Keratin Gene Expression in Mouse Skin Tumors and in Mouse Skin Treated with 12-O-Tetradecanoylphorbol-13-acetate

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ABSTRACT

Alterations in the pattern of epidermal differentiation and proliferation occur during mouse skin carcinogenesis. We have used cDNA clones corresponding to the major keratin subunits synthesized in differentiating epidermal cells (M, 67,000 and 59,000) and in proliferating epidermal cells (M, 60,000, 55,000, and 50,000) to study changes in keratin gene transcript levels in mouse epidermis exposed to tumor promoters. The same probes were used to characterize the keratin expression patterns in benign and malignant skin tumors. A single topical treatment with 12-O-tetradecanoylphorbol-13-acetate caused a rapid initial decrease in the epidermal transcript levels corresponding to the M, 67,000 and 59,000 keratin subunits. By 48 h the transcript level for the M, 67,000 keratin subunit was restored to control values, whereas the transcript levels for the M, 59,000 subunit returned to control at a slower rate. In contrast, the transcript level for the M, 55,000 subunit was increased substantially 12–48 h after treatment, the M, 50,000 subunit transcript increased to a lesser extent, and the M, 60,000 subunit message was transiently decreased at 12 h but returned to the level of solvent-treated skin by 24 h. Single exposure to the incomplete tumor promoters 4-O-methyl-12-O-tetradecanoylphorbol-13-acetate, the ionophore A23187, and mezerein induced changes in keratin gene transcripts similar to those of 12-O-tetradecanoylphorbol-13-acetate. The antipromoter fluocinolone acetonide, administered with 12-O-tetradecanoylphorbol-13-acetate, partially inhibited the decrease in the M, 59,000 and 67,000 transcripts and completely inhibited the increase in the M, 55,000 transcript. In skin papillomas produced by initiation and promotion, keratin gene expression was similar to normal skin, with the exception of a two-fold increase in the transcript levels for the M, 55,000 keratin subunit. However, in carcinomas, the transcript levels for the M, 67,000 and 59,000 subunits were only 1–3% of those observed in untreated mouse epidermis. In concert with other data, the rapid and selective loss of transcripts for differentiation-related keratins after exposure to both complete and incomplete tumor promoters is most consistent with the interpretation of these results with regard to dynamic changes. Alterations in keratin gene expression occurring after treatment of mouse skin with tumor promoters and in papillomas and carcinomas requires additional cellular changes that are most likely to be genetic in nature.

INTRODUCTION

Chemical induction of skin tumors in mice can be operationally subdivided into at least three different stages, i.e., initiation, promotion, and malignant conversion (1, 2). Tumor promotion is thought to involve the clonal expansion of initiated epidermal cells, resulting in the formation of primarily benign tumors, papillomas (3). The conversion of epidermal papillomas to carcinomas requires additional cellular changes that are most likely to be genetic in nature (2, 4).

Alternations of both epidermal cellular differentiation and skin proliferation patterns are thought to constitute essential events during multi-stage mouse skin carcinogenesis (5–7). The major differentiation products in mouse epidermal cells are keratins, a family of related structural proteins of molecular weight 40,000–70,000, which make up the subunits of the intermediate filaments in epithelial cells (8, 9). Different subsets of keratins are preferentially expressed in basal and differentiating cell layers of newborn mouse epidermis (10–13), and differential expression has been demonstrated in primary epidermal cell culture and in malignantly transformed epidermal cells (14). Since individual keratins are synthesized from different mRNAs (11, 15–18), the transcript levels of specific keratins reflect changes in the expression of genes closely regulated during differentiation.

Previous studies of keratin protein patterns during skin carcinogenesis have shown that major changes in the amount and synthesis of keratin subunits are apparent in mouse epidermis after treatment with tumor promoters and in papillomas and carcinomas (19–23). The interpretation of these results with regard to dynamic changes in skin, however, has been hampered by the high stability and slow turnover of keratin proteins (8, 24) and by differential resistance toward proteolytic modification among the subunits (24–26). In the present study, we used cDNA clones corresponding to the major keratins expressed in the differentiating layers of mouse epidermis [M, 59,000 and 67,000; Nos. 10 and 1 according to the catalogue of Moll et al. (9)] and to keratins predominantly expressed in the proliferating basal cells [M, 50,000, 55,000, and 60,000; Nos. 16, 14, and 5 according to Moll et al. (9)] to characterize the changes in keratin gene expression occurring after treatment of mouse skin with tumor promoters and in papillomas and carcinomas.

MATERIALS AND METHODS

Animal Treatments. Female SENCAR mice 7–9 weeks old, which are predisposed to tumor development (2), were used. The backs of the...
mice were shaved 24 h prior to topical treatment with 5 μg of TPA, 250 μg of 4-O-methyl-TPA, 2 μg of mezerein, 250 μg of the ionophore A23187, or 5 μg of TPA in combination with 2 μg of fluorocinolone acetonide. The doses of tumor promoters were chosen to give a comparable hyperplastic response (27-29) and were applied in 200 μl of acetone over an area of approximately 8 cm². Mice were killed at different time points after treatment, and total RNA was isolated. Repeated treatments with TPA, 2 μg twice weekly for 4 weeks with or without prior initiation with 20 μg of 7, 12-dimethylbenz[a]anthracene were carried out according to a schedule used in tumor induction experiments.

Papillomas were induced by initiation with 20 μg of 7, 12-dimethylbenz[a]anthracene followed by promotion with 2 μg of TPA twice weekly for 12 weeks, and RNA was prepared from pools of 6-12 papillomas. Carcinomas were derived from papillomas induced as above but subsequently treated with a carcinogen (20 mg of urethane i.p. once weekly) to accelerate malignant conversion. Carcinomas were also produced by subcutaneous implantation of malignantly transformed mouse epidermal cells (cell line Pam 212) (30). RNA was isolated from pools of 3-6 carcinomas.

Isolation of RNA. The epidermis was separated from the dermis by flotation on 1% trypsin at 37°C for 1 h, and total RNA was prepared from groups of 5-15 mice, essentially as described by Chirgwin et al. (31). The amount of polyadenylic acid containing RNA was determined by hybridization to 3H-polyuridylic acid (32), and the integrity of each preparation was checked by subjecting a 10-μg portion of the RNA to electrophoresis in a 1.1% formaldehyde/agarose gel, transfer of the RNA to nitrocellulose paper (33), followed by hybridization to cDNA probes specific for the M, 67,000 keratin subunit. Lane 1, control; lane 2, 12 h after TPA treatment; lane 3, 12 h after combined treatment with TPA and fluocinolone acetonide; lane 4, 72 h after TPA treatment; lane 5, 7 days after TPA treatment; lane 6, 12 h after 4-O-methyl-TPA treatment; lane 7, 48 h after 4-O-methyl-TPA treatment; lane 8, 12 h after treatment with the ionophore A23187; lane 9, 48 h after A23187 treatment; lane 10, 12 h after mezerein treatment; lane 11, 48 h after mezerein treatment.

RESULTS

Modulation of Keratin Gene Expression by Treatment with Tumor Promoters in Vivo. A single topical application of TPA (5 μg) to mouse epidermis caused a rapid and dramatic decrease in the relative level of transcripts corresponding to the M, 67,000 and 59,000 keratin subunits (Fig. 1, lane 2, and Chart 1A). Twelve h after treatment, the transcript levels were only 5% of that in untreated epidermis. The level of transcripts for these two differentiation keratins remained low for an additional 12-24 h. By 48 h after TPA treatment, the transcript levels for both genes were increasing so that the M, 67,000 transcripts were more abundant than in control epidermis, while the M, 59,000 transcript was still slightly below control values. Seven days after a single exposure, the transcript levels for both genes were essentially back to control values. Similar results were obtained in two independent experiments.

In contrast to the results with the differentiation-related keratins, the transcript levels corresponding to the M, 55,000 and 59,000 keratin subunits, preferentially synthesized in proliferating basal cells, showed no such decrease after TPA exposure. There was a slight (50%) decrease in the transcript level corresponding to the M, 60,000 keratin subunit at 12 h only (Chart 1B). A substantial increase in the transcript level for the M, 55,000 keratin subunit was observed at 24 and 48 h after treatment (Chart 1B).

Other agents which are active as promoters but without the complete promoting activity of TPA were tested by a single topical application. 4-O-methyl-TPA (250 μg), the ionophore A23187, or 5 μg of TPA in combination with 2 μg of fluocinolone acetonide, transferred to a nitrocellulose sheet, and subsequently hybridized to a cDNA clone specific for the M, 67,000 keratin subunit. Lane 1, control; lane 2, 12 h after TPA treatment; lane 3, 12 h after combined treatment with TPA and fluocinolone acetonide; lane 4, 72 h after TPA treatment; lane 5, 7 days after TPA treatment; lane 6, 12 h after 4-O-methyl-TPA treatment; lane 7, 48 h after 4-O-methyl-TPA treatment; lane 8, 12 h after treatment with the ionophore A23187; lane 9, 48 h after A23187 treatment; lane 10, 12 h after mezerein treatment; lane 11, 48 h after mezerein treatment.

Fig. 1. Analysis of mRNA corresponding to the M, 67,000 keratin subunit in adult mouse epidermis after single treatments with complete and incomplete tumor promoters. Twenty μg of total RNA were electrophoresed on a 1.1% agarose gel and transferred to a nitrocellulose sheet, and subsequently hybridized to a cDNA clone specific for the M, 67,000 keratin subunit. Lane 1, control; lane 2, 12 h after TPA treatment; lane 3, 12 h after combined treatment with TPA and fluocinolone acetonide; lane 4, 72 h after TPA treatment; lane 5, 7 days after TPA treatment; lane 6, 12 h after 4-O-methyl-TPA treatment; lane 7, 48 h after 4-O-methyl-TPA treatment; lane 8, 12 h after treatment with the ionophore A23187; lane 9, 48 h after A23187 treatment; lane 10, 12 h after mezerein treatment; lane 11, 48 h after mezerein treatment.
KERATIN GENE EXPRESSION DURING SKIN CARCINOGENESIS

Chart 1. Relative quantitation of keratin transcript levels in adult mouse epidermis after a single treatment with TPA. Total epidermal RNA was prepared from control mouse epidermis, and at various time points after TPA treatment, slot-blotts were prepared. After hybridization to the different keratin cDNA clones, the resulting autoradiograms were scanned densitometrically, and the area under each peak was calculated. The data are expressed relative to the transcript levels observed with control mouse epidermal RNA. A, transcript levels corresponding to the M, 67,000 and 59,000 keratin subunits preferentially expressed in differentiating epidermal cells; B, transcript levels corresponding to the M, 60,000, 55,000, and 50,000 keratin subunits preferentially expressed in proliferating basal cells.

A23187 (250 μg), and mezerein (2 μg) caused essentially the same pattern of changes in keratin transcripts as treatment with TPA (Chart 2). In all cases, a marked decrease in the M, 67,000 and 59,000 transcripts was observed at 12 h. However, only TPA showed the increase above control value in M, 67,000 expression at 48 h, while this message returned more slowly after treatment with the other agents. This can be seen also on the Northern blots of mRNA from epidermis treated with these agents (Fig. 1). In the case of A23187, the messages for the differentiation-related keratins remained depressed for longer than 48 h. TPA appeared to have the most pronounced and selective effect of any agent tested on the increase in the levels of the proliferation-associated M, 55,000 transcripts, but all of the agents studied produced changes similar to TPA in pattern.

Tumor promotion requires multiple exposures for the biological response of tumor formation. When TPA exposure was repeated twice weekly for 4 weeks and the keratin transcript levels measured 72 h after the last exposure, the pattern was similar to that seen at 72 h after a single exposure. Results were identical in initiated skin or skin not exposed to an initiator (Table 1).

FA is a potent antipromoting agent in mouse skin. When FA was applied simultaneously with TPA, there was a partial inhibition of the loss of the M, 67,000 and 59,000 transcripts at 12 h (Table 2 and Fig. 1, lane 3). Most striking was the marked depression of the M, 55,000 transcript level in the group treated with FA and TPA, while the expected increase in this transcript at 12 h was observed in the group treated with TPA alone. The expected increase in M, 50,000 transcript was also suppressed by FA treatment, but the suppression was less striking than that for the M, 55,000 transcript.

Keratin Gene Transcripts in Papillomas and Carcinomas. As shown in Table 1, the relative transcript levels corresponding to the keratin subunits are very similar in papillomas and normal adult epidermis, with the exception of a substantial increase in the transcript level corresponding to the M, 55,000 keratin in papillomas. In contrast, the relative transcript levels corresponding to the keratin subunits preferentially synthesized in differentiating epidermal cells (M, 67,000 and 59,000) are very low in carcinomas. The loss of these transcripts is a consistent finding in both primary carcinomas and in a transplantable carcinoma resulting from injection with cell line Pam 212. The presence and
KERATIN GENE EXPRESSION DURING SKIN CARCINOGENESIS

Table 1
Relative quantitation of keratin gene mRNAs in mouse skin after repeated treatment with TPA and in papillomas and carcinomas

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak area (% of control) for corresponding keratin subunit:</th>
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<tr>
<td></td>
<td>M&lt;sub&gt;67,000&lt;/sub&gt;</td>
</tr>
<tr>
<td>TPA (4 weeks, 2 μg twice weekly)</td>
<td>196</td>
</tr>
<tr>
<td>DMBA (20 μg once) TPA (4 weeks, 2 μg twice weekly)</td>
<td>164</td>
</tr>
<tr>
<td>Papillomas&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104</td>
</tr>
<tr>
<td>Carcinomas&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>Carcinomas&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
</tbody>
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<sup>a</sup> The data represent the mean of two separate preparations of RNA from pools of 6–12 papillomas.
<sup>b</sup> The carcinomas were derived by subcutaneous implantation of malignant transformed Pem 212 cells (I) or were induced chemically by topical applications to mouse skin as described in "Materials and Methods" (II). RNA was purified from pools of 3–6 carcinomas.

Effect of the anti-promoter fluocinolone acetonide on changes in keratin mRNA levels induced by TPA

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Peak area (% of control) for corresponding keratin subunits:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>M&lt;sub&gt;67,000&lt;/sub&gt;</td>
</tr>
<tr>
<td>TPA (5 μg)</td>
<td>11</td>
</tr>
<tr>
<td>TPA (5 μg) + FA (2 μg)</td>
<td>32</td>
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The availability of specific cDNA probes for individual epidermal keratin genes has provided the capability to study changes associated with promoter exposure at both the transcriptional and post-transcriptional levels. Previous studies with these probes (11) have indicated that the differentiation-associated keratins are expressed in suprabasal cells, while the proliferation-associated keratins are expressed in basal cells. The current studies reveal that TPA exposure causes a marked and rapid decrease in messages for the differentiation-related keratins (M<sub>67,000</sub> and 59,000). The rapid decrease in these transcripts (subsequent experiments have shown a decrease to 25% of control values by 4 h after exposure), its specificity (other keratin mRNAs, calmodulin mRNA, and certain oncogene transcripts [34] are not affected), and its occurrence prior to any change in epidermal proliferation or overall cell number (35) suggest a specific effect on cells synthesizing these messages. The rapid decrease in these transcripts is likely to involve both cessation of transcription and an acceleration of mRNA degradation in affected cells. In contrast to its effects on the differentiation-related keratins, TPA causes a substantial increase in transcripts for the proliferation-related M<sub>55,000</sub> keratin, but this is not observed until 12–24 h after treatment, and it persists for 48–72 h. Other changes include an increase in the M<sub>50,000</sub> transcript which parallels the M<sub>55,000</sub> change but is generally smaller and a transient decrease in the M<sub>60,000</sub> transcript with a rapid recovery.

The acute changes in keratin transcripts were similar after exposure to a variety of agents which induce hyperplasia and have some activity as partial or complete promoters in SENCAR mice. Thus, the specificity of these changes for complete promotion remains to be determined. Several incomplete promoters produced effects sufficiently different from those of TPA to be noteworthy. 4-O-methyl-TPA failed to increase the M<sub>55,000</sub> transcript to the level of the other agents. A23187 caused a much prolonged loss of the M<sub>67,000</sub> and 59,000 keratins, and exposure to incomplete promoters never resulted in the elevated steady-state level of the M<sub>67,000</sub> transcript found after 48 h with TPA. Furthermore, TPA was the only agent which specifically increased expression of the M<sub>55,000</sub> transcript to a much greater extent than the M<sub>50,000</sub> transcript.

Several interpretations regarding the acute response to TPA...
and other agents are possible. TPA may directly or indirectly regulate transcription of the keratin genes in specific cells. This would be consistent with previous interpretations from protein synthesis studies (19, 36), in which induction of new synthesis was inferred. Alternatively, these results could be related to promoter-induced changes in relative abundance of epidermal subpopulations, each with its own profile of keratin expression. We have shown that epidermal subpopulations are heterogeneous in their TPA responses (37, 38). Certain cells are induced to differentiate, while others are stimulated to proliferate. Furthermore, we have demonstrated that the response pattern is dependent on the state of epidermal maturation at the time of TPA exposure (39). In vivo, TPA causes a rapid loss of certain basal cells into the suprabasal compartments and an increase in the thickness of the stratum corneum within a few hours (40, 41). At the ultrastructural level, there is disruption of the normal cytoplasmic organization in epidermal cells in the more superficial layers and some fragmentation of the bundles of tonofilaments (35). The rapid loss of transcripts for the M, 67,000 and 59,000 keratins (localized in differentiating cells) could be accounted for by phorbol ester-mediated accelerated maturation of viable suprabasal cells to the fully differentiated state. Since mature squames do not transcribe RNA and are high in nuclease activity, the rapidity of message loss is most consistent with this explanation. The concomitant decrease of the M, 60,000 keratin transcripts suggests that these transcripts may exist in the same cells expressing the M, 59,000 and 67,000 gene or in the subcell of basal cells which undergoes accelerated differentiation. Since the M, 60,000 decrease is only partial, other cells are likely to be making this transcript as well. For example, the M, 60,000 gene may be initially expressed in cells within the basal layer and continue to be expressed in cells that are committed to differentiate or the M, 60,000 mRNA may persist longer in the committed cells. In situ hybridization experiments localizing mRNAs within individual cells within the epidermis, and nuclear run-off transcription experiments on purified cell populations are required to distinguish between these possibilities. The specific increase in M, 55,000 message following TPA exposure could result from an increase in a subpopulation expressing this gene. Specific increases in a dark cell population (35, 42) is a known response to promoter exposures. Alternatively promoters could shorten the cell cycle in the proliferating epidermal cell pool (43), and this could alter keratin gene expression to favor the M, 55,000 gene. Analysis of carcinomas failed to show an enhanced level of M, 55,000 transcripts, which suggests that rapid proliferation alone is not sufficient to favor expression of the M, 55,000 transcript.

Differential cellular responses to TPA as the basis for our results is supported by the studies with fluorocinclone acetoxide. This synthetic glucocorticoid antipromoter is known to be a potent inhibitor of the inflammatory and proliferative responses of keratinocytes, but it only partially inhibits the differentiative responses (44). In the current study FA only partially inhibited the loss of the M, 67,000 and 59,000 transcripts (presumably associated with accelerated differentiation) but completely inhibited the increase in the M, 55,000 message.

Previous reports based on protein studies have suggested that chronic TPA exposure results in keratin profiles similar to late effects of a single exposure, and both are characteristic of the neonatal skin keratin pattern (20, 21). Our studies at the RNA level are consistent with the similarities of transcript levels for the keratin genes at 72 h following acute or chronic exposure. However, the measured values deviate only slightly from relative transcript levels in adult untreated controls, although significant hyperplasia was observed in the treated groups. In newborn epidermis we have observed 6-10-fold higher relative transcript levels for the M, 67,000 and 59,000 keratin subunits from those of adult skin. Thus, at the RNA level there may be important differences between newborn skin and chronic TPA treatment which could be related to message synthesis or degradation.

The keratinocytes present in chemically induced benign skin tumors, papillomas, are characterized by an increased proliferative activity which includes the capacity to divide after leaving the basement membrane (5). However, at the same time they retain the ability to terminally differentiate. Consistent with this maturation competence, near-normal transcript levels for the M, 67,000 and 59,000 keratin subunits were found, whereas an increased transcript level was observed for the M, 55,000 subunit. The selection of a M, 55,000 rich cell type in papillomas is a potential explanation for this finding.

Epidermal squamous cell carcinomas show marked deviations from normal epidermis and papillomas with regard to keratin protein pattern (22, 23). The almost total loss of the differentiation-associated keratins may reflect the loss of a normal differentiation pattern encountered in these cells. It has been reported that carcinomas may contain masked transcripts for the M, 67,000 keratin subunit (23). Our results from analysis of carcinomas induced chemically or derived by implantation of malignant transformed epidermal cells do not support this but indicate that carcinomas contain very low levels of this transcript. Therefore, there appears to be a transcriptional block or a selective rapid degradation of the message precluding the synthesis of the M, 67,000 and 59,000 keratin subunits.

Our results on the differential expression of keratin genes in tumors could be used to discriminate between benign and malignant epidermal tumors. Recently, Klein-Szanto et al. (45) have suggested that loss of differentiation-associated keratins is an early marker of malignant conversion. The use of specific immunological probes for screening keratin changes could provide a relatively easy method to monitor conversion. We have recently produced antisera that are monospecific for the differentiation-associated keratins (M, 59,000 and 67,000) using synthetic peptides corresponding to unique C-terminal sequences of these keratin subunits (13). We selected this approach for antibody production after an examination of amino acid sequence data, which were deduced from the nucleotide sequences of cDNA clones for these keratins, revealed that the carboxyl-terminal sequences were unique. We are currently screening biopsies of tumors, which were produced by protocols which accelerate malignant conversion (2), to determine if the conversion of papillomas to carcinomas can be detected in early stages.

REFERENCES


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