, 2b, 2c, 3, 5, 6, 7, 8, and 52) that were also represented on gels of the SDS-DTT extracted proteins (Figure 2.2F, page 14). All of these proteins were also found in the filaments formed from mouse epidermis 48 hours after TPA treatment (Figure 2.4C), although the proportions differed from those of controls. There appeared to be a decrease in the higher-molecular-weight proteins (proteins 2a, 2b, 2c) and in protein 6. Also, some of the TPA-induced proteins (proteins 4, 40, 41, 42, 43, and 44) appeared to be able to assemble into keratin filaments. Frequently the keratin filaments formed from TPA-treated epidermis were found to be shorter than the control filaments (data not shown). It is possible that the presence of new keratin proteins in the 8 M urea extract of TPA-treated epidermis could modify various properties of the filaments.

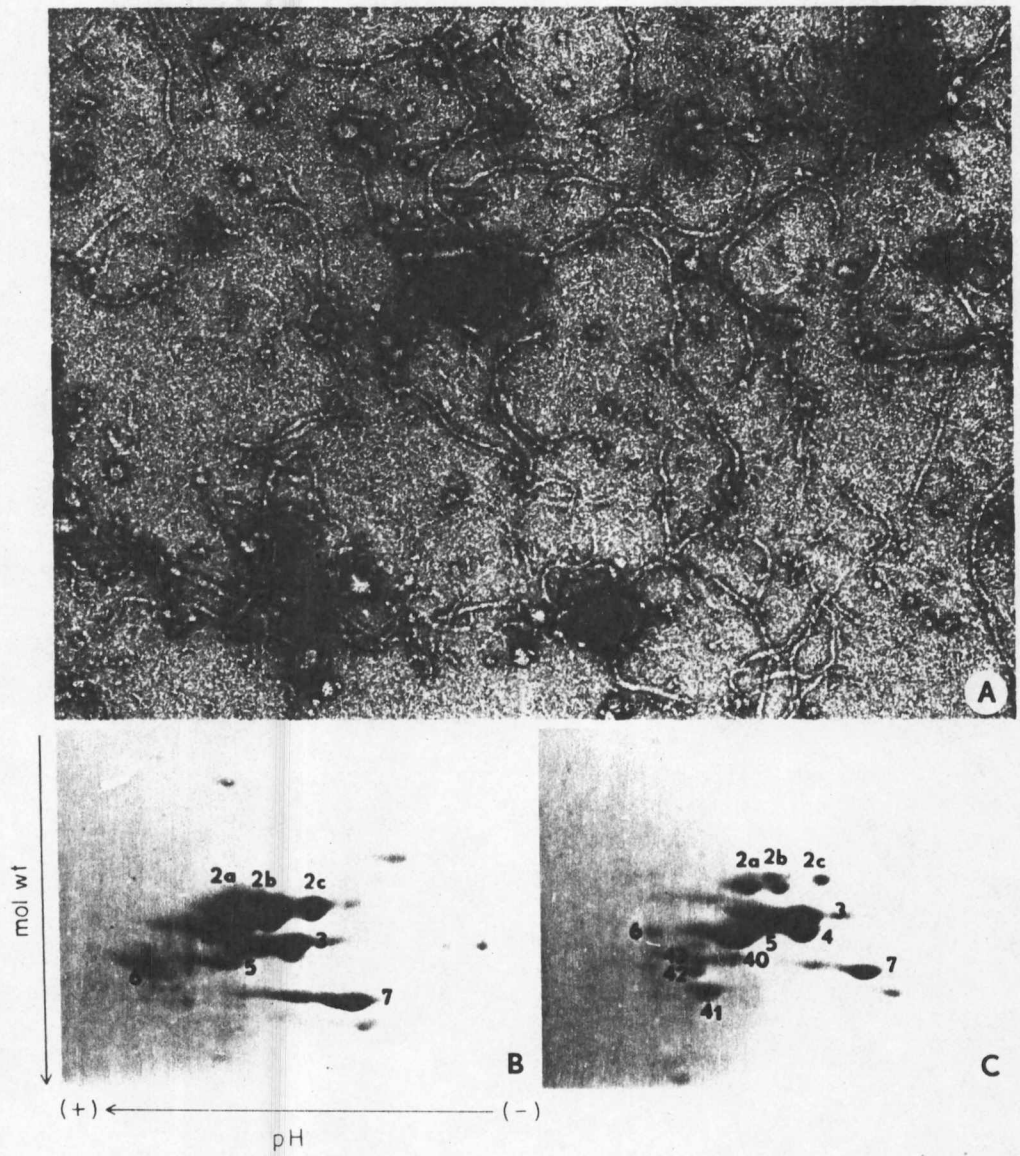

Figure 2.4. (A) Electron micrograph of keratin filaments formed in vitro. Two-dimensional electrophoresis was used to analyze the polypeptide composition of the keratin filaments formed in in vitro from (B) acetone- and (C) TPA-treated mouse epidermis.

Immunological crossreactivity. Antibodies to mammalian keratins show wide crossreactivity among species, indicating that different keratins were immunologically related (11, 17, 40). To demonstrate the immunological crossreactivity of the SDS-DTT extracted proteins, I prepared rabbit antiserum against a purified keratin isolated from the two-dimensional gels. In Ouchterlony double-diffusion tests (Figure 2.5A), the antiserum to protein 3 (M. W. 54,000) formed precipitin lines with each of the purified keratins (proteins 2a, 2b, 2c, 3, 4, 5, 6, 7, 8, 40, 41, 42, 43, and 44). The immunological crossreactivity indicated that these proteins have considerable sequence homology and provided further evidence that they are closely related epidermal keratins.

Amino acid analysis of the keratins. After the keratins were separated by two-dimensional electrophoresis, the individual proteins were cut from the stained gels and eluted from the gel pieces by electrophoresis. The purified keratins were then acid hydrolyzed, and their amino acid composition was determined (Table 2.1). The keratins that were analyzed had very similar amino acid compositions (proteins 2b, 2c, 3, 4, 6, 8, and 40). These included the major proteins present in normal epidermis as well as 2 of the new proteins (proteins 4 and 40) which were induced by tumor-promoter treatment. All the proteins had relatively high contents of serine, glutamic acid, and glycine. In addition, the keratins had relatively low levels of proline, methionine, and histidine. This composition was characteristic of keratins which had been isolated from several species (11, 36).

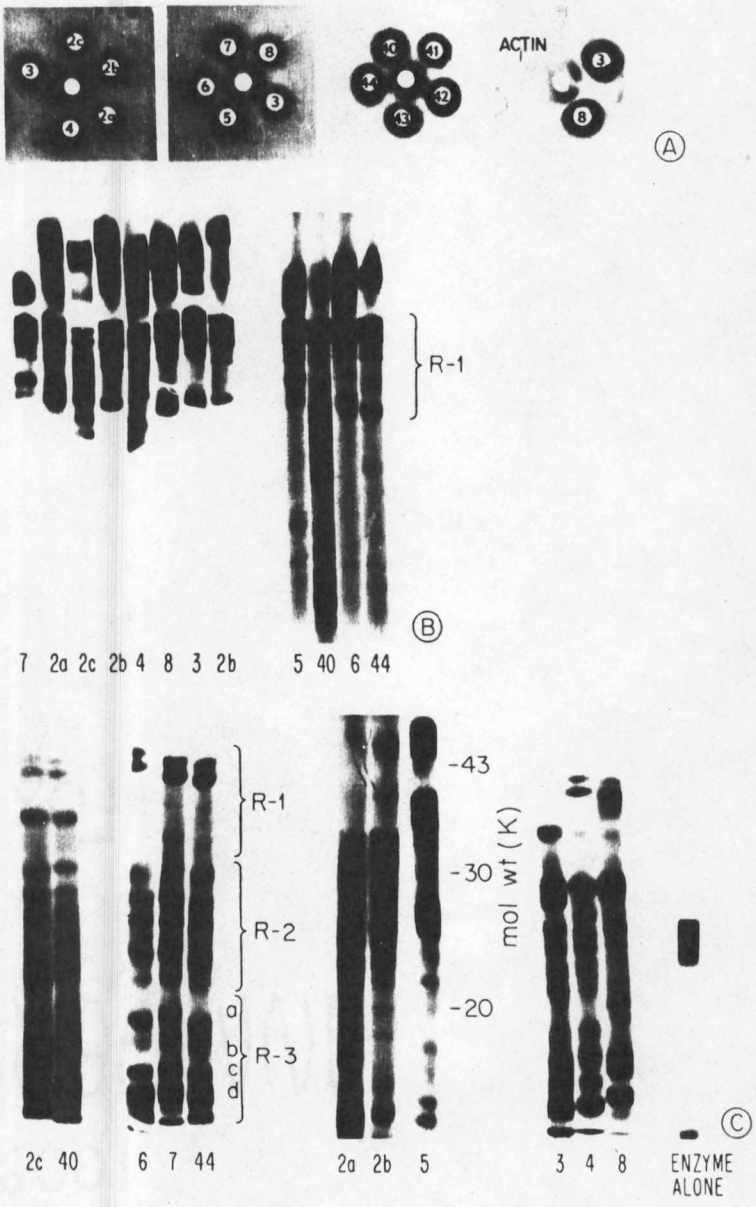

Figure 2.5. (A) Ouchterlony double diffusion tests showing immunological crossreactivity of the keratins extracted with SDS-DTT from mouse epidermis. Numbers identify the purified keratin in each well. Precipitin lines were formed against each keratin using antiserum prepared against protein 3. (B) Peptide maps produced after digestion with 20 µg staphylococcal V8 protease. Numbers identify the individual keratins. (C) Peptide maps produced 40 µg staphylococcal V8 protease. The peptide maps of the keratins which showed the greatest similarities are arranged together. R = region.

Table 2.1

Amino Acid Analysis of Isolated Epidermal Keratins of the Sencar Mouse

Amino Acid	Amino Acid Composition (mol %) of Keratins						
	2b	2c	3	4	6	8	40
Asp	7.92	7.89	8.58	8.68	9.85	8.93	8.84
Thr	3.31	3.41	4.17	4.70	5.03	4.19	3.83
Ser	13.40	13.36	9.21	8.71	10.11	9.24	11.76
Glu	14.47	12.15	15.66	13.17	15.77	16.15	15.63
Pro	1.21	1.34	1.32	1.64	0.81	1.22	1.41
Gly	24.66	25.46	19.96	12.24	12.88	17.76	18.26
Ala	4.20	4.62	6.02	7.31	6.72	5.97	5.26
Val	1.85	1.25	1.97	4.15	1.52	3.11	2.10
Met	1.64	1.98	2.71	2.61	2.98	2.88	2.27
Ile	3.12	3.51	4.11	4.34	5.30	4.03	4.16
Leu	8.10	8.19	9.06	8.32	11.0	9.43	9.13
Tyr	3.95	3.81	2.23	2.88	2.52	2.69	3.29
Phe	2.91	2.99	2.83	3.37	2.88	2.79	3.16
His	0.69	0.75	1.20	1.28	0.63	1.05	1.16
Lys	4.06	4.54	4.90	6.82	5.12	4.92	4.38
Arg	4.52	4.75	6.05	6.80	6.87	5.65	5.26

Peptide maps produced by limited proteolysis. Further evidence which indicates that the SDS-DTT extracted proteins were keratins came from analysis of the polypeptide maps produced by limited proteolysis. It was not possible to make autoradiograms of the peptide maps because these proteins could not be labeled well with [^{14}C]formaldehyde, probably because the majority of mouse keratins are acetylated at the N-terminus (36). The use of long electrophoresis tubes for limited proteolysis allowed introduction of enough protein to permit visualization of the polypeptide fragments with Coomassie blue protein stain. The purity of the individual protein gel slices was verified by an additional electrophoresis of a sample of each protein on a 10% SDS-polyacrylamide slab gel. Each protein migrated as a single band (data not shown).

The polypeptide patterns of the keratins (proteins 2a, 2b, 2c, 3, 4, 5, 6, 7, 8, 40, and 44) produced by digestion with staphylococcal V8 protease were quite similar (Figure 2.5B and C). For ease of comparison, the polypeptide patterns were divided into 3 regions according to molecular weights (Figure 2.5C). Region 1 contained polypeptides with molecular weights ranging between 30,000 and 43,000, region 2 ranged between 20,000 and 30,000, and region 3 consisted of any peptides below 20,000.

Most of the keratins were digested to only a small extent when exposed to 20 µg V8 protease (Figure 2.5B). The exception was the TPA-induced protein 40, which was more sensitive to the protease. The polypeptide pattern was primarily confined to region 1, consisting of

a major protein doublet followed by a set of 3 less distinct protein bands. Keratins underwent considerable digestion upon incubation with 40 µg V8 protease (Figure 2.5C). Although the protein profiles were very similar, particularly in regions 2 and 3, differences in the peptide maps of the individual keratins could be observed. In Figure 2.5C the peptide maps of the keratins which showed the greatest similarities are arranged together; however, the relative intensities of the separated polypeptide fragments varied among the individual keratins. For example, the polypeptide patterns of keratins 6, 7, and 44 were quite similar. The TPA-induced keratin 40 resembled the high-molecular-weight keratin 2c. Another of the TPA-induced proteins, keratin 4, shared common features with proteins 3 and 8. Keratins 2a, 2b, and 5 were grouped because they had a very distinct region 1. The polypeptide patterns obtained with the SDS-DTT extracted proteins of the mouse epidermis were very similar to those reported by Fuchs and Green (11) for human keratins. The data obtained from the peptide maps provided further evidence that proteins 2a, 2b, 2c, 3, 4, 5, 6, 7, 8, 40, and 44 were closely related, but distinct, keratin proteins.

These data indicated that the SDS-DTT extracted proteins that were visualized on the two-dimensional gels were epidermal keratins. Two of the TPA-induced proteins were fully characterized (proteins 4 and 40) and found to be epidermal keratins.

Two-dimensional analysis of the 4 M urea soluble protein components. After mice received a single application of TPA, 4 M urea extractable proteins showed considerable changes (Figure 2.6A-E). Extraction of

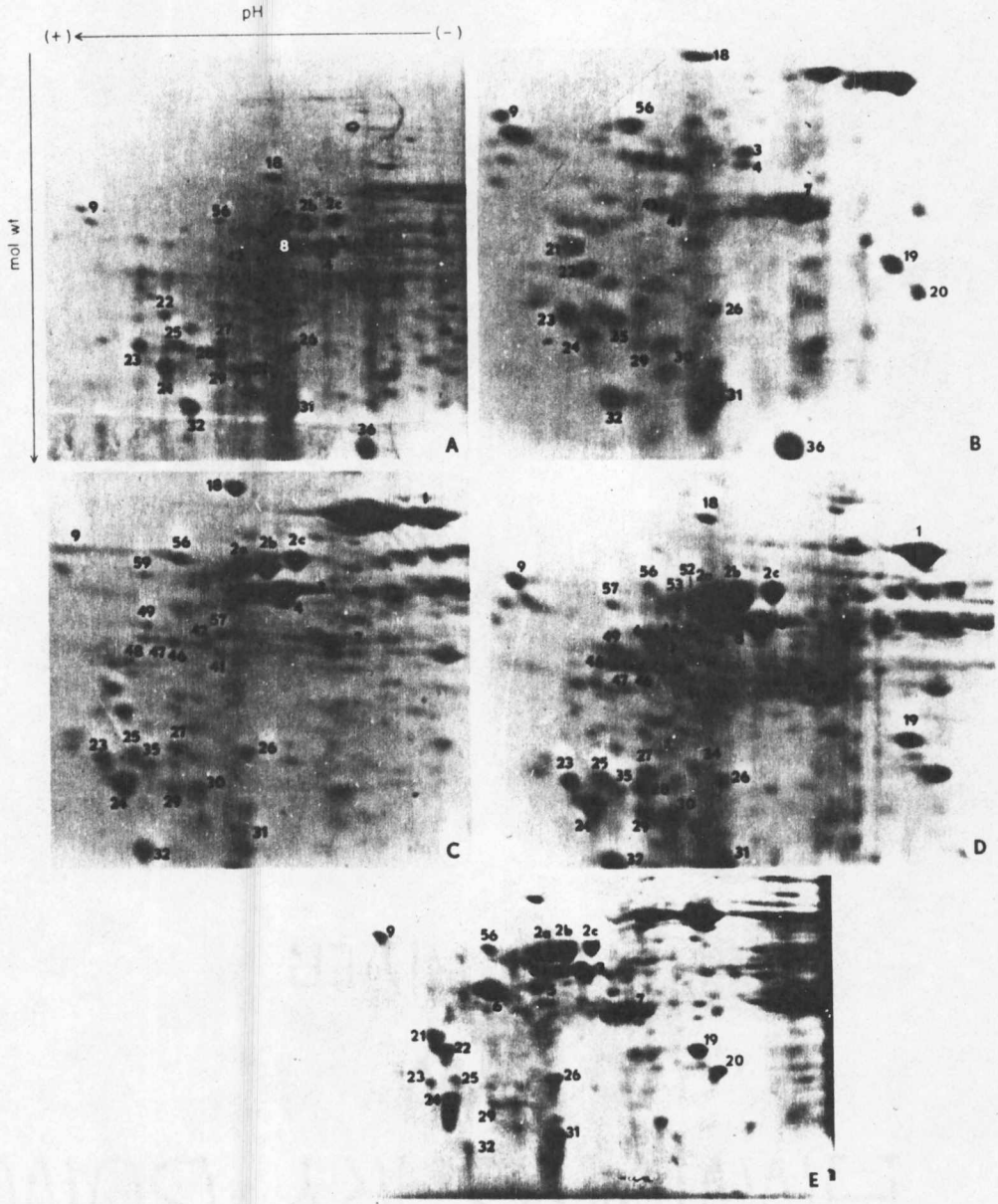

Figure 2.6. Two-dimensional gel protein profiles of the 4 M urea solubilized proteins at various times following the application of TPA. (A) 48 hours, (B) 72 hours, (C) 1 week, and (D) 2 weeks after treatment. Gel profiles of proteins extracted 48 hours after acetone treatment (E).

the epidermis with 4 M urea would solubilize most of the protein components of the living nucleated cell layers of the epidermis and remove some of the fibrous proteins from the stratum corneum which were not crosslinked by disulfide bonds (9).

At 48 hours after treatment with TPA (Figure 2.6A), the most prominent change from the control patterns (Figure 2.6E) was the loss of protein 6. In addition, several changes were seen in proteins ranging in molecular weight from 30,000 to 43,000. By 72 hours after treatment (Figure 2.6B), the modification observed in the 48 hours gels had progressed even further. Not only was protein 6 still absent, but also most of the keratin proteins had decreased or essentially disappeared (proteins 2a, 2b, 2c, 3, 4, 5, and 8). One week after TPA treatment (Figure 2.6C), when most of the gross morphological and ultrastructural changes in the epidermis have subsided (25, 28), all the keratins (proteins 2a, 2b, 2c, 3, 4, 5, and 8) except for protein 6 reappeared in the 4 M urea extracts. This was also evident 2 weeks after TPA treatment (Figure 2.6D).

Modification of keratin synthesis and/or disulfide bond crosslinking could account for the disappearance of the keratins from the urea gels. Partial support for TPA modification of keratin synthesis came from our studies of the protein profiles of isolated epidermal basal cells obtained from adult mice after TPA treatment. The most striking difference between the basal cells of the TPA and acetone groups was that TPA induced a large decrease in the higher-molecular-weight keratins (proteins 2a, 2b, and 2c) and in protein 6. Since these proteins were reduced in both 4 M urea and SDS-DTT protein extracts,

it was most likely that TPA was modifying the synthesis of these proteins in epidermal basal cells rather than altering extractability by modifying disulfide bond crosslinking (manuscript in preparation). Alteration of synthesis of the higher-molecular-weight keratin basal cells did not explain the decrease in the other keratins (proteins 3, 4, 5, 6, and 8) seen at 72 hours after treatment.

Another possible explanation for the reduction of the keratins in the 4 M urea extract was that TPA modified the reducing environment of the living epidermal cells. This reducing environment normally maintains the keratins in a reduced form that favors filament formation (17, 37). It is possible that TPA treatment changed the environment so that the keratins became prematurely crosslinked by disulfide bonds. The premature oxidation of keratin thiol groups would modify the extractability of the keratins so that 4 M urea would no longer be effective, and more stringent conditions would be required for solubilization. Preliminary data obtained in my laboratory supported this explanation. It had been demonstrated that TPA treatment of epidermal basal cells could enhance the disulfide bond crosslinking of the keratins.

Several other protein changes could be seen in the gels of the 4 M urea extracted TPA-treated epidermis (Figure 2.6A-D) compared with control gels (Figure 2.6E). At 48 or 72 hours after treatment (Figure 2.6A and B), several proteins (proteins 27, 28, 35, 36, 41, and 42) were induced by TPA which were not very distinct in the control (Figure 2.6E). These early protein alterations not only occurred during the TPA induction of maximal hyperplasia and inflammation at 48 and 72 hours,

but also were components of the one- and two-week samples (Figure 2.6C and D, page 24) in which the hyperplasia and inflammation had subsided. This indicated that although the induction of hyperplasia appeared to be necessary for the expression of these proteins, the maintenance of hyperplasia was not essential for their continued production at later times. Therefore, after many of the TPA-induced morphological and ultrastructural modifications had subsided, TPA still had a profound influence on epidermal protein expression.

In addition to the proteins induced by TPA at the early time points, several other proteins (proteins 29, 30, 34, 43, 44, 46, 47, 48, 49, and 59) became more distinct at 1 and 2 weeks after treatment (Figure 2.6C and D). The majority of the early and late protein alterations induced by TPA were also components of the 4 M urea extracts of newborn epidermis (proteins 27, 34, 35, 36, 41, 42, 43, 44, 46, 47, 48, 49, and 59) (Figure 2.3B, page 16). This was especially true at 2 weeks after TPA treatment, when both the 4 M urea and SDS-DTT extracted protein patterns (Figure 2.6D and 2.2E, page 14) were remarkably similar to the newborn profile (Figure 2.3A and B, page 16).

Analysis of protein alterations induced by the weak tumor promoters. The effects of the weakly promoting agents, mezerein, EPP, and mechanical abrasion on the mouse epidermal proteins were studied by use of two-dimensional electrophoresis (Figure 2.7A-E). By comparing the protein changes induced by TPA with those produced by weakly promoting hyperplastiogenic agents, one can determine which of these changes, if any, are essential for promotion. The weakly promoting agents produced

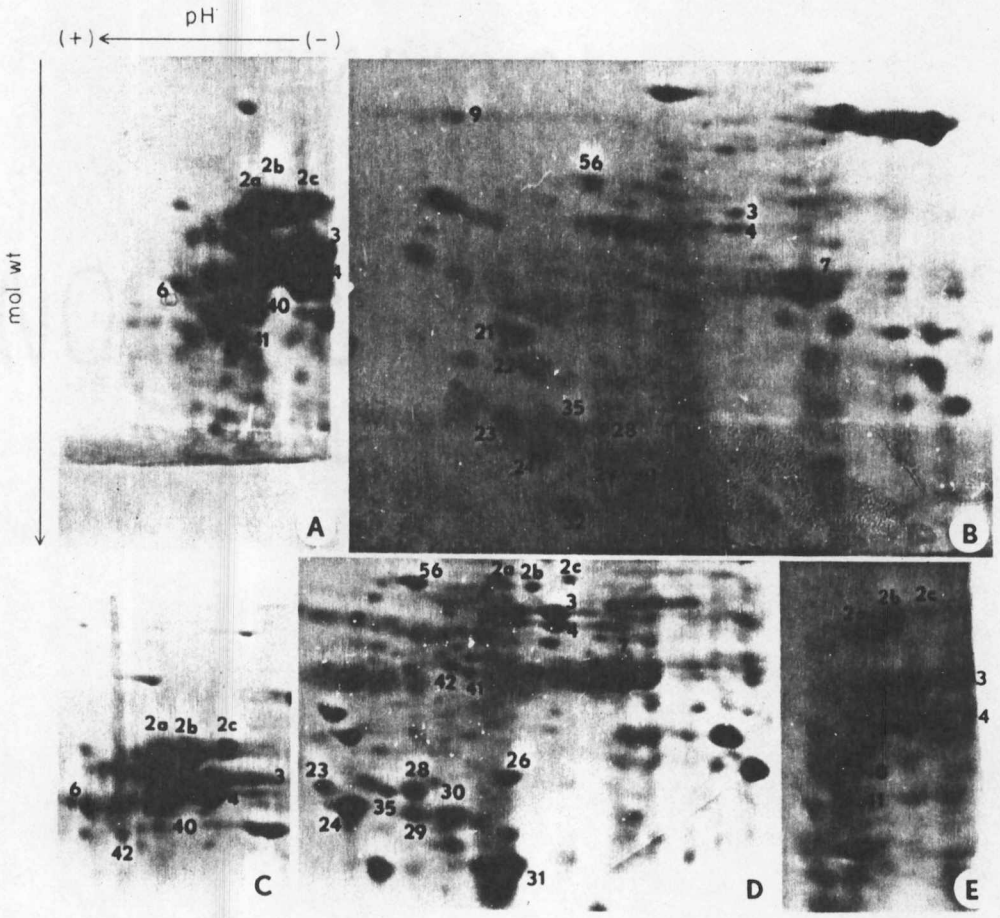

Figure 2.7. Two-dimensional gel profiles of proteins isolated from mouse epidermis after treatment with the weakly promoting hyperplasiogenic agents. Gel profiles of the SDS-DTT protein extracts (A) mezerein, 48 hours after treatment, (C) EPP, 48 hours after treatment; and (E) abrasion, 4 days after treatment. Two 4 M urea extracted protein profiles are presented: (B) mezerein, 48 hours after treatment; (D) EPP, 48 hours after treatment.

protein changes similar to those induced by TPA. However, not only did the time of appearance of these protein changes differ for each compound, but also, the relative amounts of the proteins often varied. Even though dosages of the compounds were chosen to give comparable hyperplasia, slight differences in the intensity of the hyperplasia that was induced might account for the observed differences in the protein profiles.

Protein changes induced by mezerein were essentially identical to those induced by TPA. However, the protein modifications occurred earlier after mezerein treatment than after TPA treatment. This is not surprising, because mezerein is a more potent hyperplasiogenic agent than TPA. At 48 hours after mezerein treatment, the profiles of the 4 M urea and SDS-DTT extracted protein (Figure 2.7A and B) were identical to those of the samples obtained 72 hours after TPA treatment (Figures 2.2C, page 14, and 2.6B, page 24). As observed with TPA, new keratin proteins were induced in the SDS-DTT extract, the number of keratins solubilized with 4 M urea decreased, basal cell protein patterns were modified (data not shown), and newborn proteins appeared. These data suggest that the majority of protein changes induced by TPA are components of the second stage of promotion.

EPP protein modifications not only appeared at later times, but were usually less prominent than those induced by TPA. Unlike TPA and mezerein treatments, at 12 hours after treatment with EPP produced no protein modifications (data not shown). By 48 hours after EPP application, the changes observed in the SDS-DTT extracted proteins (Figure 2.7C)

were both quantitatively and qualitatively less than those observed with TPA or mezerein. The 4 M urea extracted proteins (Figure 2.7D) at 48 hours resembled the TPA 4 M urea extract at this time (Figure 2.6A, page 24). As observed with TPA, there was a decrease in keratins 2a, 2b, 2c, and 6 in the urea extract of the EPP-treated epidermis. This observation was supported by a similar significant decrease in the higher-molecular-weight keratins (2a, 2b, and 2c) and protein 6 in the basal cells isolated from EPP-treated epidermis (data not presented).

Four days after abrasion, at a time when maximal hyperplasia was induced, the SDS-DTT extracted proteins were quite similar to those seen after treatment with TPA or mezerein (Figure 2.7E). There was a marked reduction of the higher-molecular-weight keratins (proteins 2a, 2b, and 2c) and new keratins (proteins 4, 40, 41, 42, 43, and 44) were induced.

Protein modifications similar to those induced by TPA were observed at 1 and 2 weeks after treatment with the hyperplasiogenic agents, especially mezerein and abrasion (data not shown). Differences in the protein profiles and in the concentration of the proteins could result from slight variations in the degree of hyperplasia that was induced following treatment with a hyperplasiogenic agent, and may or may not be significant with regard to promoting ability.

Discussion

The data presented in this report demonstrate that TPA and other hyperplasiogenic agents induce numerous protein modifications which

include major changes among the epidermal keratins. The keratin modification progresses with time after TPA treatment, resulting in a keratin pattern which resembles that of newborn epidermis.

The proteins solubilized with SDS-DTT from either acetone- or TPA-treated mouse epidermis are primarily keratins. The characterization is based on 7 parameters: extractability, isoelectric point, molecular weight, filament formation, antibody crossreactivity, peptide maps, and amino acid composition.

Of particular interest is the TPA-induced modification of the polypeptide composition of the epidermal keratin filaments formed in vitro. There are 2 possible explanations: (1) the TPA induction of the lower-molecular-weight new keratins may affect the normal filament protein composition, or (2) the stoichiometric ratios of certain keratins may be altered so that their incorporation into filaments is modified. Evidence which indicates that modifications of the size of keratin proteins and their stoichometric ratios can alter the properties of the keratin filaments has been reported by a number of investigators (11, 18, 37). For example, Steinert and co-workers (37) studying filament formation in vitro, found 5 combinations of keratin proteins which did not result in significant filament formation. In addition, maximum yields of filaments could be obtained only when a higher-molecular-weight and a lower-molecular-weight keratin were present in a stoichiometric 1:2 ratio. It is possible that the new keratins (proteins 4, 40, 41, 42, 43, and 44) induced by TPA are incompatible in filament formation with the higher-molecular-weight

keratins (proteins 2a, 2b, and 2c). In addition, the stoichometric ratios appear to be altered after TPA treatment due to a large increase in the proteins found in the lower-molecular-weight regions (proteins 3, 4, 8, 40, 41, 42, 43, and 44) and a decrease in the higher-molecular-weight keratins as observed in isolated basal cells (unpublished data).

By modifying the keratins, which are the major differentiation product of the epidermis, TPA can alter the properties of the filaments that are formed from these proteins and thus modify their cytoplasmic function; this can account for some of the morphological and biochemical alterations seen after TPA treatment. This theory is supported by the belief that intermediate filaments, of which keratins form one class, are involved in the mechanical integration of various components found in the cytoplasm. They may play an essential role in regulation of cell shape as well as be influenced by the metabolic state of the cells (17).

Of the several protein changes appearing in the 4 M urea extracts of TPA-treated adult epidermis, one of the most striking is the substantial decrease in the keratin proteins which can be solubilized. A partial explanation of this reduction in the extractable keratins comes from our observation that TPA induces a large decrease in the higher-molecular-weight keratins (proteins 2a, 2b, and 2c) and protein 6 in isolated epidermal basal cells obtained from adult skin following treatment (unpublished data). Another explanation is that TPA could modify the reducing environment of the epidermal cells so that the keratins become crosslinked prematurely by disulfide bonds; this cross-linking would prevent their extraction with 4 M urea. Reiners and

Slaga, in my laboratory, have found that TPA and mezerein enhance disulfide bond formation in isolated basal cells. Premature oxidation of keratin thiol groups to disulfide bonds can have profound effects on the physical properties (11) and thus the cellular function of the filaments. Raick (24) provided evidence which indicated that TPA modified keratin filament structure. He found that as early as 12 hours after TPA treatment of mouse skin, there was fragmentation of the tonofilament bundles. Other work by Steinert and co-workers (37) showed that the maintenance of keratin thiol groups in their reduced forms were essential for long-filament formation in vitro. They found that the rapid formation of interchain disulfide links inhibited the assembly of keratins into long filaments. It is possible that premature oxidation of keratin sulfhydryl groups could result in shorter tonofilaments in vivo, causing the fragmentation that was seen by Raick with the electron microscope. But, most importantly, this early crosslinking of the keratins would prevent their extraction with 4 M urea and explain their absence on the two-dimensional gels. Therefore, TPA appears both to alter the synthesis of certain keratins and to modify their extractability, possibly by premature formation of disulfide bonds.

To determine if these protein changes were crucial for tumor-promoting activity, we studied the effects of the weakly promoting agents, mezerein, EPP, and mechanical abrasion, on mouse epidermal proteins. In general, the weak promoting agents, especially mezerein and abrasion, produced protein changes very similar to those induced by TPA. This indicates that the majority of the protein modifications induced by TPA are associated with its hyperplasiogenic properties.

However, slight differences in the temporal sequence of protein induction as well as relative amounts were observed among the various compounds. This is particularly true with the early protein modifications induced by EPP, which are both quantitatively and qualitatively less than those observed with TPA, mezerein, and abrasion. This decrease in epidermal responsiveness to EPP with regard to protein modifications is not surprising, since there are numerous observations which indicate that many of the biochemical alterations induced by EPP are usually quantitatively less than that produced upon treatment with TPA or mezerein. For example, it has been shown that the EPP-induced increases in ornithine decarboxylase activity and polyamine levels are significantly lower than those induced by TPA and mezerein. The decreased response of the epidermis to EPP could be due to greater cytotoxicity leading to cell death. Preliminary results in our laboratory indicate that EPP is extremely cytotoxic to isolated adult basal cells, whereas TPA and mezerein are not. Raick and Burdzy (28) suggest that the weak promoting action of EPP may result from its ability to cause focal areas of necrosis in the epidermis. In addition, Raick (27) reports that EPP induced fewer tumors in a three-stage system (dimethylbenzanthracene initiation followed by limited TPA treatments and then repetitive EPP applications) than did dimethylbenzanthracene followed by only limited TPA treatment. The decreased tumor yield may have been the result of EPP's toxic necrotic effects.

Three observations may explain the protein changes induced by the hyperplastic agents:

1. The conversion of adult mouse epidermis to a multilayered epithelium resembling neonatal skin (4, 24) after treatment with hyperplasiogenic agents may result in the expression of other biochemical features which are characteristic of this newborn-like state. Numerous observations indicate that TPA treatment of adult mouse skin can induce newborn-like morphological and biochemical features such as the induction of certain proteins (4, 5), a decrease in histidase activity (7), and similar G1 chalone (16) and β-adrenergic receptor (20) characteristics.

2. Alterations in the rate of cell maturation and cell turnover in mouse epidermis by hyperplasiogenic agents could modify protein synthesis and processing (10, 12, 41). Fuchs and Green (12) stated that if the rate of migration of the epidermal cells is altered, changes in the synthesis and processing of the keratins could occur by modification of the time required for maturation. There is evidence that TPA can alter the rate of migration and turnover of mouse epidermal cells (10).

3. Extensive proteolysis and/or abnormal protein synthesis could also result in alterations of mouse epidermal proteins. Steinert and co-workers (38) suggested this explanation for the keratin alterations that are found in a number of disorders of epidermal keratinization in humans. Further support is the fact that TPA does induce neutral proteases in mouse epidermis (42).

In conclusion, it appears that the majority of protein modifications induced by the tumor promoter TPA are due to induction of hyperplasia alone. What may be important for promoting action is the relative rates of synthesis of specific epidermal proteins which are critical factors involved in controlling epidermal proliferation and differentiation.

The protein modifications which appear to be specific to tumor promotion, or at least the second stage of promotion, may be related to the degree by which TPA, mezerein, EPP, and abrasion induce hyperplasia and the subsequent dedifferentiation to a more embryonic state.

CHAPTER III

EFFECTS OF INHIBITORS OF TUMOR PROMOTION ON 12-O-TETRADECANOYLPHORBOL-13-ACETATE-INDUCED KERATIN MODIFICATION IN MOUSE EPIDERMIS

Introduction

Tumor promotion in mouse skin has been shown to consist of at least two stages (32). The first stage of promotion can be brought about by limited applications of 12-O-tetradecanoylphorbol-13-acetate (TPA). When this is followed by repetitive treatments with mezerein, a weak promoter which induces similar biochemical and morphological effects as TPA, the second stage of promotion can be accomplished.

Inhibitors of tumor promotion, such as the antiinflammatory steroids, retinoids, and protease inhibitors, have contributed significantly to the understanding of the mechanisms involved in tumor promotion (57, 58, 59). The effects of these inhibitors on the first and second stages of promotion have been studied (64). The protease inhibitor tosylphenylalanylchloromethyl ketone (TPCK) has been found to be an effective inhibitor of stage one, whereas retinoic acid (RA) specifically inhibited stage two. The antiinflammatory steroid fluocinolone acetonide (FA) has been found to be a potent inhibitor of both stages of promotion.

My laboratory recently compared the protein changes induced by TPA in adult mouse epidermis with those induced by the weakly promoting

hyperplasiogenic agents, mezerein, ethylphenylpropiolate, and mechanical abrasion, by use of two-dimensional gel electrophoresis (52). I found that TPA, as well as the weak promoters, induced substantial modifications of the epidermal keratins. The majority of protein changes induced by TPA treatment appeared to be associated with its hyperplasiogenic properties and not necessarily to reflect its promoting activity.

To further investigate the role that hyperplasia plays in the induction of the observed keratin modifications, I studied the effects of several inhibitors of tumor promotion and cycloheximide on the TPA-induced protein changes. The agents that prevented the induction of hyperplasia, i.e., the antiinflammatory steroid FA and cycloheximide, also prevented the appearance of the TPA-induced protein modifications; whereas RA and TPCK, which had little effect on TPA-induced hyperplasia, were found to have essentially no influence on the protein changes.

Materials and Methods

Chemicals. TPA was obtained from Dr. P. Borchert (University of Minnesota, Minneapolis, Mn.). FA was received from Syntex Laboratories, Inc. (Palo Alto, Ca.), RA was supplied by Hoffmann-LaRoche, Inc. (Nutley, N. J.), TPCK and cycloheximide were acquired from Aldrich Chemical Company, Inc. (Milwaukee, Wi.). All reagents for SDS-polyacrylamide electrophoresis were received by Bio-Rad Laboratories (Richmond, Ca.). The isoelectric focusing ampholytes were obtained from LKB (Durham, N. C.).

Treatment of animals and protein extraction. Eight-week-old female SENCAR mice raised at Oak Ridge National Laboratory were shaved at least

2 days before treatment. Groups of 10 animals were treated with either TPA (5 µg), TPA (5 µg) plus FA (2 µg), TPA (5 µg) plus RA (10 µg), TPA (5 µg) plus TPCK (10 µg) in 0.2 ml acetone or with acetone alone. Cycloheximide (2 mg) in 0.2 ml acetone was applied 12 hr after treatment with TPA (5 µg). This dose of cycloheximide resulted in 95% inhibition of protein synthesis. The animals were killed by cervical dislocation at various times following treatment. Excess hair was removed from the backs with a chemical depilatory (Nair).

The epidermis was removed by scraping with a razor blade. The epidermal proteins were isolated by a modification of the procedures used by Fuchs and Green (11) and Dale and Ling (9). The epidermises were homogenized in 4 M urea and 1 mM phenylmethylsulfonylfluoride using a Polytron PT-10 homogenizer for 45 seconds. The homogenate was centrifuged at 35,000 rpm for 30 minutes and the pellets were resuspended in 2% sodium dodecylsulfate (SDS) 10 mM dithiohreitol (DTT) followed by an incubation at 37° C for 1 hour. The SDS-DTT extract was then centrifuged at 35,000 rpm for 30 minutes. The 4 M urea supernatant containing the more readily soluble proteins of the epidermis and the SDS-DTT supernatant containing the keratins were dialyzed against distilled water, lyophilized, and stored at -20° C until two-dimensional electrophoresis was performed.

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (23). The pH range used for the isoelectric focusing dimension was pH 4-6. This particular pH range was chosen because it allowed maximal separation of

individual keratins (39, 52). The second dimension utilized 8 or 10% SDS-acrylamide slab gels. Following electrophoresis, the proteins were fixed in 12% trichloroacetic acid, then stained in a solution of 0.1% Coomassie blue, 7% acetic acid, and 50% ethanol. The gels were destained in a solution of 7% acetic acid and 10% ethanol and photographed.

Results

The effects of the tumor promotion inhibitors FA, RA, and TPCK on the TPA-induced protein modifications were studied at various times following treatment of adult SENCAR mouse skin. Extraction of the epidermis with 4 M urea was used to remove most of the protein components that were not crosslinked by disulfide bonds (9). The remaining proteins, which were predominantly the epidermal keratins, were solubilized with SDS-DTT. Recently my laboratory showed that TPA and other hyperplasiogenic agents induced major changes in the SDS-DTT extracted proteins identified as keratins on the basis of their extractability, molecular weight, isoelectric point, assembly into filaments *in vitro*, immunological crossreactivity, amino acid composition, and peptide maps (52).

As early as 12 hours after a single treatment, TPA (Figure 3.1B) induced a new protein (protein 4), with an approximate molecular weight of 51,000 daltons, that was absent in the SDS-DTT extracts of acetone controls (see Figure 3.1A). At all time points that were studied following treatment with acetone alone, the protein pattern remained unchanged. A representative two-dimensional gel profile, that of 48 hours after treatment with acetone, is shown in Figure 3.1 A. As previously reported, protein 4 has been characterized and shown to be a keratin (52).

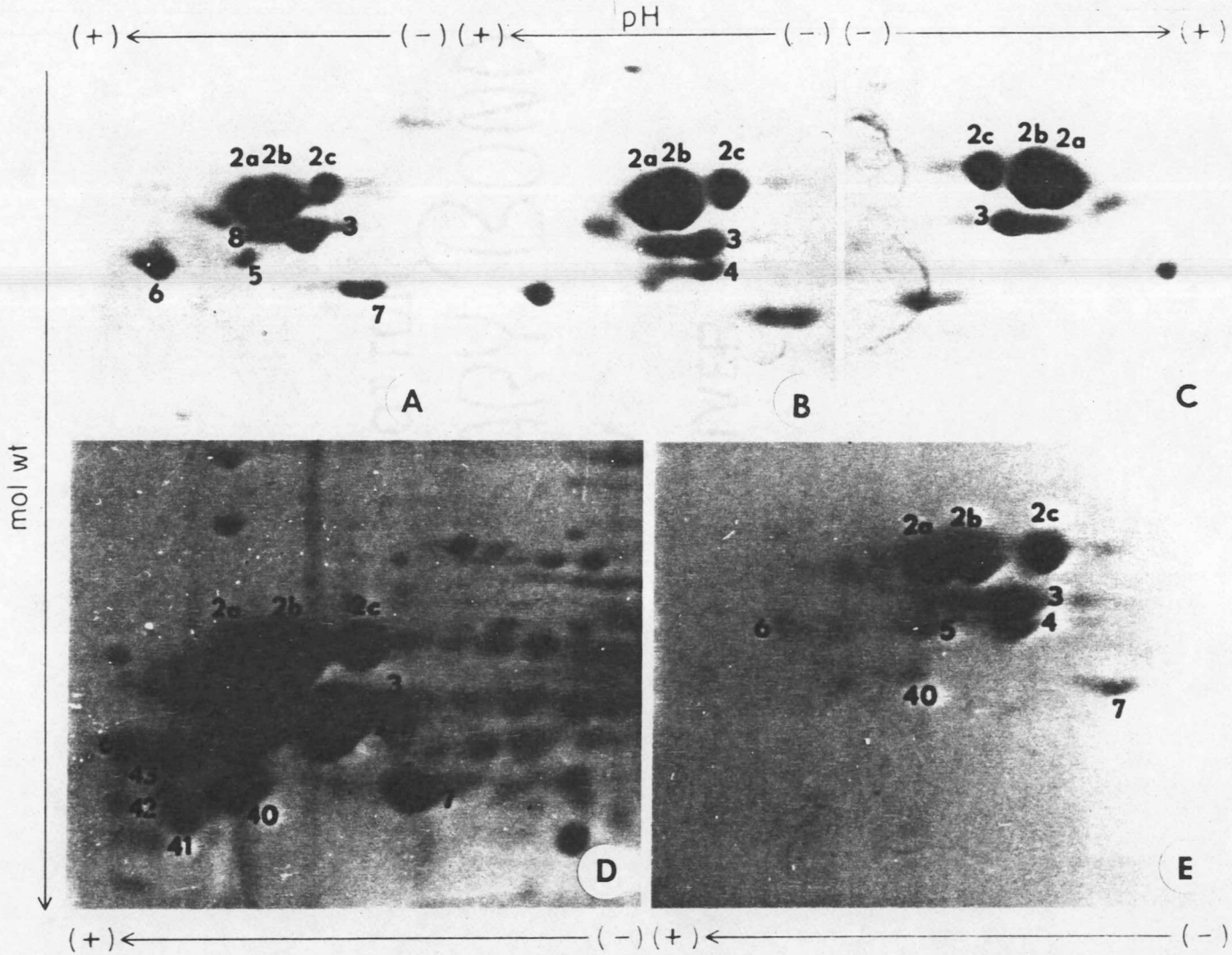

Figure 3.1. Two-dimensional protein patterns of SDS-DTT extracts of adult SENCAR mouse epidermis. (A) acetone control, (B) TPA at 12 hours posttreatment, (C) TPA + FA at 12 hours, (D) TPA at 48 hours posttreatment, and (E)TPA + FA at 48 hours. The pH gradient of the isoelectric focusing dimension ranged between pH 4.0 and pH 6.0. The keratins were found to migrate between pH 5.3 and pH 5.8. The molecular weights of the keratins ranged between 43,000 and 62,000 daltons. The second dimension utilized 8% SDS polyacrylamide slab gels for (B) and (C), and 10% SDS-polyacrylamide slab gels for (A), (D), and (E).

The antiinflammatory steroid FA, when administered simultaneously with TPA (Figure 3.1C), was able to prevent the induction of protein 4 at 12 hours. RA was also found to inhibit the induction of protein 4; however, TPCK had no influence (data not shown). These inhibitors did not induce any significant modifications when administered alone to adult mouse epidermis (data not shown).

By 48 hours posttreatment with TPA (Figure 3.1D), there was a substantial increase in protein 4, as well as the induction of several minor proteins (proteins 40, 41, and 42) ranging in molecular weights between 45,000 to 50,000 daltons. As with protein 4, evidence had been obtained that indicated these minor proteins were also keratins (52). FA was able to cause a significant inhibition of all of these protein modifications (Figure 3.1E). In contrast, RA did not inhibit the TPA-induced protein changes at 48 hours (Figure 3.2C). At 48 hours TPCK had no influence on the induction of protein 4 by TPA (Figure 3.2B); however, there appeared to be a slight reduction in the minor proteins (proteins 40, 41, and 42).

In addition to the tumor-promotion inhibitors, the effects of cycloheximide on the TPA-induced protein changes were also studied. Cycloheximide was administered 12 hours after TPA treatment, a time at which maximal protein synthesis had been found to be induced (24). At 48 hours posttreatment with TPA, cycloheximide (Figure 3.2A) inhibition of the TPA-induced protein modifications was comparable to that observed with FA. This indicated that protein synthesis was necessary for the appearance proteins 4, 40, 41, and 42 after TPA treatment.

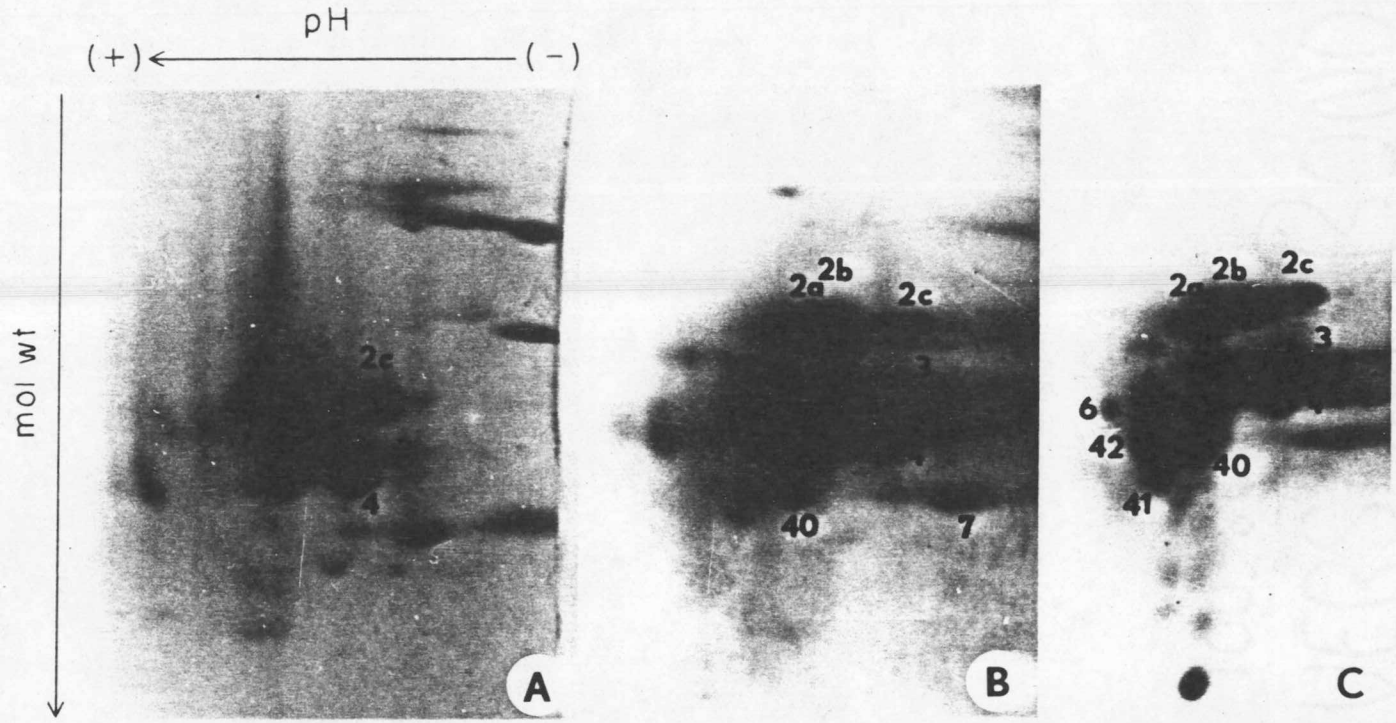

Figure 3.2. Two-dimensional protein patterns of SDS-DTT extracts of adult SENCAR mouse epidermis treated with TPA and various tumor promotion inhibitors or cycloheximide at 48 hours posttreatment. (A) TPA + cycloheximide, (B) TPA + TPCK, and (C) TPA + RA. The second dimension was run on 10% SDS polyacrylamide gels.

Two weeks following a single treatment with TPA (Figure 3.3A) the keratin pattern was remarkably similar to that seen in newborn epidermis (52). Keratins 2a and 2b were found to consist of at least two proteins each: a high molecular weight component of 62,000 daltons (proteins $2a_1$ and $2b_2$) and a lower molecular component of 60,000 daltons (proteins $2a_1$ and $2b_2$). In general, very low amounts of the higher molecular weight components ($2a_1$ and $2b_1$) were detected in untreated adult mouse epidermis. However, these proteins were found to be more abundant in newborns and in TPA-treated adult epidermis 2 weeks following a single application. In addition, similarities between TPA-treated adult and newborn epidermis were noted among a group of proteins designated 52, 53, and 54 (52).

FA was quite effective in preventing the TPA-induced newborn-like protein modifications seen at 2 weeks (Figure 3.3D). Not only was there a reduction in proteins $2a_1$ and $2b_1$, but also the proteins 52, 53, and 54 were decreased. However, these TPA protein modifications were not affected by RA (Figure 3.3B) or TPCK (Figure 3.3C).

Discussion

Recently, my laboratory demonstrated that TPA, as well as several weak promoters, induces substantial modification of mouse epidermal keratins. This indicates that these protein changes may be a general response to any hyperplasiogenic agent, promoting or nonpromoting. If hyperplasia is responsible for the observed protein changes, then any agent that prevents the induction of hyperplasia should also prevent

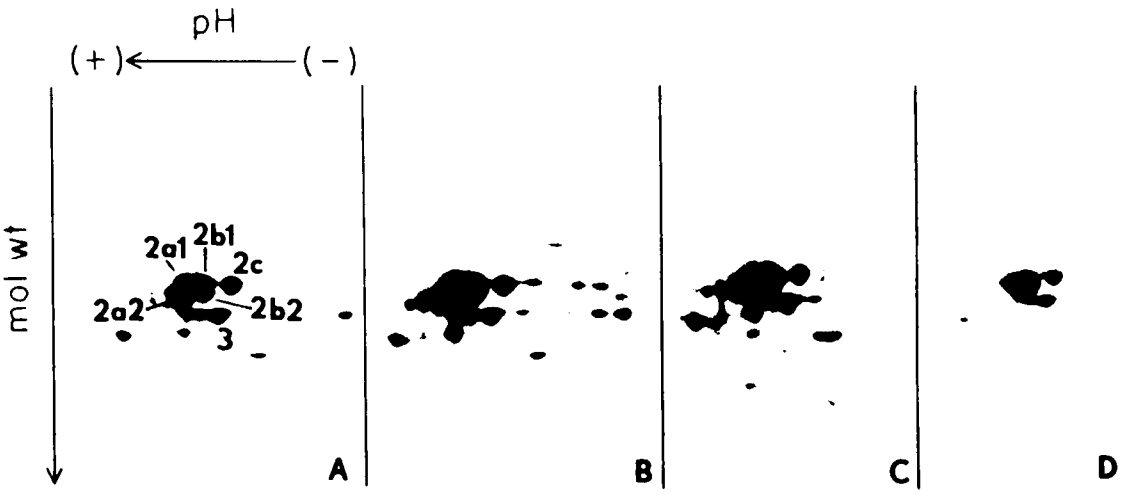

Figure 3.3. Two-dimensional protein patterns of SDS-DTT extracts of adult SENCAR mouse epidermis two weeks following treatment with TPA and various tumor promotion inhibitors. (A) TPA alone, (B) TPA + RA, (C) TPA + TPCK, and (D) TPA + FA. The concentration of SDS-polyacrylamide used for the second dimension was 10%.

the appearance of these protein modifications. This has been found to be the case. The protein changes induced by TPA were found to be prevented by FA and cycloheximide, both of which significantly reduce the induction of epidermal hyperplasia. The TPA-induced protein modifications were not substantially altered by TPCK and RA, which have little effect on the hyperplasia.

The ability of the antiinflammatory steroid to block the TPA-induced proteins changes was not surprising. It has been reported that FA inhibits skin tumor promotion through its ability to inhibit macromolecular synthesis, which consequently prevents the hyperplasia (57, 66).

The mechanism by which the retinoids inhibit tumor promotion may be due to either their influence on epithelial differentiation (58) and/or their ability to inhibit the induction of ornithine decarboxylase activity (67, 68, 69). Recently Haddox and co-workers (68) suggested that the major mechanism by which vitamin A inhibits ornithine decarboxylase induction is through a transcriptional block. In addition, vitamin A produced a 20% inhibition of general protein synthesis as well as a delay in DNA synthesis. Interfering with early critical macromolecular events important for promotion may be one mechanism by which retinoids act. An early transient inhibition of protein synthesis may explain why RA is able to inhibit the TPA induction of protein 4 at 12 hours but not at 48 hours posttreatment.

Protease action may play an important role in tumor promotion. Evidence that supports this are the observations that TPA-induced

neutral proteases in mouse epidermis (42) and protease inhibitors, such as TPCK, inhibited tumor promotion by TPA (59). Although protease inhibitors have little effect on TPA-induced epidermal hyperplasia, they do inhibit the inflammatory response where proteolytic activity is necessary for its induction (59, 65). Since many of the TPA keratin modifications have been associated with the induction of hyperplasia, it can be predicted that TPCK should have little influence on these protein changes because it does not interfere with hyperplasia. This is found to be true for the induction of protein 4 by TPA at 48 hours; however, the minor keratins (proteins 40, 41, and 42) were slightly reduced, indicating that these proteins may arise from proteolysis. Support for this comes from the work of Steinert and co-workers (38) who suggested that proteolytic enzymes or abnormal protein synthesis were responsible for the keratin modifications that are observed in a number of human skin disorders of keratinization.

It is also possible that hyperplasiogenic agents may induce new mRNA species which code for new keratins. Fuchs and Green (11, 12) stated that the modification of the environment of a keratinocyte can influence the program of keratin synthesis which is directed by changes in the mRNA populations. Therefore, it is possible that hyperplasiogenic agents modify the environment of the epidermal cells such that they can exist in a hyperplastic state that triggers a new program of keratin synthesis. FA's ability to prevent both the hyperplasia and the subsequent protein modifications indicated that it may be able to prevent the epidermal cells from responding to the environmental changes induced by the hyperplasiogenic agents. Similarly, cycloheximide may

interfere with the implementation of any changes in the program of keratinization by preventing the synthesis of necessary proteins. By not interfering with the induction of epidermal hyperplasia, TPCK and RA probably cannot substantially modify any changes in the program of keratin synthesis that appear to be associated with the hyperplastic state.

In fact, hyperplasiogenic agents may create an environment that resembles that of newborn epidermis. Support for this is the induction of newborn-like keratin patterns in adult mouse epidermis 2 weeks following treatment with hyperplasiogenic agents. In addition, there have been several reports that indicate that tumor promoters can induce several other morphological and biochemical characteristics in adult mouse skin that mimic the newborn phenotype (5, 7, 16, 20, 24).

As observed with the early protein modifications, hyperplasia also appears to be necessary for the induction of the newborn keratin patterns in adult skin. If hyperplasia is inhibited by the anti-inflammatory steroid FA, the keratin changes are also inhibited; whereas TPCK and RA, which do not modify the hyperplasia, were found not to interfere with the appearance of the newborn-like keratin patterns. This provides further support for the observation that the induction of hyperplasia results in a new pattern of keratin expression.

In conclusion, further evidence is presented using inhibitors of tumor promotion that indicates the major keratin modifications are associated with the induction of a hyperplastic state and are not necessarily related to tumor promoter ability. What may be critical

to promotion is that potent tumor promoters, such as TPA, may be able to make these changes in keratin expression permanent in certain critical cells whereas the majority of weakly promoting hyperplasiogenic agents may only induce transient changes. Induction of a newborn phenotype may provide an environment that would give initiated basal cells a growth advantage.

CHAPTER IV

MODIFICATION OF KERATINS IN SENCAR MOUSE BASAL KERATINOCYTES BY THE POTENT TUMOR PROMOTER 12-O-TETRADECANOYLPHORBOL-13-ACETATE AND OTHER WEAKLY PROMOTING HYPERPLASIOGENIC AGENTS

Introduction

Mouse skin carcinogenesis has been divided into at least two stages: initiation, which is induced by a single application of a subthreshold dose of a carcinogen; and promotion, which is brought about by repetitive treatment with a noncarcinogenic tumor promoter (45, 49, 62). The most potent and widely studied skin tumor promoters are the phorbol esters. The potent phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces numerous cellular and biochemical responses. Those responses that appear to correlate best with promotion are the induction of epidermal hyperplasia, the stimulation of ornithine decarboxylase followed by elevation of polyamine levels, and the induction of dark basal keratinocytes which may represent primitive stem cells (34, 49, 54).

Recently, Slaga and co-workers (32, 49, 54) demonstrated that promotion consists of multiple stages. The first stage can be accomplished by limited treatment with TPA. The second stage is accomplished by repetitive treatment with mezerein, a weak promoter that induces cellular responses similar to those produced by TPA (19). These results indicate that most of the biochemical and morphological responses

associated with promotion are components of the second stage. Another very weak promoter, ethylphenylpropiolate (EPP), is not able to act as a second-stage promoter. This may be because EPP does not induce many of the biochemical responses seen with TPA and mezerein, even though it induces hyperplasia and inflammation (54). Preliminary evidence is presented which indicates that EPP may be effective in a third stage of promotion in which the induction of cellular proliferation may be the critical event.

Recently, my laboratory obtained evidence which indicated that TPA as well as several weakly promoting hyperplasiogenic agents modified the epidermal keratins (52). To determine if these agents were influencing keratin expression in a particular cell population of the epidermis, I investigated the protein changes induced in basal keratinocytes which were isolated from treated adult mouse epidermis. This paper provides evidence that TPA as well as the weakly promoting hyperplasiogenic agents, mezerein and EPP, induced major modifications of keratins in mouse epidermal basal cells as determined by two-dimensional electrophoresis.

Materials and Methods

Chemicals. TPA and mezerein were obtained from Dr. P. Borchert (University of Minnesota, Minneapolis). EPP was from Aldrich Chemical Company (Milwaukee, Wi.). All reagents for SDS-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories (Richmond, Ca.). The isoelectric focusing ampholytes were obtained from LKB (Durham, N. C.).

Treatment of animals, isolation of basal cells, and protein extraction. Female SENCAR mice (7-8 weeks old) raised at Oak Ridge National Laboratory were shaved at least 2 days before treatment. Groups of 10 animals were treated topically with either TPA (5 μg), mezerein (2 μg), EPP (13.9 mg) in 0.2 ml acetone, or acetone alone. The doses of the compounds were chosen so that comparable hyperplasia would be induced (unpublished data). Animals were killed by cervical depilatory (Nair), and the animals were washed with Betadine and 70% ethanol. The skin of the back was removed, and the connective tissue and fat were scraped from the underside. The epidermis was separated and the basal cells were isolated by the technique described by Yuspa and Harris (63). The adult skin was floated at room temperature for 3 hours on 0.25% trypsin in Hanks' balanced salt solution. The epidermis was removed as a sheet by gentle scraping with a spatula. The epidermal sheets were then gently stirred in Enriched Waymouths media for 30 minutes, which released the basal cells into the medium. To reduce contamination with larger, more differentiated cells, we did not chop the epidermis prior to stirring. Large clumps of tissue were removed by 2 filtrations through nylon mesh. Cells were collected by centrifugation and washed twice with phosphate-buffered saline. Microscopic examination showed that approximately 80% of the cells were basal cells. This is further suggested by the fact that a large number of the cells dissolved in 2% SDS, demonstrating that the keratins were not crosslinked by disulfide bonds and that cornified envelopes were not present, which indicates that these were basal cells. For separation of newborn epidermis, the Yuspa and Harris (63) procedure was used.

Epidermal proteins were isolated by a modification of the procedures used by Fuchs and Green (11) as well as Dale and Ling (9). Cell pellets were mixed with a solution of 4 M urea and 1 mM phenylmethylsulfonyl-fluoride and homogenized in a Polytron PT-10 homogenizer for 45 seconds. The homogenate was centrifuged at 35,000 rpm for 30 minutes, and the pellets were resuspended in 2% SDS-10 mM dithiothreitol (DTT), then incubated at 37° C for 1 hour. The SDS-DTT extract was centrifuged at 35,000 rpm for 30 minutes. The 4 M urea supernatant containing the more readily soluble proteins of the epidermis and the SDS-DTT supernatant containing the more insoluble proteins, which were predominantly the keratins, were then dialyzed against distilled water, lyophilized, and stored at -20° C until two-dimensional electrophoresis was performed.

Two-dimensional electrophoresis. Two-dimensional electrophoresis was performed by the method of O'Farrell (23). The isoelectric focusing dimension was run over a narrow acidic pH range (pH 4-6) in 5.5-mm glass electrophoresis tubes. This particular pH range was chosen because it would allow maximal separation of the individual keratins which have been shown to have isoelectric points within this pH range (8). The second dimension utilized 10% SDS-polyacrylamide slab gels. Following electrophoresis, the proteins were fixed in 12% trichloroacetic acid, then stained in a solution of 0.1% Coomassie blue, 7% acetic acid, and 50% ethanol. Gels were destained in a solution of 7% acetic acid and 10% ethanol, then photographed.

Results

Two protein extracts, 4 M urea and SDS + DTT, were analyzed by two-dimensional electrophoresis. Mice were killed 48 hours after their skin was treated with TPA, mezerein, EPP, or acetone, and the epidermal basal cells were isolated as described in Methods. The 4 M urea extract removed the more soluble epidermal proteins that were not stabilized by disulfide bonds (9, 52). The proteins remaining following urea extraction were solubilized with 2% SDS-DTT. This would efficiently extract any proteins, particularly the keratins, that were crosslinked by disulfide bonds.

The majority of the SDS-DTT extracted proteins visualized on a two-dimensional gel were the epidermal keratins, as previously reported (52). Criteria used for the identification of the keratins were: (1) extractability, (2) isoelectric points between pH 5.3 and 5.8, (3) molecular weights between 45,000 and 62,000, (4) assembly into filaments *in vitro*, (5) immunological crossreactivity, (6) amino acid composition, and (7) peptide maps.

Analysis of the SDS-DTT solubilized proteins of isolated basal cells showed major changes in the epidermal keratins upon promoter treatment. The most striking difference between the basal cells of the TPA (Figure 4.1A) and the acetone control (Figure 4.1B) groups was that TPA induced a large decrease in the high-molecular-weight keratins, proteins 2a (60,000), 2b (60,000), and 2c (62,000). There was also a slight reduction in a keratin with molecular weight 49,000 (protein 6). Another keratin of molecular weight 49,000 (protein 5) which had an

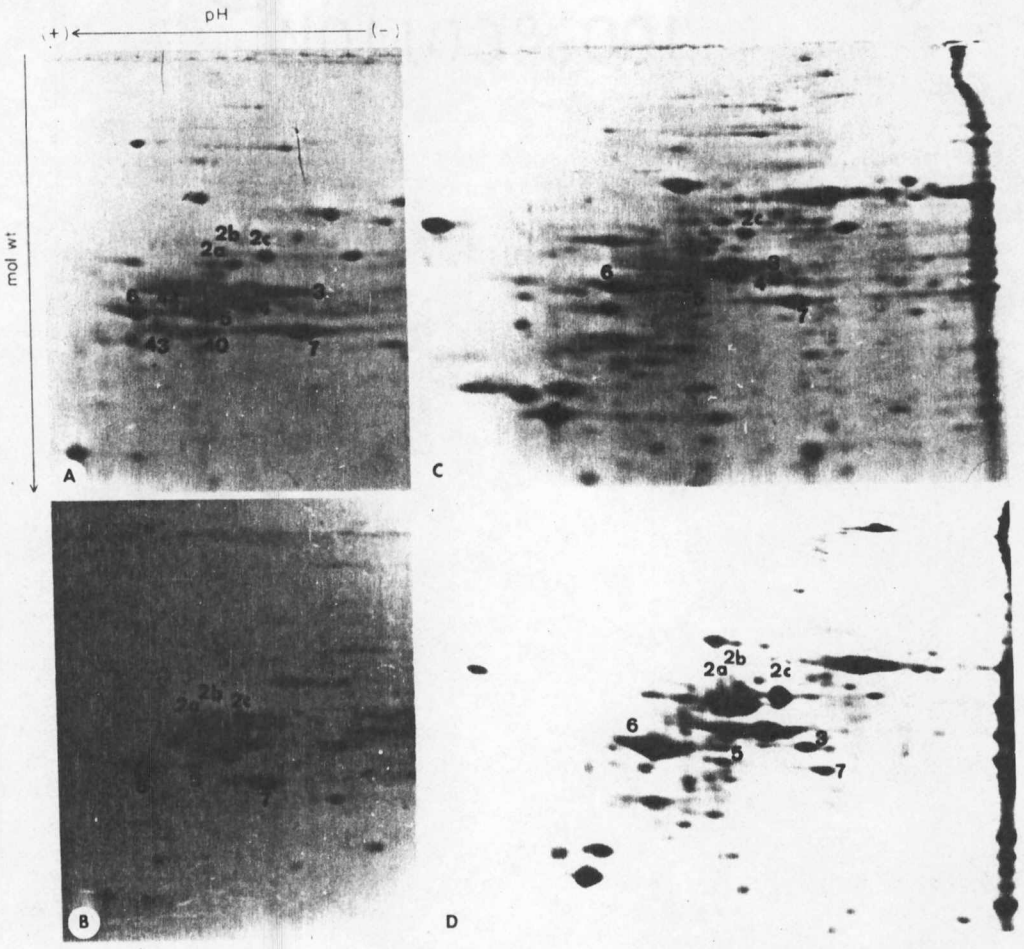

Figure 4.1. Two-dimensional protein patterns of both the 4 M urea and SDS-DTT protein extracts of basal cells isolated from adult epidermis 48 hours after treatment with TPA or acetone. The pH gradient of the isoelectric focusing dimension ranged between pH 4.0 and 6.0. Keratins were found to migrate between pH 5.3 and 5.8. The molecular weight of the keratins ranged between 43,000 and 62,000. SDS-DTT extracted proteins of the basal cells isolated following treatment of adult skin with (A) TPA or (B) acetone were predominantly the keratins. 4 M urea extracts of the (C) TPA and (D) acetone treatment groups contained the more readily solubilized proteins which were not crosslinked by disulfide bonds.

isoelectric point more basic than that of protein 6 was found to be slightly increased. In addition to these protein changes, several minor proteins (proteins 4, 40, and 43) ranging in molecular weights between 47,000 and 51,000 daltons appeared in the extracts from the TPA-treated basal cells.

Similar protein modifications were also detected in the 4 M urea extracts of basal cells isolated from TPA (Figure 4.1C) when compared with keratinocytes of acetone-treated epidermis (Figure 4.1D). There was a significant decrease in the high-molecular-weight keratins 2a, 2b, and 2c. In addition, the lower-molecular-weight (49,000) keratin (protein 6) was reduced compared with controls.

Comparison of the protein changes induced by TPA with those produced by weakly promoting hyperplasiogenic agents allows the role that these protein modifications play in tumor promotion to be investigated. The weakly promoting compounds studied produced protein alterations similar to those induced by TPA. The two-dimensional gel profiles of the SDS-DTT extracted proteins for EPP (Figure 4.2A) and mezerein (Figure 4.2B) showed a marked reduction in the high-molecular-weight keratins (proteins 2a, 2b, and 2c). In addition, there was a decrease in keratin 6 and an increase in keratin 5. Also, as seen with TPA, several new keratins (proteins 4, 40, and 43) were detected.

Treatment of adult mouse skin with hyperplasiogenic agents has been shown to convert it to a multilayered epidermis resembling neonatal skin (4, 24, 28). Therefore, it was possible that newborn basal cells might have keratin patterns similar to those induced in adult epidermis by

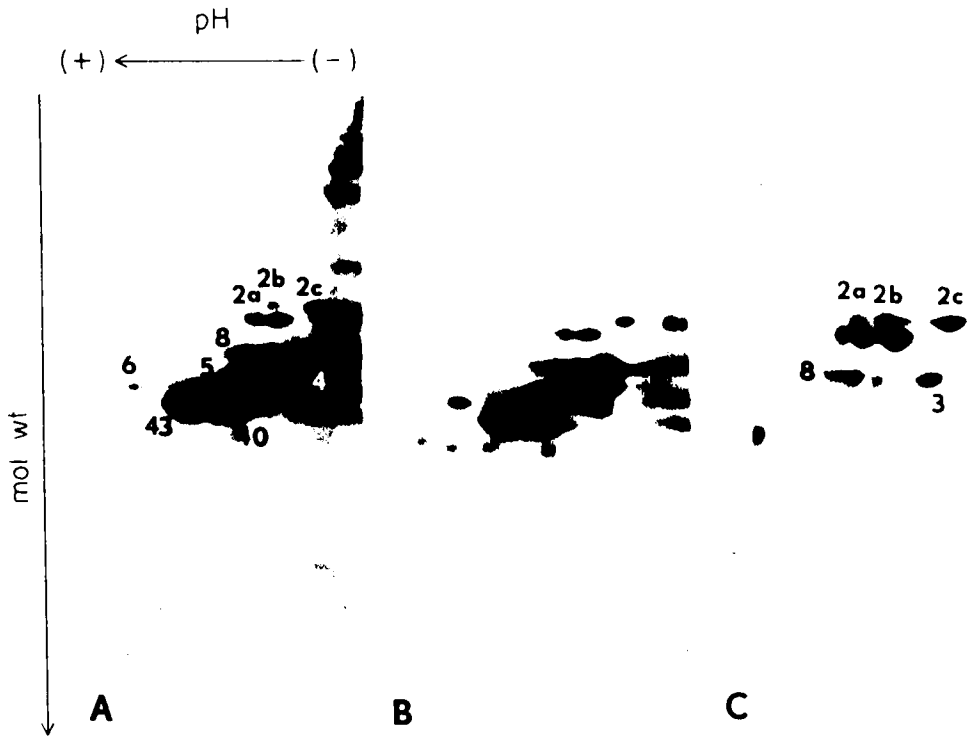

Figure 4.2. Two-dimensional gel protein profiles of the SDS-DTT extracts of basal cells isolated after treatment of adult skin with (A) EPP or (B) mezerein. SDS-DTT extracted proteins of newborn basal cells are also shown (C).

hyperplasiogenic agents. This was found not to be the case. The SDS-DTT extract of the basal cells isolated from newborn SENCAR mice (Figure 4.2C) resembled that of adult acetone controls in that all the high-molecular-weight keratins (proteins 2a, 2b, and 2c) were present and that protein 4 was absent. The newborn protein extracts differed from adult acetone controls in that proteins 5 and 7 were reduced or absent.

Discussion

Data presented in this report demonstrate that TPA and other hyperplasiogenic agents induce major changes in the keratins of epidermal basal cells. The most notable change was the decrease in the high-molecular-weight keratins (proteins 2a, 2b, and 2c). The fact that these proteins were reduced in both the 4 M urea and SDS-DTT extracts indicated that the hyperplasiogenic agents were influencing the synthesis of these proteins rather than altering extractability through modification of disulfide bond crosslinking.

Two other consistent protein changes were a decrease in keratin 6 and an increase in keratin 5. Although they had the same molecular weight, keratins 5 and 6 differed in their isoelectric points, keratin 6 being more acidic than protein 5. Since keratins have been shown to be phosphorylated (39), it was possible that keratin 6 could represent a phosphorylated derivative of keratin 5. Evidence obtained in my laboratory indicated that keratins 6 and 8 were phosphorylated (data not shown). It will require further investigation to determine whether synthesis or posttranslational modification is responsible for these observed protein changes.

In addition to modification of keratins which occur normally, the hyperplasiogenic agents induced new proteins (proteins 4, 40, and 43) in the basal cells. In my previous studies, proteins 4 and 40 were completely characterized and found to be epidermal keratins (52).

The keratin pattern was also investigated in basal cells isolated from newborn mice. Since it had been shown that hyperplastic agents induce a multilayered epidermis resembling that of newborn mice (4, 24, 28) it was possible that the basal cell keratin patterns would be similar in treated adults and newborn mice. This was found not to be the case. The newborn basal cells contained all the high-molecular-weight keratins as did the adult acetone controls.

However, high-molecular-weight keratins were absent in the basal cells obtained from hyperplastic mouse epidermis, as they were in human basal cells (12). Recently, Fuchs and Green (12) demonstrated that there were substantial changes in keratin synthesis during the migration of human epidermal cells from the basal to the stratum corneum layers. The human basal cells were found to synthesize only the small keratins. Similarly, the keratin patterns of cultured epidermal cells from a number of species were found to consistently lack the higher-molecular-weight keratins (11, 12, 50). Winter and co-workers (50) also demonstrated a reduction of the high-molecular-weight keratins in malignant skin tumors. I have found that the keratin patterns of mouse squamous cell carcinomas resemble those induced in epidermal basal cells by the hyperplastic agents (unpublished data). This suggests that the cells of a carcinoma may be locked into a basal cell pattern of keratin expression.

In conclusion, TPA and other hyperplasiogenic agents induce major changes in the pattern of keratin synthesis in epidermal basal cells. This could be a natural physiological response to changes in the cellular environment. Fuchs and Green (12) suggested that the program of keratin synthesis could be changed when the environment of a cell is modified, as is observed upon cell culture or alteration of epidermal-dermal interactions. Hyperplasiogenic agents, in inducing regenerative hyperplasia, may also modify the basal cell environment so that a new program of keratin synthesis is triggered. The key to tumor promotion may be that strong promoters such as TPA may enhance the ability of initiated cells to become permanently fixed at the basal cell stage of differentiation, whereas, weakly promoting hyperplasiogenic agents may induce only transient changes.

CHAPTER V

KERATIN MODIFICATIONS IN EPIDERMIS, PAPILLOMAS, AND CARCINOMAS DURING TWO-STAGE CARCINOGENESIS IN THE SENCAR MOUSE

Introduction

Mouse skin carcinogenesis has been divided into at least 2 stages: initiation, which is induced by a single application of a subthreshold dose of a carcinogen, and promotion, which is brought about by repetitive treatment with a noncarcinogenic tumor promoter (45). Treatment of mouse skin with these promoters, especially TPA, the most potent phorbol ester tumor promoter, causes inflammation and hyperplasia (49) and the induction of numerous other morphological and biochemical alterations such as stimulation of DNA, RNA, and protein synthesis (24, 45); alterations of cell morphology and differentiation (24, 27); decreased responsiveness to β-adrenergic agonist (20) and Gl chalone (16); increased prostaglandin synthesis (43); increased histone synthesis and phosphorylation (29); acceleration of phospholipid synthesis (31); and induction of dark basal keratinocytes (15). The effects of phorbol ester tumor promoters on specific proteins, primarily enzymes, have also been investigated. A few of these observed enzyme changes are an increase in ornithine decarboxylase activity (22), an increase in phosphodiesterase activity (21), an increase in neutral proteases (42), an increase in phospholipase activity (47), and a decrease in histidase activity (7). In addition, TPA has been shown to induce 2 proteins

which have been associated with the production of keratohyalin granules (4, 5).

Recently, my laboratory has shown that TPA and several weakly promoting hyperplasiogenic agents modify the epidermal keratins (52). These keratins, which are the major differentiation production of the epidermis, are thought to be involved in the regulation of cell shape and function as mechanical integrators of various cytoplasmic components (11, 13, 17, 40). Some of the morphological and biochemical alterations attributed to TPA treatment may be due to the modification of the keratins, which may alter their normal cytoplasmic function. To gain understanding of the role of these keratin modifications in tumor promotion, I investigated the keratin polypeptide patterns of mouse epidermis, papillomas, and carcinomas during an initiation-promotion experiment by use of two-dimensional gel electrophoresis. Because the keratin polypeptide patterns of papillomas, carcinomas, and uninvolved epidermis were found to be quite distinctive, changes in keratin synthesis may serve as a marker of malignant transformation in mouse epidermis.

Materials and Methods

Chemicals. TPA was obtained from Dr. P. Borchet (University of Minnesota, Minneapolis). DMBA was ordered from Sigma Chemical Company (St. Louis, Mo.). All reagents for SDS-polyacrylamide electrophoresis were received from Bio-Rad Laboratories (Richmond, California). The isoelectric focusing ampholytes were obtained from LKB (Durham, N. C.).

Treatment of animals and protein extraction. Female Sencar mice (7 to 8 weeks old) raised at Oak Ridge National Laboratory were shaved at least 2 days before treatment. There were 4 experimental groups: (1) a group of 100 animals which received multiple twice-weekly treatments with 1 µg TPA, (2) a group of 100 animals which were initiated with 10 nmol DMBA, followed 1 week later by repetitive 1 µg TPA treatments, (3) a group of 50 animals which received repetitive acetone treatments, (4) a group of 50 animals which were initiated with 10 nmol DMBA only. At certain intervals during the initiation-promotion experiment, 4 animals from each group were killed by cervical dislocation. A chemical depilatory (Nair) was applied for 5 minutes to remove excess hair. The skins were removed, and the epidermis was scraped off with a razor blade. If tumors were present, these were removed prior to scraping of the epidermis. Papillomas from 4 animals were pooled according to size: small, those measuring less than 0.3 cm; and large, those greater than 0.3 cm. Carcinomas were not pooled; instead, protein patterns from individual carcinomas were studied. Tissue samples from papillomas and carcinomas were fixed in 10% formalin for histological evaluation.

Epidermal proteins were isolated by a modification of the procedures used by Fuchs and Green (11, 13) and Dale and Ling (9). Prior to extraction, epidermal tumors were thoroughly chopped with scissors. The epidermis and tumors were placed into a solution of 4 M urea and 1 mM phenylmethylsulfonylfluoride and homogenized with a Polytron PT-10 homogenizer for 45 seconds. The homogenate was centrifuged at 35,000 rpm for 30 minutes, and the pellets were resuspended in 2% SDS-10 mM DTT,

then incubated at 37° for 1 hour. The 2% SDS-DTT extract was centrifuged at 35,000 rpm for 30 minutes. The 4 M urea supernatant containing the more readily soluble proteins of the epidermis and the SDS-DTT supernatant containing the more insoluble proteins which were predominantly the keratins were then dialyzed against distilled water, lyophilized, and stored at -20° until two-dimensional electrophoresis was performed.

Two-dimensional electrophoresis. The method of O'Farrell (23) was employed. The isoelectric focusing dimension was run over a narrow acidic pH range (pH 4.0 to 6.0) in 5.5-mm glass electrophoresis tubes. This particular range was chosen because it would allow maximal separation of the individual keratins which have been shown to have isoelectric points within this pH range (39). The second dimension utilized 10% SDS-polyacrylamide slab gels. After electrophoresis, the proteins were fixed in 12% trichloroacetic acid, then stained in a solution of 0.1% Coomassie blue, 7% acetic acid, and 50% ethanol. Gels were destained in a solution of 7% acetic acid and 10% ethanol.

Results

The more soluble proteins, those which were not crosslinked by disulfide bonds, were removed with 4 M urea (9, 52). SDS-DTT was used to efficiently solubilize the epidermal keratins that were stabilized by disulfide bonds. Keratin changes that were observed in the SDS-DTT extracts could also be detected in the 4 M urea extracts which solubilized keratins not crosslinked by disulfide bonds. However, keratin modifications were best visualized using the SDS-DTT extracts because many

of the nonkeratin proteins which were not relevant to this investigation were removed during the 4 M urea extraction. The majority of the SDS-DTT extracted proteins visualized on the two-dimensional gels have been shown to be the epidermal keratins based on their extractability, isoelectric point, molecular weight, ability to assemble into filaments in vitro, amino acid composition, peptide map, and immunological cross-reactivity.

Keratin modifications that occurred during initiation and promotion were investigated in 4 experimental groups: (1) DMBA initiation alone, (2) DMBA initiation followed by repetitive TPA treatments, (3) repetitive TPA treatments, and (4) repetitive acetone treatments (Figure 5.1A-D). No protein alterations were observed in mouse epidermis after multiple treatments with the solvent acetone. A representative two-dimensional gel of the SDS-DTT extracted proteins of acetone treated epidermis is shown in Figure 5.1A. The major proteins (proteins 2a, 2b, 2c, 3, 5, 6, 7, and 8) which are visualized on the gels have been previously shown to be epidermal keratins (52). DMBA initiation also did not modify the epidermal keratins. The epidermal protein extracts from initiated animals resembled that of acetone controls throughout the experiment (data not shown).

Since it is possible that the response of mouse epidermis to tumor promoters may change after repetitive treatments, the abilities of tumor promoters to induce keratin modifications were studied after multiple applications. The epidermal keratin modifications induced by repetitive TPA treatment were essentially identical to those observed with a single

TPA application (52). For example, all the protein changes induced after a single TPA treatment (proteins 4, 40, 41, 42, 43, and 44) were also observed in protein extracts of mouse epidermis after 27 twice-weekly TPA treatments (Figure 5.1B). These TPA-induced proteins ranging in molecular weights between 45,000 and 51,000 with isoelectric points between pH 5.3 and 5.4 have been characterized and shown to be epidermal keratins (52).

To determine if these TPA-induced protein changes were reversible after multiple applications, I removed one group of animals from treatment after they received 44 TPA applications. At 12 days after the last TPA treatment (Figure 5.1D), there was a significant reduction among the TPA-modified proteins (proteins 4, 40, 41, 42, 43, and 44). As observed with a single TPA treatment, the keratin profile at this time resembled that of the newborn (52).

The protein patterns that were studied in the initiation-promotion group were those found in papillomas, carcinomas, and the surrounding uninvolved epidermis. In all cases, the protein changes observed in the initiated uninvolved epidermis which surrounded the papillomas and carcinomas (Figure 5.1C) were similar to the protein patterns in uninitiated mice receiving repetitive TPA treatments alone (Figure 5.1B). The TPA-induced keratins were among the proteins which were extracted from the epidermis of the initiated and promoted animals.

At various times during the experiments, papillomas which arose on the initiated-promoted animals were pooled according to size and extracted for protein as described in "Methods." The TPA-induced

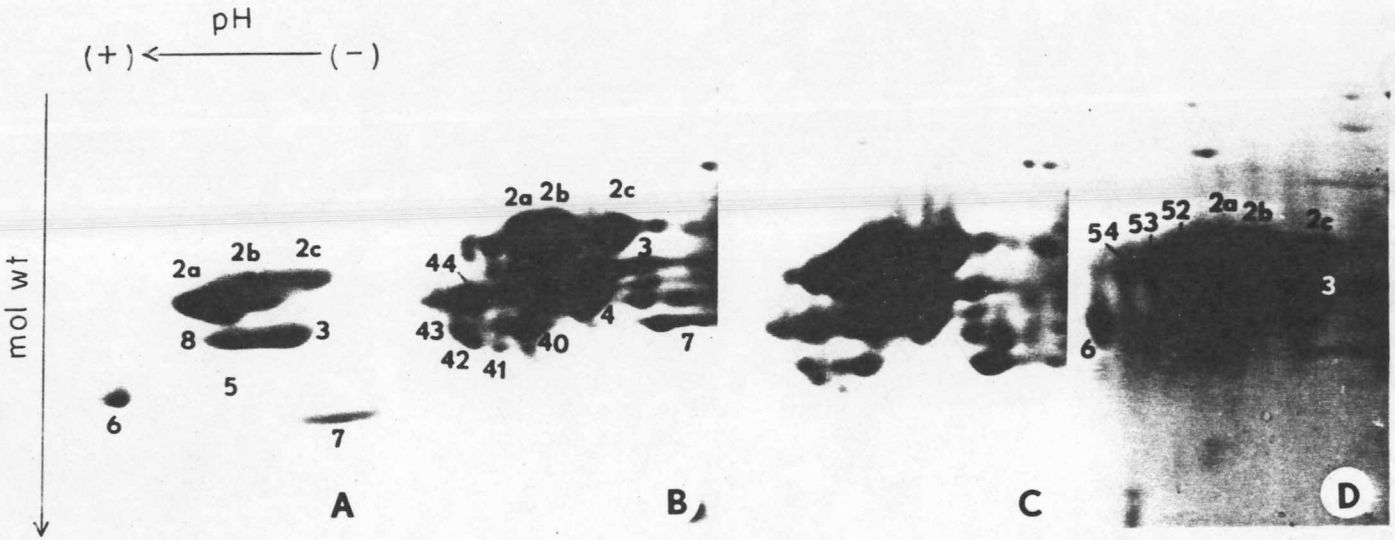

Figure 5.1. Two-dimensional protein patterns of SDS-DTT extracts of mouse epidermis after (A) 27 treatments with acetone, (B) 27 treatments with 1 µg TPA, and (C) DMBA initiation plus 27 treatments with 1 µg TPA. Protein patterns of animals which were removed for 12 days after 44 TPA treatments are alwo shown (D). The pH gradient of the isoelectric focusing dimension ranged between pH 4.0 and 6.0. Keratins migrated between pH 5.3 and 5.8. Molecular weights of the keratin ranged between 43,000 and 62,000.

keratin modifications observed in the epidermis were found to be even more pronounced in the epidermal papillomas. Representative gels are shown in Figure 5.2A-F. All the papillomas contained a very distinctive protein 4 which was quite heterogeneous along the isoelectric focusing dimension. In fact, in some papillomas (Figure 5.2C and F) protein 4 was even more predominant than protein 3, a major keratin of normal skin. The other TPA-induced proteins were also represented to some extent. The most striking feature of the gel profiles of the epidermal keratins of papillomas was observed in the region of proteins 2a and 2b. There were multiple discrete protein spots along the isoelectric focusing dimension toward the more acidic pH range (note Figure 5.2A-C). This is indicative of protein charge heterogeneity, possibly due to phosphorylation. From my previous work, it had been demonstrated that proteins 2a and 2b consist of at least 2 components, a higher-molecular-weight protein of 62,000 ($2a_1$ and $2b_1$) and a lower-molecular-weight component of 60,000 ($2a_2$ and $2b_2$) (52). Acetone controls (Figure 5.1A page 68) contained only a small amount of the higher-molecular-weight components ($2a_1$ and $2b_1$). However, proteins $2a_1$ and $2b_1$ were found to be present in newborn epidermis as well as to be induced by TPA in adult epidermis. In some papillomas (Figure 5.2A-C) both protein components of 2a and 2b were present; however, unlike the newborn and TPA-treated adult epidermis, these proteins were quite heterogeneous in charge. In other papillomas (Figure 5.2D-F) there appeared to be selective loss of the higher-molecular-weight (62,000) component ($2a_1$ and $2b_1$). This was found not to be related to the size of the papilloma. However, as

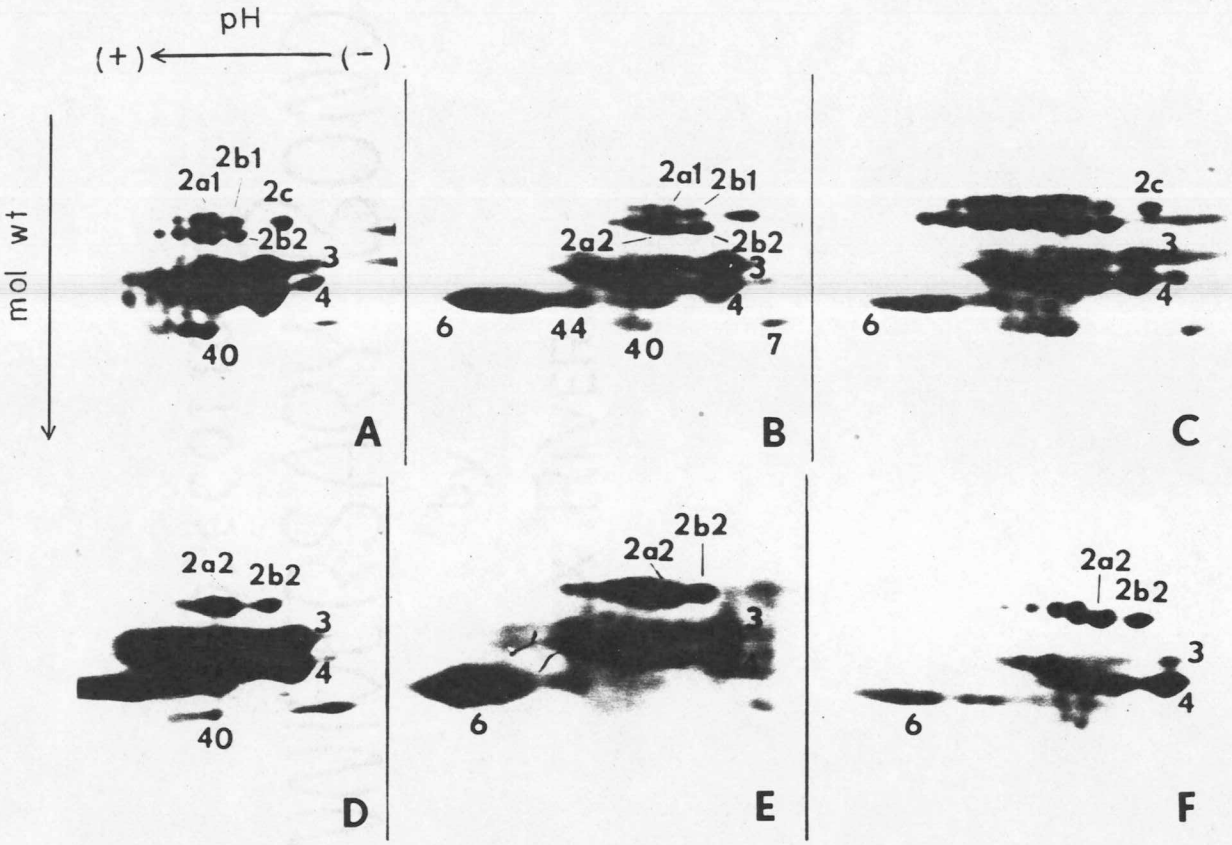

Figure 5.2. Two-dimensional protein patterns of the SDS-DTT extracted proteins of papillomas arising during two-stage mouse skin carcinogenesis. (A) Small papillomas obtained from initiated mice after 13 TPA treatments, (B) small papillomas obtained after 16 TPA treatments, (C) large papillomas obtained after 27 TPA treatments, (D) small papillomas obtained after 27 TPA treatments, (E) small papillomas obtained after 44 TPA treatments, (F) large papillomas obtained after 83 TPA treatments.

the experiment progressed, the loss of this group of keratins was observed more frequently than at earlier times. Although there were protein features which appeared to be distinctive for the papillomas, individual variations were noted among all the protein profiles of papillomas that were examined.

The protein profiles of the carcinomas which arose after 6 months of promotion were distinguishable from those of the surrounding epidermis and the papillomas. The protein feature which characterized the carcinomas was the significant reduction in both groups of high-molecular-weight keratins (62,000 and 60,000) (Figure 5.3A-C). All the carcinomas contained protein 4. As seen with the papillomas, protein 4 was occasionally more predominant than protein 3 (Figure 5.3B).

To determine if the loss of the high-molecular-weight keratins upon malignancy was a feature limited to two-stage carcinogenesis, I studied the keratin profile of a carcinoma induced by a complete carcinogenic dose of DMBA. The loss of the high-molecular-weight keratins was quite evident in the protein profile of the DMBA-induced carcinoma (Figure 5.3D). Interestingly, protein 4 was not very abundant; however, small amounts of the minor keratins were observed.

Recently, my laboratory has demonstrated that TPA induces a significant reduction in the high-molecular-weight keratins in adult epidermal basal cells (53). As seen in Figure 5.3E, the protein profiles of the TPA-treated basal cells were similar to those of the carcinomas, in that the low-molecular-weight keratins represent the predominant keratin species.

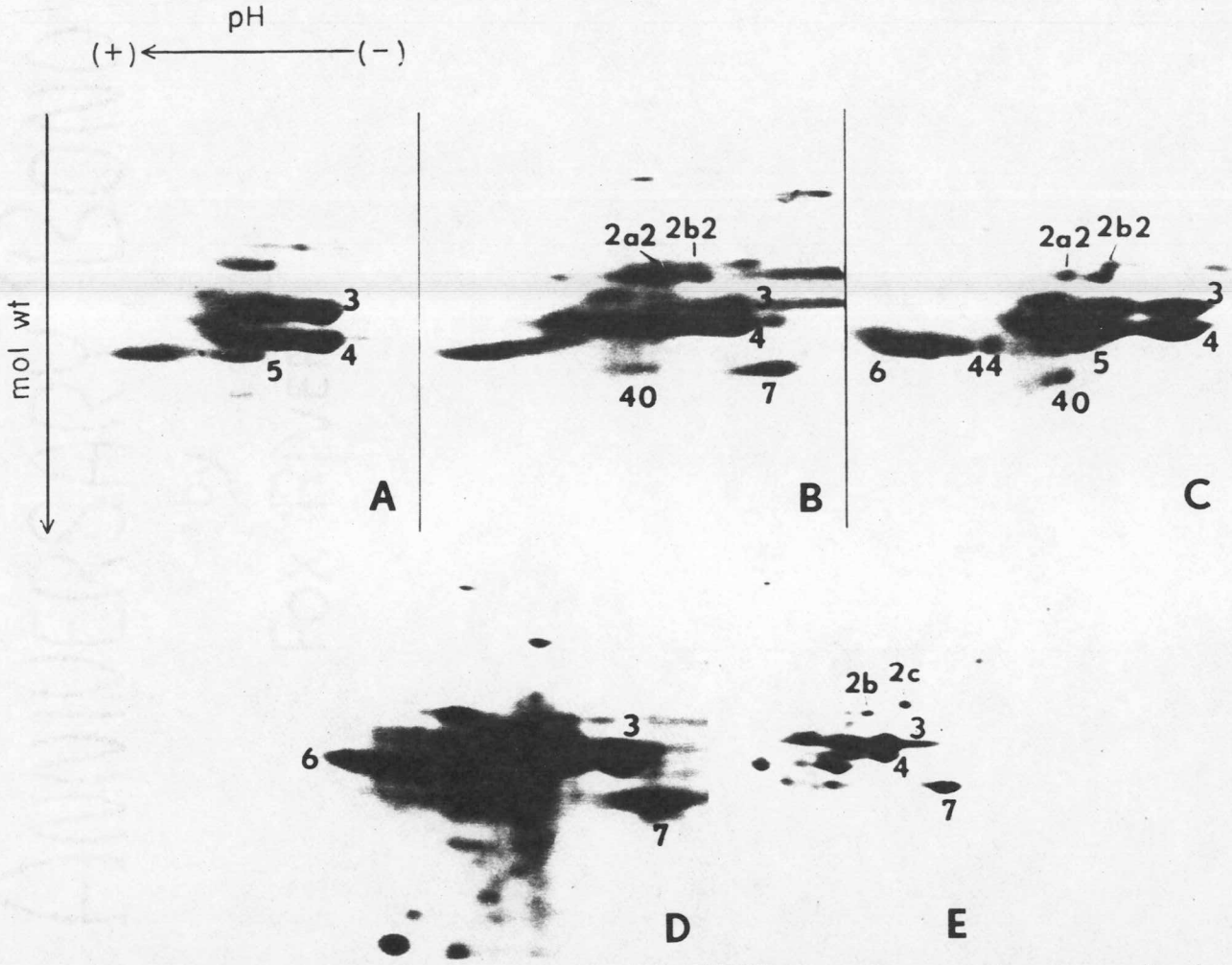

Figure 5.3. Comparison by two-dimensional electrophoresis of the keratin patterns found in carcinomas during two-stage carcinogenesis with those found in a carcinoma induced by complete carcinogenesis and with those found in basal cells after TPA treatment. (A) Carcinoma obtained from an initiated mouse after 44 TPA treatments, (B) carcinoma obtained after 57 TPA treatments, (C) carcinoma obtained after 80 TPA treatments, (D) carcinoma obtained after complete carcinogenesis with DMBA, (E) keratin patterns obtained from basal cells isolated from adult mouse epidermis 48 hours after a single TPA treatment.

Discussion

The keratin polypeptide patterns of mouse epidermis, papillomas, and carcinomas were found to be characterized by the presence of distinct keratin modifications. The modifications found in initiated and noninitiated epidermis after repetitive TPA treatments were very similar to those observed after a single TPA application. These keratin changes were also observed in papillomas. However, the distinguishing feature of the papillomas was the presence of high-molecular-weight keratins which had considerable charge heterogeneity. A significant reduction in these high-molecular-weight keratins was the most striking protein alteration observed in the carcinomas. Modification of the epidermal keratins may serve as a biochemical marker for malignant progression in mouse epidermis.

The epidermal keratin modifications induced by repetitive TPA treatments in both initiated and noninitiated mouse skin were essentially identical to those observed with a single TPA application, which indicated that the epidermal response is not altered after prolonged treatment. In addition, these keratin modifications were found to be reversible when TPA treatment was discontinued.

The charge heterogeneity exhibited by the high-molecular-weight keratins was a consistent feature of papillomas. Since keratins have been shown to be phosphorylated (40), it is possible that these multiple protein spots represent phosphorylated derivatives of the major keratins (proteins 2a, 2b, and 2c) having different numbers of phosphate groups. Preliminary evidence obtained in my laboratory indicated that papillomas

have elevated levels of protein kinases which are capable of phosphorylating the keratins. At early time points the papillomas generally contained both groups of high-molecular-weight keratins (60,000 and 62,000). A selective loss of the 62,000-molecular-weight keratin components was observed in some papillomas, becoming more frequent as the experiment progressed. I have previously demonstrated that TPA-treated adult epidermis and newborn epidermis contain both groups of high-molecular-weight keratins (52). The papillomas which also contain both groups of high-molecular-weight keratins resemble the newborn pattern except for the greater charge heterogeneity. Although no charge heterogeneity was observed, the keratin patterns of fetal epidermis at 17 days of gestation resembled that of some of the papillomas in which the 62,000-molecular-weight proteins were decreased or absent (data not shown). These changing keratin patterns may reflect different states of embryonic keratin gene expression that may occur during promotion. It is possible that the papillomas which have a selective loss of one group of keratins may represent a more advanced stage in the progression to malignancy. Preliminary data supporting this have been obtained in studies of both keratin patterns and γ-glutamyltranspeptides (GGT) activity in individual papillomas. Recently, DeYoung and co-workers (46) demonstrated that malignant squamous carcinomas had elevated GGT activities, suggesting that GGT may serve as a marker of epidermal neoplasia. In my laboratory, I have found that in addition to carcinomas, a small percentage of papillomas have increased GGT activities. The majority of these

GGT-positive papillomas are found to lack the 62,000-molecular-weight keratins. Papillomas demonstrating elevated GGT activity as well as loss of certain keratins may be on the verge of malignancy.

The changes in the high-molecular-weight keratins were even more significant in the carcinomas, in which both the 62,000- and 60,000-molecular-weight components were substantially reduced. These protein alterations were shown not to be limited to carcinomas induced by two-stage carcinogenesis; they were also observed in carcinomas induced by a complete carcinogenic dose of DMBA. Winter and co-workers (50) have also recently demonstrated that mouse skin malignant tumors specifically lack keratin components with molecular weights greater than 61,000.

The carcinoma keratin profiles were very similar to the patterns observed in basal cells following a single TPA treatment. This may indicate a basic defect in the program of differentiation in the carcinoma. By maintaining a basal cell pattern of gene expression, the cells of the carcinoma may be able to escape normal differentiation and growth controls. Several investigators have presented evidence which indicates that transformed epidermal cells do have basic defects in their program of differentiation (48, 51). Yuspa and co-workers (51) demonstrated that transformed epidermal cells continue to proliferate when exposed to a calcium environment which promotes terminal differentiation in normal cells. Cell lines derived from human squamous cell carcinomas have a partial defect in terminal differentiation as measured by their survival and cornified envelope formation in semisolid media (48).

In conclusion, changes in keratin synthesis may serve as a biochemical marker for malignant transformation in mouse epidermis. Modification of the program of keratins synthesis in mouse epidermal basal cells may be one mechanism by which TPA promotes. TPA may induce changes that allows initiated basal cells to become fixed into a basal pattern of differentiation, resulting in a permanent growth advantage.

CHAPTER VI

CONCLUSION

Tumor promoters induce numerous cellular and molecular changes in mouse skin, only some of which are probably necessary for promotion. Many of these biochemical responses are associated with the induction of inflammation and hyperplasia. Distinguishing the critical biochemical events induced only by tumor promoters and not by nonpromoting inflammatory hyperplasiogenic agents has been very difficult. The purpose of this dissertation was to determine if the phorbol ester tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induced any protein changes in mouse epidermal proteins which were specific to promotion. Two-dimensional gel electrophoresis was the technique used for studying the proteins. This allowed for the separation of proteins on the basis of their isoelectric points as well as their molecular weights. To determine the specificity of the TPA-induced protein changes to tumor promotion, the protein modifications induced by several weakly promoting inflammatory hyperplasiogenic agents were studied; the effects of several inhibitors of tumor promotion on the TPA-induced protein changes were investigated; and the protein alterations that occurred throughout an initiation and promotion experiment were analyzed.

Evidence obtained by comparing the TPA-induced protein modifications to those induced by weakly promoting hyperplasiogenic agents and by studying the effects of the inhibitors of promotion on the TPA-induced

protein changes indicated that most of the protein modifications, particularly among the epidermal keratins, were associated with the induction of hyperplasia and not related to promoting activity. Although these protein changes were not specifically induced by tumor promoters, they were features of papillomas and carcinomas associated with carcinogenesis. The role that these protein modifications play in tumor promotion is most likely the same as that of hyperplasia, in that the protein changes are probably necessary for promotion; however, they are not sufficient. Other critical events induced by promoters probably must accompany these protein changes in order for promotion to occur.

TPA and the other hyperplasiogenic agents were found to induce proteins in adult epidermis which were also present in newborn tissue. There have been several reports which indicated that tumor promoters induced biochemical changes in adult epidermis which were characteristic of the newborn state (5, 7, 16, 20, 24). Ultrastructural studies have shown that normal adult mouse epidermis, consisting of only one to two cell layers was converted to a multilayered epidermis resembling neonatal skin (24). Balmain and co-workers (5) demonstrated that TPA induced several proteins, two of which were shown to be components of fetal and newborn tissues. Following TPA treatment of adult epidermis, histidase activity was lowered to a level which resembled that found in fetal epidermis (7). Further similarities between TPA treated adult- and newborn-epidermis were shown to be their response to epidermal chalone G1 (16) and to β-adrenergic agonists (20). The appearance of fetal

characteristics in adult mouse epidermis upon the induction of hyperplasia was consistent with Uriel's (61) observations that fetal patterns may appear transiently in normal tissue undergoing regenerative growth or hyperplasia. He suggested that retrodifferentiation or dedifferentiation may represent a normal physiological process necessary for maintaining the integrity of cells against deleterious agents. In general, some characteristics of cells undergoing retrodifferentiation was a loss of cytoplasmic organization and complexity, the synthesis of embryo-fetal type antigens and enzymes, and the restriction of the expression of genes necessary for maturation to the fully differentiated state. For example, after partial hepatectomy or liver poisoning in rats, there was an increase in the synthesis of fetal-type antigens and isozymes such as α-fetoproteins, esterases, cell surface antigens, glucose-ATP-phosphotransferase, and phosphofructokinase. As described above, TPA-treatment of adult mouse epidermis can also result in the reappearance of several fetal biochemical and ultrastructural characteristics. According to Uriel, retrodifferentiation was normally counterbalanced by a process of redifferentiation which restored the mature adult state. He postulated that tumor cells may arise by escaping redifferentiation; thus giving them a preferential growth advantage. This may be the key to tumor promotion; promoters may enhance the ability of initiated cells to escape redifferentiation and become permanently fixed at a less differentiated state. Support for this comes from the observation that carcinomas were found to have keratin patterns resembling the basal cell state, indicating that they had lost the ability to express the

high molecular weight keratins that are characteristic of epidermal cells at a more advanced stage of terminal differentiation.

Modification of epidermal keratins were among the major protein alterations which were observed in TPA treated mouse epidermis, papillomas and carcinomas. Variation of the keratin polypeptide composition of keratin filaments could have profound influence on the cytoplasmic function of these filaments. Alterations in filament structure may be responsible for some of the morphological and biochemical responses that are observed in these tissues (11, 12, 17, 60). Lazarides (17, 60) suggested that a cell regulates the function of the intermediate filaments, of which keratin filaments are a class, by controlling the ratios and quantity of the different polypeptide constituents of the filaments. There is evidence which indicates that the regulation of the expression of different filament subunits is controlled at the mRNA level (11, 12, 60). For example, Fuchs and Green (11, 12) have demonstrated that during the maturation of human epidermal cells, substantial changes in the pattern of keratin synthesis occurred which was due to changes in the keratin mRNAs. The human epidermal basal cells contained only the mRNAs that coded for the low-molecular-weight keratins; whereas, the superficial more differentiated cells possessed the mRNAs coding for the high molecular weight keratins. The particular pattern of keratin synthesis that is found in epithelial cells can be influenced by changes in their environment. For example, cells of different stratified squamous epithelia that normally have their own distinct keratin patterns were found upon culturing, to synthesize similar keratins regardless of origin (12). This may be a possible

explanation for the observed changes in mouse epidermal keratins upon treatment with hyperplasiogenic agents. These agents may modify the cellular environment such that a new program of keratin synthesis is initiated which probably is primarily controlled by changes in the mRNA populations. Another important point to consider is that not all epidermal cells may respond in the same way to the hyperplasiogenic agents. For instance, initiated cells may respond with a program of keratin synthesis that may directly or indirectly contribute to a growth advantage. The keratin patterns of carcinomas were distinctly different from papillomas and epidermis. The high-molecular-weight keratins which are characteristic of epidermal cells committed to terminal differentiation were present in epidermis and to varying degrees in most papillomas. However, the carcinomas expressed the basal cell keratin pattern, having only the low molecular weight keratins which indicated a possible defect in differentation. Maintaining a basal cell environment that is conducive to proliferation and not to differentiation that leads to cell loss would allow initiated cells to eventually evolve into carcinomas. Alterations of differentiation may be critical for the carcinogenic process. Changes in keratin patterns appear to be indicative of corresponding changes occurring in the state of differentiation of epidermal cells. Therefore, keratins may be potential markers for studying malignant progression.

There are several research directions that could be followed to pursue this project: (1) to determine if the changes in keratin patterns are due to changes in the mRNA populations, (2) to determine

if the charge heterogeneity observed among the papilloma keratins is due to phosphorylation, and possibly what consequences this charge heterogeneity has on filament structure and orientation, (3) to look for alterations in differentiation by studying keratin patterns of distinct and isolated cell populations obtained from epidermis, carcinomas and papillomas using Percol density gradient centrifugation. The effects of various agents such as carcinogens, inhibitors, and promoters on keratin expression in these cell populations could be studied. Perhaps, chemotherapeutic agents may be found that would induce carcinoma cells to enter terminal differentiation. This could be monitored by the appearance of the high-molecular-weight keratins that are indicative of cells that are committed to terminal differentiation.

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VITA

Karen Gray Nelson was born in Martinsville, Virginia, on March 9, 1953. She attended Robert E. Lee High School in Springfield, Virginia, and graduated in June 1971. She received her Bachelor of Science degree in Biology in June 1975 at Virginia Polytechnic Institute and State University. In September of that year, she entered the doctoral program at The University of Tennessee—Oak Ridge Graduate School of Biomedical Sciences at the Biology Division, Oak Ridge National Laboratory. She acquired a Ph.D. degree in August 1981 and subsequently accepted a postdoctoral research position in carcinogenesis at University of North Carolina at Chapel Hill.