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Genetics

v-Ha-*ras* transgene abrogates the initiation step in mouse skin tumorigenesis: Effects of phorbol esters and retinoic acid

(cancer/oncogene/epidermis/promotion/progression)

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ABSTRACT **Experimental carcinogenesis has led to a concept that defines two discrete stages in the development of skin tumors: (*i*) initiation, which is accomplished by using a mutagen that presumably activates a protooncogene, and (*ii*) promotion, which is a reversible process brought about most commonly by repeated application of phorbol esters. We have created a transgenic mouse strain that carries the activated v-Ha-*ras* oncogene fused to the promoter of the mouse embryonic α-like, ζ-globin gene. Unexpectedly, these animals developed papillomas at areas of epidermal abrasion and, because abrasion can also serve as a tumor-promoting event in mutagen-treated mouse skin, we tested these mice for their ability to respond to phorbol ester application. Within 6 weeks virtually all treated carrier mice had developed multiple papillomas, some of which went on to develop squamous cell carcinomas and, more frequently, underlying sarcomas. We conclude that the oncogene "preinitiates" carrier mice, replacing the initiation/mutagenesis step and immediately sensitizing them to the action of tumor promoters. In addition, treatment of the mice with retinoic acid dramatically delays, reduces, and often completely inhibits the appearance of promoter-induced papillomas. This strain has use in screening tumor promoters and for assessing antitumor and antiproliferative agents.**

The two-stage mouse epidermal model for tumorigenesis provides compelling evidence that some tumors can pass through several stages of increasing virulence before emerging as a lethal malignancy (1–5). In this system, tumor development depends upon two discrete steps, initiation and promotion, which differ from one another in very fundamental ways. Initiation, generally induced by a potent mutagen, is an irreversible process that constitutes the first step. This step can be followed days, weeks, or even months later by a reversible step involving multiple applications of one of several tumor-promoting compounds, most commonly the ester phorbol 12-myristate 13-acetate (PMA). This treatment, delivered over several weeks, finally leads to the formation of papillomas, some of which progress to form carcinomas. Interestingly, abrasion or mechanical disruption of the skin can also promote papilloma and carcinoma formation in mutagen-treated mice, presumably by inducing regenerative epidermal hyperplasia.

The initiation step is generally held to create cryptic carcinogenic mutations in epidermal stem cells that remain dormant pending some epigenetic intervention, called promotion, which induces papilloma formation. Initiation is highly correlated with activation of the c-Ha-*ras* oncogene; for example, v-Ha-*ras*-bearing retroviruses can serve in place of mutagens as initiators (6–9). The role of the promoter is less well understood, but the activating effect of PMA on protein kinase C (10–12) suggests that promotion might operate through this kinase, which, among other actions, induces transcription of an array of genes, including the protooncogenes c-*myc*, c-*fos*, and c-*sis* (13–18). In this regard, it is noteworthy that v-*fos* and v-*ras* can cooperate to convert normal keratinocytes to squamous cell carcinoma (19). Given that promotion can be accomplished by using nonphorbol ester irritants as well as by wound healing, more complicated models, including effects on surrounding cells (20), cannot be ruled out.

Because the c-Ha-*ras* protooncogene seems a major target in the papilloma system, it should be possible to create a transgenic mouse in which the first step of this two-step process—namely, activation of the Ha-*ras* gene, has already been accomplished. Such a gene, inherited through the germ line and expressed in appropriate cells, should constitute a promoter-sensitive system, no longer requiring initiators. Furthermore, this system should also allow us to focus on the role that promotion plays in tumorigenesis and progression.

In the course of studies designed to understand development of the embryonic hematopoietic system, we created a series of transgenic mice that carry a fusion gene consisting of the promoter of the embryonic α-like, ζ-globin gene driving the activated v-Ha-*ras* oncogene. We noted that mice of one of these strains, TG.AC, developed papillomas at areas of skin abrasion, and this prompted us to test the possibility that this line would respond directly to tumor promoters. Indeed, >90% of mice bearing the v-Ha-*ras* transgene developed papillomas within 6 weeks of starting PMA application. This line, described below, provides direct evidence for the involvement of the mutant v-Ha-*ras* oncogene as an initiator in the two-stage epidermal tumorigenesis system. It also provides a powerful and convenient model for assaying potential tumor promoters and for testing potential antitumor and antiproliferative agents that might act on the c-Ha-*ras* or protein kinase C pathways.

MATERIALS AND METHODS

Plasmid Constructs. The transgene was formed from two components. (*i*) The 0.95-kilobase (kb) DNA segment located just 5′ to the initiating ATG of the ζ-globin gene (21) was obtained by Bal-31 deletion from the *Xba* I site within the first ζ-globin exon. This fragment was cloned into pGEM-3. (*ii*) A 2.5-kb *Bam*HI fragment was obtained from a plasmid construct (made by B. Velan) that carried the v-Ha-*ras* coding sequence followed by a simian virus 40 (SV40) splice/polyadenylylation signal. This fragment was cloned into the *Bam*HI site in the polylinker of the plasmid described above. The v-Ha-*ras* sequence used contains activating mutations in codons 12 (Gly → Arg) and 59 (Ala → Thr) and was subcloned from plasmid pA-9 (22). In addition to the v-Ha-*ras* coding region, pA-9 contains 5′ and 3′ untranslated sequences. An

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Abbreviations: PMA, phorbol 12-myristate 13-acetate; SV40, simian virus 40.

additional plasmid, pASV, consisting of the pGEM-4 vector into which an 0.8-kb DNA segment encoding the SV40 splice/adenylylation signals was cloned, provided both an antisense RNA for RNase protection assays (see below) and a DNA probe to detect transgenic mice.

Preparation of Primary Keratinocytes. Inbred FVB mice were sacrificed by using CO_2 narcosis; back fur was closely shaved; and skin was dissected, removed, and cut into 1-cm^2 patches. After being washed in phosphate-buffered saline, the skin patches were placed fur-side-down in a Petri dish and scraped with a scalpel to remove as much of the underlying tissue as possible. The scraped patches were placed in fresh Petri dishes containing Dispase II (25 mg/ml) (Sigma) in Dulbecco's modified Eagle's medium (no serum) and incubated for 90 min at 37°C at which point the epidermis was carefully peeled off as a thin, transparent sheet and incubated at 37°C for 45 min in phosphate-buffered saline/0.25% trypsin (Sigma)/0.02% EDTA to produce a single-cell suspension of keratinocytes. The cells were carefully decanted, avoiding pieces of tissue; spun down; and washed twice with phosphate-buffered saline.

Transgenic Mice. The DNA fragment used for microinjection into mouse ova was isolated from plasmid sequences by digestion with the restriction endonuclease *Eco*RI, subsequent agarose gel electrophoresis, and electroelution of the excised band (see Fig. 1*A*). Transgenic mice were generated in inbred FVB mice (Taconic Farms) by standard techniques (23). Founders and carriers were identified by hybridizing tail-biopsy DNA with the pASV probe as described below.

Skin Treatments. Five micrograms of PMA (Sigma) dissolved in 100 μl of acetone was applied with a micropipet to the shaven lower back of treated mice. Treatments were generally three times per week for 4–6 weeks. Five micrograms of retinoic acid dissolved in 50 μl of dimethyl sulfoxide was applied as above. When mice were treated with both reagents, the PMA was always applied first. Control mice were treated with solvents alone.

RNA Analysis. RNA levels were analyzed in total RNA samples prepared from tissue treated according to Chirgwin *et al.* (24) by using the CsCl-sedimentation gradient modification. RNA yield was determined after resuspension in sterile H_2O by UV absorption at 260 nM. A radiolabeled, antisense RNA probe (pGEM-4 vector) was synthesized by using SP6 RNA polymerase to transcribe an ≈200-base pair (bp) sequence in the *Dra* I-linearized pAS4 plasmid. The probe covered the polyadenylylation region of the SV40 sequence used to construct the transgene. An ≈120-bp sequence is protected by the 3′ end of the transgene mRNA (see Fig. 1*B*). RNase protection assays were done as described by Melton *et al.* (25) by using 10–20 μg of total RNA per assay.

Pathology. The animals were inspected for tumors at least twice each week. Complete autopsies were performed, and both gross and microscopic evaluations were done. Tissues were fixed in Omnifix or formalin, dehydrated, blocked in paraffin, and sectioned at 10 μm on an AO microtome. The tissue sections were mounted on glass slides and were stained with hematoxylin/eosin. The tissues were photographed at ×63 and ×100 on a Zeiss Photoscope with Technical Pan (Kodak) film at ASA 50 and developed for moderate contrast.

RESULTS AND DISCUSSION

Design of the Transgene and Its Expression in Carrier Strains. To explore the regulation of genes expressed early in embryonic development, we fused a transgene consisting of a 0.95-kb DNA segment immediately 5′ of the initiation codon of the mouse embryonic ζ-globin gene (21) to the v-Ha-*ras* oncogene and introduced the fused gene into fertilized 12-hr embryos derived from inbred FVB mice (Fig. 1*A*). The mouse ζ-globin gene is expressed in primitive,

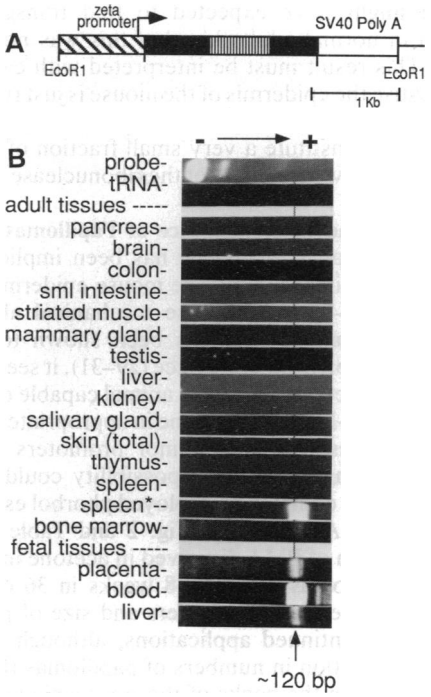

FIG. 1. Structure and expression of a fusion transgene carrying the ζ-globin promoter driving v-Ha-*ras*. (*A*) Diagrammatic representation of the transgene carrying the ζ-globin promoter region fused 5′ to the coding sequences of v-Ha-*ras*. The diagonal striped box represents a 0.95-kb DNA segment encoding the 5′ region of mouse embryonic ζ-globin gene, including the site of transcription initiation (arrow). The filled box with vertical stripes is the v-Ha-*ras* gene, the solid region is untranslated flanking sequences, whereas the open box is an SV40 sequence carrying splice/polyadenylylation sites. The fragment was cut with *Eco*RI and gel isolated before nuclear injection. (*B*) Expression of the v-Ha-*ras* transgene in various tissues of embryonic and adult transgenic mice. Transgene expression is shown as the result of an RNase protection assay with an ≈200-bp radiolabeled, antisense probe derived from the 3′ end of the SV40 segment of the transgene (see *A*). Expected size of the 3′ end of the transgene mRNA is ≈120 bp. The asterisk next to the second spleen sample indicates that this spleen was enlarged with foci of lymphoproliferation as discussed in text.

nucleated erythrocyte precursors that appear in the blood islands of the yolk sac of mouse embryos at day 7 of gestation and disappear from the circulation a few days before birth (26, 27). The dimensions of the promoter are not known, and a goal of these transgenic experiments was to define this putative promoter region. Another goal, in view of the lack of embryonic erythroid cell lines, was to derive such lines from tumors induced by the activated v-Ha-*ras* oncogene, which has been shown to induce tumors in various tissues of several lines of transgenic mice (28).

Four lines of transgenic mice carrying 3–10 copies of the fusion gene were made. As measured by RNase protection of an antisense probe, three of these showed no expression, whereas the fourth, TG.AC, expressed the transgene in day-12 embryonic blood, the hematopoietic fetal liver, and placenta, in addition to expressing low amounts in the adult bone marrow (Fig. 1*B*). The TG.AC line also displayed an unusual and unexpected phenotype that prompted us to investigate it further. When male TG.AC littermates were caged together, they would occasionally fight and, at the site of skin abrasions, develop squamous papillomas. Young female mice do not generally fight and fail to develop papillomas, although older male and female animals do go on to develop papillomas at the site of chronic skin abrasions, such as the genitalia, the eye lids, nares, and mouth. In view of this

result, we might have expected to find transgene RNA expression in normal adult skin, but this was not the case (Fig. 1*B*). This result must be interpreted with caution (see below) because the epidermis of the mouse is just two to three cell layers in thickness, and target cells expressing the transgene may constitute a very small fraction of the tissue assayed, well below sensitivity of the ribonuclease protection assay.

Promoter-Dependent Appearance of Papillomas. Because the activated c-Ha-*ras* oncogene has been implicated as a potential initiator in the two-stage mouse epidermal tumorigenesis model (6–9) and because mechanical abrasion of mutagen-treated mouse skin has been shown to promote papilloma formation in initiated mice (29–31), it seemed likely that we had produced a transgenic animal capable of expressing the activated v-Ha-*ras* transgene in appropriate epidermal stem cells and responsive to tumor promoters without a mutagenic initiating event. This possibility could easily be tested by using the commonly employed phorbol ester, PMA, as the promoter. As shown in Fig. 2 and Table 1, thrice-weekly application of PMA dissolved in acetone induced the eruption of papillomas within 6–8 weeks in 36 of 37 mice bearing the transgene. The numbers and size of papillomas increased with continued applications, although there was considerable variation in numbers of papillomas (from 10 to >100) occurring on the backs of these isogenic and equivalently treated mice. A small proportion (<3%) remained free of papillomas. None of seven nontransgenic treated mice developed papillomas, and two of eight acetone-treated transgenic controls developed just two solitary papillomas, possibly as a consequence of skin shaving or fighting. Three of the five untreated, transgenic males originally caged together developed papillomas as their abrasions began to heal (Table 1). All had developed papillomas by 8–9 weeks after they were separated from one another.

Progressive changes in the skin were observed as a function of PMA application. After 1 week of PMA treatment the epidermis of PMA-treated mice was 5–10 cells thick, whereas the untreated epidermis is only two to three cells thick (Fig. 3 *A* and *B*). In this respect, there was no difference between wild-type and transgenic animals. By the fourth week of treatment, the transgenic skin differed from wild type in that some of the PMA-treated group had focal, slightly raised, flaky skin lesions. By the fifth week, many of the PMA-treated transgenic mice had raised focal nodules on treated areas that progressed to papillomas. By 6 weeks, the epidermal hyperplasia and hyperkeratosis remained (Figs. 3*F* and 4*A*), and gross differences between treated wild-type and transgenic skin emerged. The transgenics had raised, focal regions with nodular to papillary, acanthotic proliferations of the epidermis and accentuated hyperkeratosis. These papillomas also expressed large amounts of transgenic mRNA as measured by RNase protection (Fig. 5). The flatter, nodular lesions had proliferations of epidermal cells that pushed into the underlying dermis as inverted papillomas or keratoacanthomas. Significantly, the PMA-treated wild-type skin continued to be hyperplastic and hyperkeratotic but did not have the focal areas of proliferative atypia (Fig. 3*E*).

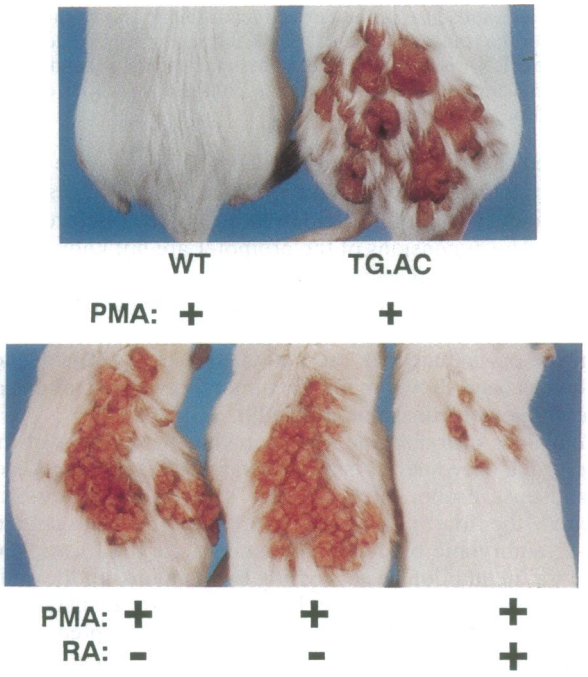

FIG. 2. Papillomas occurring in transgenic mice treated with PMA and PMA with retinoic acid. (*Upper*) Photographs of wild-type (WT) and transgenic (TG.AC) mice both treated with thrice-weekly application of PMA for 4 weeks. (*Lower*) Photographs of transgenic mice treated thrice weekly with PMA or PMA and retinoic acid (RA) according to the protocol indicated.

Table 1. Occurrence of papillomas and malignant skin tumors in transgenic mice treated with combinations of PMA and retinoic acid

Treatment	Strain	Papilloma occurrence, no/*n*: Time after beginning of treatment, weeks 5–6	8–9	10–14	Malignant skin tumor
None	WT	0/7	0/7	0/7	0
None	TG.AC	0/7	0/7	0/7	0
PMA	WT	0/7	0/7	0/7	0
PMA	TG.AC	36/37	36/37	**36(27)**/37	12/37 at 8–12 mo
RA	WT	0/2	0/2	0/2	0
RA	TG.AC	0/5	0/5	0/5	0
PMA + RA	WT	0/4	0/4	0/4	0
PMA + RA	TG.AC	0/12	3/12	5/12	3/12 at 8–15 mo
Abrasion	WT	0/3	0/3	0/3	0/3
Abrasion	TG.AC	3/5	**5**/5	**5**/5	5/5 at 9 mo

Boldface numbers indicate the number of animals having >20 papillomas each. RA, retinoic acid; WT, wild type.

As noted above, it was puzzling that these papillomas arose in the *apparent* absence of expression of the v-Ha-*ras* transgene in the skin of susceptible transgenic animals (see Figs. 1 and 5). In view of the fact that the skin, particularly as sampled for these studies, carried only the thickness of a few epidermal cells (see Fig. 3 *A* and *B*) and a large mass of underlying dermis containing fat and connective tissue, it would be possible to miss a transcript expressed in a tiny proportion of the cells sampled. Accordingly, we assayed cells that were relatively enriched for epidermal cells and keratinocytes. Fig. 5 shows that untreated transgenic skin exhibits no protected antisense sequences. By contrast, skin treated with PMA, which induces epidermal hyperplasia (see Fig. 3*F*) and thus increases the number of epidermal cells 3- to 5-fold, shows clear, albeit low-level expression of the transgene (Fig. 5). Because the transgenic mRNA was possibly induced directly by PMA treatment rather than by epidermal hyperplasia, isolated keratinocytes derived from PMA-treated and untreated transgenic skin were assayed for transgene expression. As shown in Fig. 5, both keratinocyte preparations express the transgene at about the same level, indicating that constitutive expression of the transgene occurs in the absence of phorbol ester treatment. The apparent absence of expression in untreated adult skin (Fig. 1*B*) is thus probably a reflection of the relatively small number of target cells and the sensitivity of the assay.

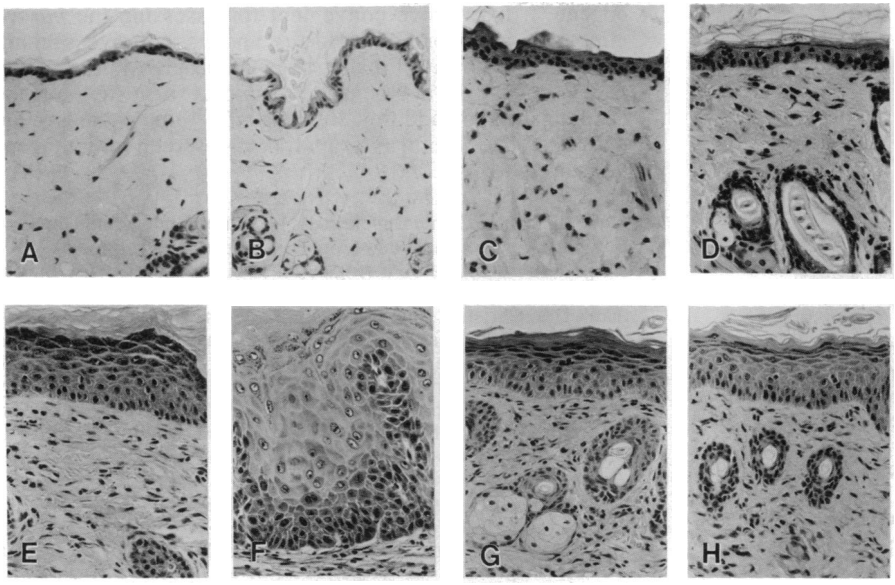

FIG. 3. Histopathology of skin from wild-type (FVB) and transgenic mice treated with various combinations of PMA and retinoic acid. Photomicrographs of skin from matched pairs of wild-type (*A*, *C*, *E*, and *G*) and transgenic (*B*, *D*, *F*, and *H*) mice illustrate untreated controls (*A* and *B*) and skin treated with retinoic acid (*C* and *D*), PMA (*E* and *F*), or PMA and retinoic acid (*G* and *H*). Treatment was three times weekly for 6 weeks in accordance with described protocols. These micrographs permit comparison of the relative thickness of the epidermis and the number of inflammatory cells in the dermis with each treatment in transgenic mice matched with a wild-type control. The most striking differences are observed by comparing hyperplasia in the PMA-treated wild-type skin (*E*) with the transgenic skin (*F*). The focus in the PMA-treated transgenic epidermis is thicker, and the cells have an atypical, vesicular chromatin pattern (×140).

Occurrence of Malignant Skin Tumors. After a 4- to 6-week course of PMA treatment, the animals were observed at weekly intervals for over a year. In the first weeks after treatment terminated, the papillomas continued to increase in size and numbers, but in the ensuing months, many of the papillomas desiccated, some sloughed off and, in a few cases, the skin returned to a normal appearance. However, in many mice an occasional papilloma or flat raised lesion developed into a large ulcerated, disc-like tumor associated with metastases to local lymph nodes. Twelve of the 37 animals treated with PMA in the transgenic group developed malignant skin tumors within 6–12 mo of initiating PMA treatment (see Table 1). Because not all mice reached 12 mo posttreatment, it is likely, given more time, that additional mice would have developed malignancies. Five of the five transgenic males originally caged together and thus subject to abrasions developed malignant tumors by 9 mo of age. Interestingly, there is a poor correlation between number of papillomas a mouse develops and occurrence of malignant skin tumors.

Some of the epidermal lesions progressed to frankly invasive squamous cell carcinomas with keratin pearls, dyskeratosis, and increasing cellular atypia (Fig. 4*D*). These tumors infiltrated deeply into the underlying dermis. In a significant number of cases, metastases of malignant squamous cells were found in regional lymph nodes, indicating the malignant potential of the epidermal lesions. The larger, more rapidly growing lesions, most typically associated with surface ulceration, proved to be composed of poorly differentiated spindle cells that grew in interlacing bundles and had a very high mitotic rate (Fig. 4 *B* and *C*). These tumors were associated with a generous infiltrate of granulocytes and, in particular, eosinophils. These tumors also frequently metastasized to regional lymph nodes.

Some mice with papillomas had disc-shaped soft tissue masses under the papillomas (Fig. 4*B*). In most cases, afflicted mice had more than one of these disc-shaped masses, which proved to be early spindle cell tumors (Fig. 4 *B* and *C*). When these combinations of papillomas with underlying spindle cells were transplanted into nude mice, the resulting tumor turned out to be composed of spindle and not squamous cells. These spindle cell sarcomas, as with the squamous papillomas, express large amounts of the v-Ha-*ras* transgene (Fig. 5). The tumors could be further distinguished because papillomas expressed keratin 1, 10, 6, and 16 mRNAs [data not shown (32)], whereas the sarcomas did not. A number of transgenic mice also showed mild to severe splenomegaly. This result was true of all mice that developed skin malignancies. These spleens contained lymphoproliferative lesions and, while they are poorly understood, these lesions may be responsible for the transgene expression (Fig. 1*B*) in enlarged spleens of adult animals.

Anomalous Expression of the Transgene and the Occurrence of Nonskin Tumors. Expression of a transgene in epidermal cells seems anomalous, given that the ζ-globin gene is normally expressed in embryonic erythrocyte precursors (26, 27). Indeed, the specific properties of the TG.AC transgene may be a consequence of the specific site of the transgene and/or manner of genomic integration. Although the version of the ζ-globin promoter used in these studies is probably incomplete, its expression in fetal blood, placenta, and liver (Fig. 1*B*) suggests that it does retain an element of tissue specificity. Even low-level expression in adult bone marrow (Fig. 1*B*) fits the range of hematopoietic cells of mesodermal origin in which this promoter might be active. Given both the anomalous and expected range of tissue expression and the oncogenic nature of the v-Ha-*ras* oncogene, it might be expected that nonskin tumors would arise in the transgenic

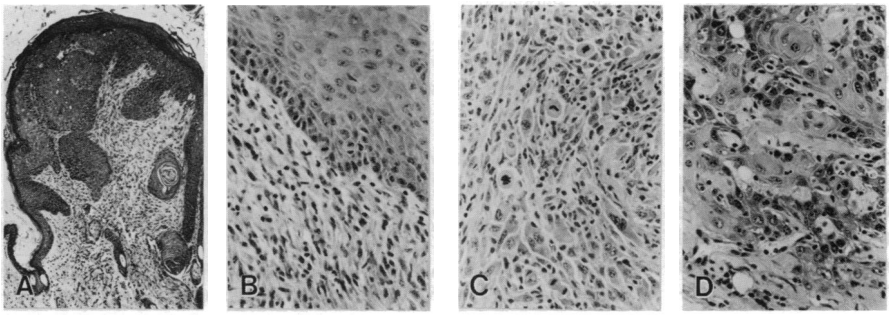

FIG. 4. Benign and malignant skin tumors in transgenic mice. (*A*) Benign squamous papilloma with inflammatory infiltrate in underlying dermis that arose after 6 weeks of PMA treatment (×90). (*B*) Higher magnification of similar lesion, showing the junction between an epidermal papilloma and a spindle cell neoplasm in dermis (×140). (*C*) Cytological features of a sarcoma that can be compared with invasive squamous cell carcinoma (*D*), both from PMA-treated transgenic mice (×140).

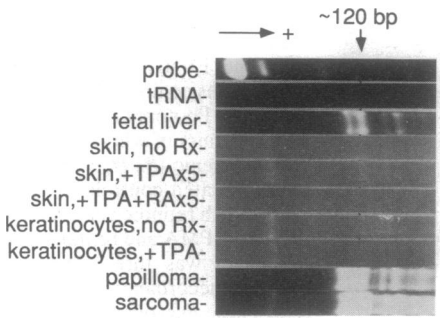

FIG. 5. Expression of transgene mRNA in skin, keratinocytes, and tumors of transgenic mice. Transgene mRNA expression is shown as an ≈120-bp RNase protected, radiolabeled antisense probe derived from the SV40 3′ region of the transgene (see Fig. 1*A*). The larger band represents full-length transcript. Various tissues and treatments are indicated (no Rx, no treatment; x5, five treatments).

TG.AC line. Indeed, treated and untreated older transgenic mice of this strain do develop other malignancies that will be considered in detail elsewhere.

Retinoic Acid Inhibits the Development of Papillomas in PMA-Treated Transgenic Mice. The papillomas that arise in these transgenic mice in response to tumor promotion indicate that the activated v-Ha-*ras* transgene is sufficient as the initiating step in this process. These genetically preinitiated mice provide a model system in which to examine the isolated effects of tumor promoters and agents that interfere with their action. The retinoids represent a class of compounds shown to block phorbol ester-induced tumor promotion in the mutagenized mouse epidermis (for reviews, see refs. 33, 34). Although their mechanism of action is poorly understood, the retinoids have been suggested to block promotion through their ability to modulate squamous differentiation in keratinocytes (33). Indeed, mutagenized mouse skin has been used for many years to identify vitamin A derivatives with antikeratinizing properties (35). More recent evidence (for review, see ref. 15), pointing to the role of retinoic acid and its family of receptors in embryonic morphogenesis and transcription regulation, underscores the potent biologic effects of this family of agents.

If the transgenic mouse is relevant to the mouse epidermal initiation/promotion system, it should also be protected from PMA-induced tumor promotion by retinoic acid applications. As shown in Fig. 2 and Table 1, concurrent applications of retinoic acid (dissolved in dimethyl sulfoxide) substantially delayed and reduced the numbers or entirely prevented the occurrence of papillomas in transgenic mice treated with PMA. At the concentration used, retinoic acid by itself (see Table 1) does not induce papillomas, and treatment with dimethyl sulfoxide alone does not block papilloma formation (data not shown). Further, retinoic acid ameliorates the epidermal hyperplasia seen in response to PMA in both transgenic and wild-type mice (Fig. 3 *D* and *H*), consistent with the reduced amount of transgene mRNA seen in the skin of mice treated with both PMA and retinoic acid (Fig. 5). Interestingly, retinoic acid treatment does not seem to suppress the occurrence of malignant skin tumors (Table 1).

A Mouse Epidermal Tumorigenesis Model. It would appear that the TG.AC transgenic mice offer a convenient model in which to screen for tumor promoters and agents that block the effect of tumor promoters. Moreover, because the response to phorbol esters is consistent and relatively rapid and because these compounds act through the activation of protein kinase C, this system may be useful in assessing the action of compounds that block the action of this kinase and its second-messenger pathway. Additionally, because the system depends on the presence of activated v-Ha-*ras* oncogene, it may prove convenient for assessing the *in vivo* effects of inhibitors of v-Ha-*ras* and its putative second-messenger pathway. Finally, because progression to malignancy occurs in a stochastic fashion, suggestive of the accumulation of additional mutagenic "hits," the system may also prove useful as a rapid, *in vivo* screen for mutagens and carcinogens.

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