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## Short Communication

# Proto-oncogene expression during two-stage carcinogenesis in mouse skin

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**The level of expression of the cellular homologues to the oncogenes *ras*$^{\mathrm{Ha}}$, *ras*$^{\mathrm{Ki}}$, *fos*, *myc*, *abl* and *raf* was determined in chemically induced tumors, in mouse skin and in mouse epidermis after treatment with a tumor promoter. No significant increase in expression was found for any of the above proto-oncogenes. The expression of *abl* was reduced in both papillomas and carcinomas and after repeated applications of 12-O-tetradecanoylphorbol-13-acetate (TPA). Treatment of primary mouse epidermal cells with TPA *in vitro* failed to induce any marked alterations in proto-oncogene expression. Cell lines derived by treatment of epidermal cells with chemical carcinogens showed expression levels similar to untreated primary epidermal basal cells. These results suggest that increased expression of the proto-oncogenes under study is not required for tumor induction in mouse skin. The lower level of expression of *abl* in tumors and after repeated applications of TPA may indicate a role for this gene in some aspect of epidermal proliferation or differentiation.**

Chemical carcinogenesis in mouse skin can be subdivided into three stages, initiation, promotion and malignant conversion (1,2). Initiation appears to be a rare but essentially irreversible event, whereas repeated applications of promoter are necessary to allow the expansion of initiated cells resulting in the formation of premalignant lesions (papillomas). Only a few of the papillomas will eventually be converted to carcinomas. The precise biochemical mechanisms involved at each stage are not known.

Recently, Balmain and coworkers (3,4) have shown the presence of an activated (transforming) Harvey-*ras* (*ras*$^{\mathrm{Ha}}$) oncogene in many chemically induced mouse skin papillomas and carcinomas. The presence of an active *ras*$^{\mathrm{Ha}}$ in both benign and malignant lesions indicates that activation of *ras* $^{\mathrm{Ha}}$ alone is not sufficient to complete malignant development assuming that *ras*$^{\mathrm{Ha}}$ activation is involved in some phase of carcinogenesis. Similar conclusions were reached in studies with murine sarcoma virus infected mouse epidermal cells (5). Thus it is likely that additional changes are necessary during skin carcinogenesis. A mechanism by which the normal control of cellular proliferation and differentiation might be disrupted is the inappropriate expression of regulatory genes. It is presently thought that many of the known cellular homologues of oncogenes (proto-oncogenes) may represent such regulatory genes (6,7). In the present work we studied the expression of eight proto-oncogenes during different stages of tumor development in mouse skin and after treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) and in cells transformed in culture.

Total RNA was prepared from pools of papillomas induced in female SENCAR mice by initiation with 7,12-dimethylbenz[a]anthracene (DMBA) (20 $\mu$g) followed by promotion with TPA (2 $\mu$g twice weekly) for 12 weeks and from pools of carcinomas derived from papillomas induced as above but subsequently treated with a carcinogen (urethane, 20 mg i.p. once weekly) to accelerate malignant conversion (1). The tumors were excised, ground in liquid nitrogen and extracted in guanidinium thiocyanate buffer (8). Carcinomas were also obtained by subcutaneous implantation of malignantly transformed mouse epidermal cells (cell line PAM 212) (9). Untreated female SENCAR mice (7–9 weeks old) were used as controls. The epidermis was separated from dermis by flotation on 1% trypsin at 37°C for 1 h and total RNA was prepared as described above. The amount of poly A containing RNA was determined by hybridization to [$^3$H]poly U (10). The integrity of each RNA preparation was determined by assaying a portion of the RNA (10 $\mu$g of total) for transcripts of the 60 kd and 67 kd keratin genes by Northern blot analysis (11). The detection of intact transcripts of these genes was a good indication of the stability of RNA from both basal and suprabasal compartments of the epidermis and proliferating and differentiated cells within tumors. The 60 kd keratin gene is predominantly expressed in proliferating

**Table I.** Relative quantitation of proto-oncogene expression in mouse skin papillomas and carcinomas and in mouse epidermis after treatment with the tumor promoter TPA[a].

| Sample | Peak area[b] (% of control) | | | | | |
|---|---|---|---|---|---|---|
| | *ras*$^{\mathrm{Ha}}$ | *ras*$^{\mathrm{Ki}}$ | *fos* | *myc* | *abl* | *raf* |
| Papillomas | 88 | 84 | 46 | 74 | 16 | 56 |
| Carcinomas I[c] | 107 | 57 | 35 | 84 | 18 | n.d. |
| Carcinomas II[c] | 92 | 134 | 139 | 78 | 34 | 107 |
| TPA (5 $\mu$g, once) | | | | | | |
| 12 h | 92 | 95 | 153 | 76 | 63 | 50 |
| 24 h | 79 | 55 | 50 | 133 | 42 | 41 |
| 48 h | 96 | 80 | 66 | 132 | 63 | 66 |
| 72 h | 130 | 50 | 61 | 76 | 77 | 53 |
| 7 day | 126 | 53 | 103 | 86 | 87 | 59 |
| TPA (4 weeks, 2 $\mu$g twice weekly) | 132 | 66 | 48 | 87 | 21 | n.d.[d] |
| DMBA (20 $\mu$g, once) TPA (4 weeks, 2 $\mu$g twice weekly) | 127 | 72 | 27 | 41 | 35 | n.d. |

[a]Total RNA was purified from a pool of papillomas or carcinomas and from mouse epidermis following treatment with TPA. The autoradiograms were scanned densitometrically and the area under each peak calculated. The probes used were: v-*ras*$^{\mathrm{Ha}}$, v-*ras*$^{\mathrm{Ki}}$, c-*myc*, v-*fos*, v-*abl* and v-*raf*.
[b]The data represent the mean of two separate preparations compared to the mean of two separate preparations of RNA from normal mouse skin.
[c]The carcinomas were induced chemically (I) or (II) derived by subcutaneous implantation of malignantly transformed epidermal cells (cell line PAM 212) (9).
[d]n.d. = not done

*Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; DMBA, 7,12-dimethylbenz[a]anthracene.

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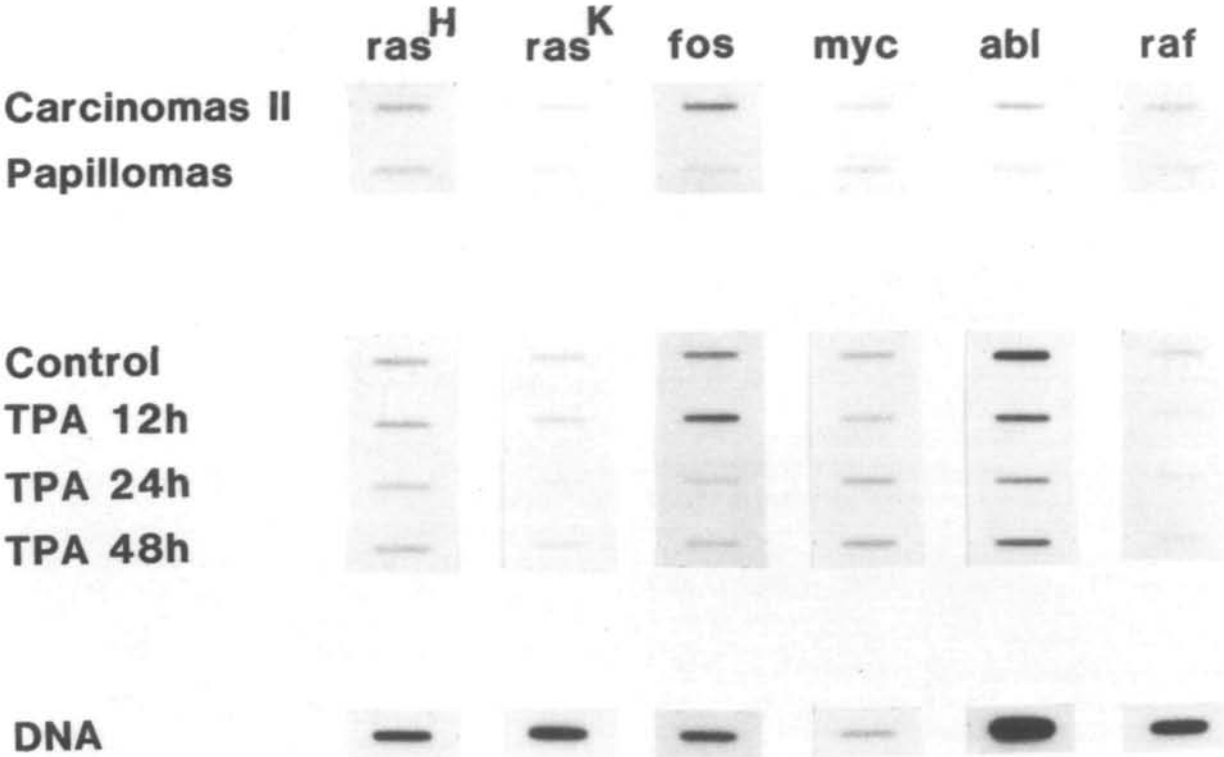


**Fig. 1.** Slot-blot analysis of proto-oncogene expression in carcinomas, papillomas and mouse epidermis after a single exposure to TPA (5 $\mu$g). The papilloma and carcinoma preparations represent pools of tumor tissue. The carcinomas used as a source for RNA were in this case derived by implantation of malignantly transformed epidermal cells (cell line PAM 212) (9). Total RNA was isolated, normalized for Poly A content, and equivalent amounts were loaded on each slot. SENCAR spleen DNA (10 $\mu$g) was applied to each blot as an internal standard.

**Table II.** Relative quantitation of proto-oncogene expression in primary mouse epidermal basal cells treated with TPA or induced to differentiate by $Ca^{2+}$ and in epidermal cell lines[a]

| Sample | | Peak area (% of primary basal cells[b]) | | | | |
|---|---|---|---|---|---|---|
| | | *ras*$^{Ha}$ | *ras* | *fos* | *myc* | *abl* |
| High $Ca^{2+}$ (1.2 mM) | 48 h | 124 | 186 | 101 | 83 | 101 |
| TPA (0.1 $\mu$g/ml)[c] | 24 h | 109 | 81 | 74 | 129 | 66 |
| | 48 h | 87 | 53 | 78 | 112 | 51 |
| | 96 h | 97 | 78 | 156 | 116 | 97 |
| Line 308[d] | | 57 | 87 | 135 | 78 | 75 |
| Line F | | 56 | 41 | 71 | 56 | 63 |
| Line D | | 110 | 84 | 81 | 176 | 95 |

[a]Total RNA was purified from each cell type and autoradiograms of the slot-blots were scanned densitometrically.
[b]Mouse epidermal cells grown under low $Ca^{2+}$-conditions (0.05 mM) are considered to represent basal cells.
[c]TPA exposure was in 0.5 mM $Ca^{2+}$ for 24 h. Medium was changed daily.
[d]Cell lines were derived by carcinogen exposure as described in (21,22) and grown in 1.2 mM $Ca^{2+}$ medium.

basal cells and the 67 kd keratin gene is only expressed in differentiated suprabasal cells (11). In addition, to ensure that transcripts of the appropriate size were detected with each oncogene probe, Northern blot analysis was performed on RNA isolated by the above technique from control epidermis (15 $\mu$g of poly A enriched). Intact transcripts of 1.4 kb (*ras*$^{Ha}$), 2 and 5 kb (*ras*$^{Ki}$), 2.2 kb (*fos*), 2.4 kb (*myc*), 5.0 kb (*abl*) and 3.2 kb (*raf*) were detected. Slot blots were prepared using the Minifold II (Schleicher and Schull, Dassel, FRG). Briefly, equal amounts of poly A containing RNA from each sample were dissolved in 100 $\mu$l $H_2O$ and 300 $\mu$l 6.15 M formaldehyde, 10×SSC (1.5 M NaCl, 0.15 M sodium citrate), incubated at 65°C for 15 min and loaded onto the slot blotter in two-fold dilutions (sample corresponding to 1, 2 and 4 ng of poly A). On each blot, 10 $\mu$g of SENCAR spleen DNA was applied as an internal standard. The nitrocellulose filters were then baked, prehybridized and hybridized to the following $^{32}P$-labeled (nick-translated) probes; v-*ras*$^{Ha}$, *Bgl*I-*Sal*I, 0.4 kb (12), v-*ras*$^{Ki}$, *Hinc*II-*Hinc*II, 1.0 kb (13); c-*myc*, *Cla*I-*Eco*RI, 2.7kb (14); c-*myb*, *Eco*RI-*Eco*RI 1 kb (15); c-*fes*, *Bam*HI-*Bam*HI, 3.8 kb (16); v-*fos*, *Pst*I-*Pvu*II, 1 kb (17), v-*raf*, *Xho*I-*Sst*II, 0.7 kb (18); v-*abl*, *Bgl*II-*Bgl*II, 1.6 kb (19). The autoradiograms were scanned at 615 nm and the peak areas calculated. The concentration of RNA assayed was shown by control experiments to be within a range suitable for linear quantitation. Replicative determinations in the same RNA sample gave values that differed by less than a factor of 2. However, assays performed on RNA preparations that were isolated on different occasions gave values that in some cases differed by slightly more than a factor of 2. Therefore, differences of <2- to 3-fold were considered to be within the range of experimental variation.

In both papillomas and carcinomas the levels of expression of *ras*$^{Ha}$, *ras*$^{Ki}$, *fos*, *myc* and *raf* were similar to the levels found in untreated epidermis (Table I) whereas the expression of *abl* was reduced approximately 5-fold. No expression of *myb* or *fes* could be detected in this or subsequent experiments. To answer the question whether or not alterations of expression of any of these proto-oncogenes might be part of the response of mouse epidermis to tumor promoters, we treated groups of female SENCAR mice (n=10) topically with TPA (5 $\mu$g) and



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isolated RNA after 12, 24, 48, 72 h and 7 days. Again expression levels were found to be comparable to control (Table I and Figure 1). Repeated treatments with TPA (2 μg twice weekly for 4 weeks), according to a schedule used in tumor induction experiments, caused a reduction in the expression of *abl* but did not significantly alter the expression of $ras^{Ha}$, $ras^{Ki}$, *fos* or *myc* (Table I). These results with repetitive TPA were identical in previously untreated skin, or skin exposed to an initiator. Similar results were also observed for skin exposed to weak or incomplete tumor promoters such as 4-O-methyl-TPA, A23187 (a calcium ionophore) and mezerein at concentrations which produce hyperplasia (data not shown). The expression levels of these four proto-oncogenes and *abl* were similarly not altered in primary mouse epidermal basal cells treated with TPA *in vitro* or basal cells induced to terminally differentiate by switching to high $Ca^{2+}$-medium (Table II) (20). Nor were any significant alterations in expression levels detected in three different non-tumorigenic epidermal cell lines which were derived by treatment with chemical carcinogens *in vivo* or *in vitro* (21,22), Table II. We have previously suggested that such cell lines represent initiated cells (21,22).

The present results indicate that increased expression of the proto-oncogenes $ras^{Ha}$, $ras^{Ki}$, *fos, myc, abl, raf, fes* or *myb* is not required for tumor induction in mouse skin. However, we can at present not rule out the possibility that the expression of one or more of these proto-oncogenes is increased in a subpopulation of cells which exist in papillomas and carcinomas or in a small subpopulation of epidermal cells after treatment with TPA. Since tumors were pooled in this study, it is also possible that there is variable expression among individual papillomas or carcinomas. Indeed, varying levels of $ras^{Ha}$ expression have been demonstrated among chemically induced papillomas (4), but the significance for carcinogenesis remains to be determined since an activated $ras^{Ha}$ oncogene was also detected in these papillomas. A second possibility that has not been addressed in the present study is that these genes may have a variable pattern of expression during the cell cycle and this is distrupted during carcinogenesis (23). Expression of *abl* and *fos* have previously not been described in epidermis although the presence of transcripts in whole skin has been reported (24). The level of expression of both genes is developmentally regulated during mouse embryogenesis (24) indicating a possible role in the control of cell differentiation and proliferation. The consistently lower expression level of *abl* in papillomas and in carcinomas and in epidermis repeatedly treated with TPA may implicate a regulatory function in epidermal cell differentiation. Further studies are required to verify this hypothesis and to define the role, if any, of the activation of known oncogenes other than $ras^{Ha}$ in mouse skin carcinogenesis.

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