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Oncogene activation in human benign tumors of the skin (keratoacanthomas): Is *HRAS* involved in differentiation as well as proliferation?

(squamous cell carcinoma/DNA polymerase chain reaction amplification/direct DNA sequencing)

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ABSTRACT **_In vitro_ DNA amplification followed by oligonucleotide mismatch hybridization was used to study the frequency of *HRAS* mutations in the benign self-regressing skin tumors keratoacanthomas and in squamous cell carcinomas. We used freshly obtained keratoacanthomas as well as Formalin-fixed paraffin-embedded tissues from both types of tumors. DNA from 50 samples of each tumor type was analyzed for activating mutations involving codons 12 and 61. A relatively high percentage (30%) of *HRAS* mutations was found in the keratoacanthomas compared with 13% in the squamous cell carcinomas. The most frequent mutation identified is the A·T-to-T·A transversion in the second position of codon 61. The present findings demonstrate the involvement of the *HRAS* oncogene in human benign tumors. Moreover, they indicate that an activated *HRAS* oncogene is not sufficient to maintain a neoplastic phenotype and argue against a role of *HRAS* in the progression of skin tumorigenesis.**

Oncogenes are altered cellular genes implicated in carcinogenesis by disrupting the control of cell proliferation, differentiation, or both. *ras* genes (H-, K-, and N-*ras*) code for similar proteins of molecular weight 21,000 whose oncogenic potential can be elicited by point mutations resulting in single amino acid substitutions (1). Most studies involving *ras* oncogenes have searched for them in a large variety of malignancies in human and animal model systems. In humans, *ras* genes (*RAS* in human gene nomenclature) are found activated in 10–15% of the malignant tumors analyzed (2). However, the frequency of *ras* gene activation can differ according to tumor type. In colon carcinoma, the frequency of *KRAS* mutations is 40% (3, 4). More recently, by use of the DNA polymerase chain reaction (PCR) method for DNA amplification, over 90% of pancreatic carcinomas analyzed were found to contain activated *KRAS* genes (5, 6).

In animal model systems, Balmain *et al.* (7) have shown that some papillomas induced in mouse skin with the dimethylbenzanthracene (DMBA) initiation and phorbol 12-tetradecanoate 13-acetate promotion protocol contained activated H-*ras* genes. We have also reported H-*ras* activation in keratoacanthomas (KAs) induced in rabbit ears by treatment with DMBA (8). The H-*ras* gene is the member of the family that has been found to be activated most frequently in animal tumors of epidermal origin (9, 10).

The appearance of premalignant lesions, some of which progress to malignancy, is an important characteristic of the development of many tumors, and activated oncogenes have been recently reported in colonic tumors in different stages of progression (3, 4, 11). Although we have previously shown *HRAS* activation in one human KA (8), oncogene activation in clearly benign human tumors has not been well documented.

KAs are benign skin tumors that undergo complete spontaneous regression which is apparently unrelated to an immunological reaction (12). In general, KAs do not evolve into squamous cell carcinomas (SCCs) but, to some extent, KAs resemble SCCs histologically in their initial proliferative phase, which is associated with keratinocytic atypia. However, a KA differs from an SCC in that after several weeks the initial proliferative phase stops and the keratinocytes undergo complete keratinization, with formation of a large keratinous plug that is later sloughed off.

In an attempt to understand the specific role of *ras* activation in the early stages of tumorigenesis we have studied the frequency of *HRAS* mutations in these two types of tumors with clearly different biological behaviors.

MATERIALS AND METHODS

Tumor Samples. Formalin-fixed and paraffin-embedded samples of 50 KAs and 50 SCCs from the last 2 years, as well as 12 samples of fresh KA tissue, were obtained from the Dermatopathology Section and the Department of Dermatology, New York University Medical Center. Tissue sections (6 μm) from each sample were stained with hematoxylin/eosin and examined.

DNA Extraction and Tumorigenicity Assay. High molecular weight DNAs from fresh tumors were isolated and transfected into NIH 3T3 mouse cells, and these were injected into *nude* mice as described (8).

PCR-Mediated Amplification. (*i*) From fresh tumor DNA: *HRAS* sequences spanning 110 base pairs (bp) across codon 12 and 219 bp across codon 61 were amplified *in vitro*, using the oligonucleotide primers shown in Fig. 1. One microgram of DNA and 20 pmol of each of the two primers were added to a 50-μl reaction mixture containing 1 unit of *Thermus aquaticus* DNA polymerase (*Taq* polymerase) as described (13). Thirty cycles of denaturation (94°C, 15 sec), annealing (55°C, 1 min), and extension (72°C, 1 min) were done with an automated DNA thermal cycler (Perkin–Elmer/Cetus).

(*ii*) From paraffin-embedded tissue: DNA amplification from Formalin-fixed and paraffin-embedded tissue was done essentially as described by Shibata *et al.* (14). Serial sections (6 μm) were cut from paraffin blocks of tumors. Normal skin obtained from plastic surgery patients was used as a control. Samples were deparaffinated with xylene and the tissue pellets were desiccated under reduced pressure and heated to

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Abbreviations: PCR, polymerase chain reaction; KA, keratoacanthoma; SCC, squamous cell carcinoma; DMBA, dimethylbenzanthracene.

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Oligonucleotide primers

	5′ primer (sense)	3′ primer (antisense)
H-*ras*-12	CTGAGGAGCGATGACGGAAT-72 bp	AGTGGGGTCGTATTCGTCCA
H-*ras*-61	TGAGCCCTGTCCTCCTGCAG-179 bp	GCCAGCCTCACGGGGTTCAC

Probes

H-*ras*-12 GTGGGCGCCGGCGGTGTGG normal

AGC
CGC
TGC

GAC
GCC
GTC

H-*ras*-61 CCGCCGGCCAGGAGGAGTA normal

AAG
TAG
GAG

CCG
CGG
CTG

CAY (Y = C and T)

FIG. 1. Sequences of synthetic oligonucleotide primers and probes used.

100°C for 10 min in 50 μl of H_2O. After addition of 50 μl of a mixture containing buffer, dNTPs, 50 pmol of each primer, and 1 unit of *Taq* polymerase, the samples were cycled as described above, except that 40 cycles were used instead of 30.

Oligonucleotide Hybridization. Amplified DNA was applied to a nylon filter (ICN) by using a slot-blot minifold II (Schleicher & Schuell) and hybridized to a panel of 20-mer synthetic oligonucleotide probes (Fig. 1) synthesized with an Applied Biosystems synthesizer and labeled with [^{32}P]ATP and T4 polynucleotide kinase (15). Prehybridization, hybridization, and washing of filters with 3 M tetramethylammonium chloride solution were as described (16).

DNA Sequencing. Direct sequencing of amplified DNA fragments was done according to Higuchi *et al.* (17). The DNA fragment was isolated from a 4% Nusieve agarose gel (FMC) and resuspended in 10 mM Tris·HCl/1 mM EDTA (pH 7.5) (TE) buffer. In 12 μl of TE buffer, approximately 0.5 pmol of PCR product was mixed with 1 pmol of one of the same primers used in the amplification, previously end labeled with [^{32}P]ATP and T4 polynucleotide kinase. The primer–template mixture was heated at 95°C for 5 min and immediately placed on ice. After 2 min of incubation at 55°C, 2.8 μl was added to 3.15 μl of each of four termination mixes, and these solutions were incubated at 50°C for 5 min with 2 units of Sequenase (United States Biochemical) (17). The samples were then electrophoresed on a 6% polyacrylamide/7 M urea gel and autoradiographed.

RESULTS

Correlation Between *HRAS* Mutations and Tumorigenicity in KAs. In the present study we first tested for the presence of *HRAS* mutations in 12 human KAs (designated HK1 to HK12). DNAs from these tumors were transfected into NIH 3T3 mouse cells, and the transfected cells were injected into *nude* mice. Cells transfected with the DNA of two KAs, HK3 and HK9, gave rise to tumors in *nude* mice. Southern blot analysis of the DNAs of the transformants revealed the presence of human repetitive *Alu* sequences as well as extra bands corresponding to human *HRAS* sequences (18, 19) (data not shown).

To search for mutations that might explain this transforming activity, we then analyzed the same tumors by PCR–oligonucleotide hybridization. The first and second exons of the *HRAS* gene were amplified *in vitro* and 1/10th of the amplification reaction mixture from each tumor DNA as well as normal control DNAs was slot-blotted onto a nylon membrane and hybridized with a mixture of oligonucleotides. These probes were specific for any mutation occurring in either the 12th or the 61st codon of *HRAS* (Fig. 1). The HK3 tumor was positive for a mutation in the second position of codon 61 and the HK9 was positive for a mutation in the first position of codon 12. To establish the exact nature of the mutations, the nucleotide sequence was determined in these two tumors by direct sequence analysis of the PCR products. As shown in Fig. 2 the HK3 tumor had an A-to-T transversion in codon 61, as expected from analysis of transformants (8). The mutation identified in the tumor HK9 was a G-to-A transition in the first position of codon 12. Taken together, these results show a perfect correlation between *RAS* mutations identified after PCR amplification and those detected by gene transfer assay.

Frequent *HRAS* Activation in Human Benign Tumors (KAs). To determine the frequency of *HRAS* activated oncogenes in KAs, we examined a larger number of samples by using PCR and oligonucleotide mismatch hybridization. This extended analysis required the use of a substantial number of samples available only in the form of Formalin-fixed paraffin-embedded tissue. By PCR amplification this material can be screened for the presence of viral sequences (14) or *RAS* mutations (5).

The first and second exons of the *HRAS* gene were amplified *in vitro* from 50 samples of KAs. Amplification from Formalin-fixed and paraffin-embedded tissues is uneven, but the amount of amplified DNA can be monitored by measuring the signal produced when the slot blot is hybridized with a probe that gives equal signals with the normal and mutated *HRAS* alleles (data not shown). Samples that did not hybridize in this initial blot were not included in the analysis, as it was assumed that there was a failure of amplification in those cases. On the basis of densitometric analysis of the first blot, a second slot blot was prepared, correcting for differences in PCR amplification. This standardized filter was hybridized with different oligonucleotides containing all possible activating changes in codons 12 and 61 (Fig. 1).

The results are shown in Fig. 3. Four mutations in codon 12 and 10 mutations in codon 61 were identified. These

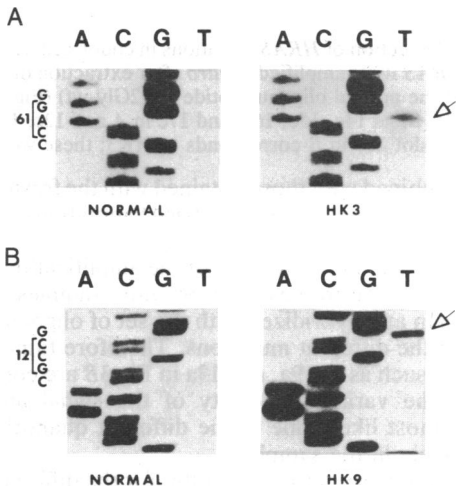

FIG. 2. Direct sequencing of amplified fragments of *HRAS* from human KAs. (*A*) Region of 12 bp around codon 61 corresponding to normal skin and human KA 3 (HK3). The sense primer used for the *in vitro* amplification was employed as a sequencing primer. (*B*) Region of 12 bp around codon 12 corresponding to normal skin and HK9. The antisense primer was the sequencing primer in this case. Arrows indicate bands corresponding to mutated base pairs.

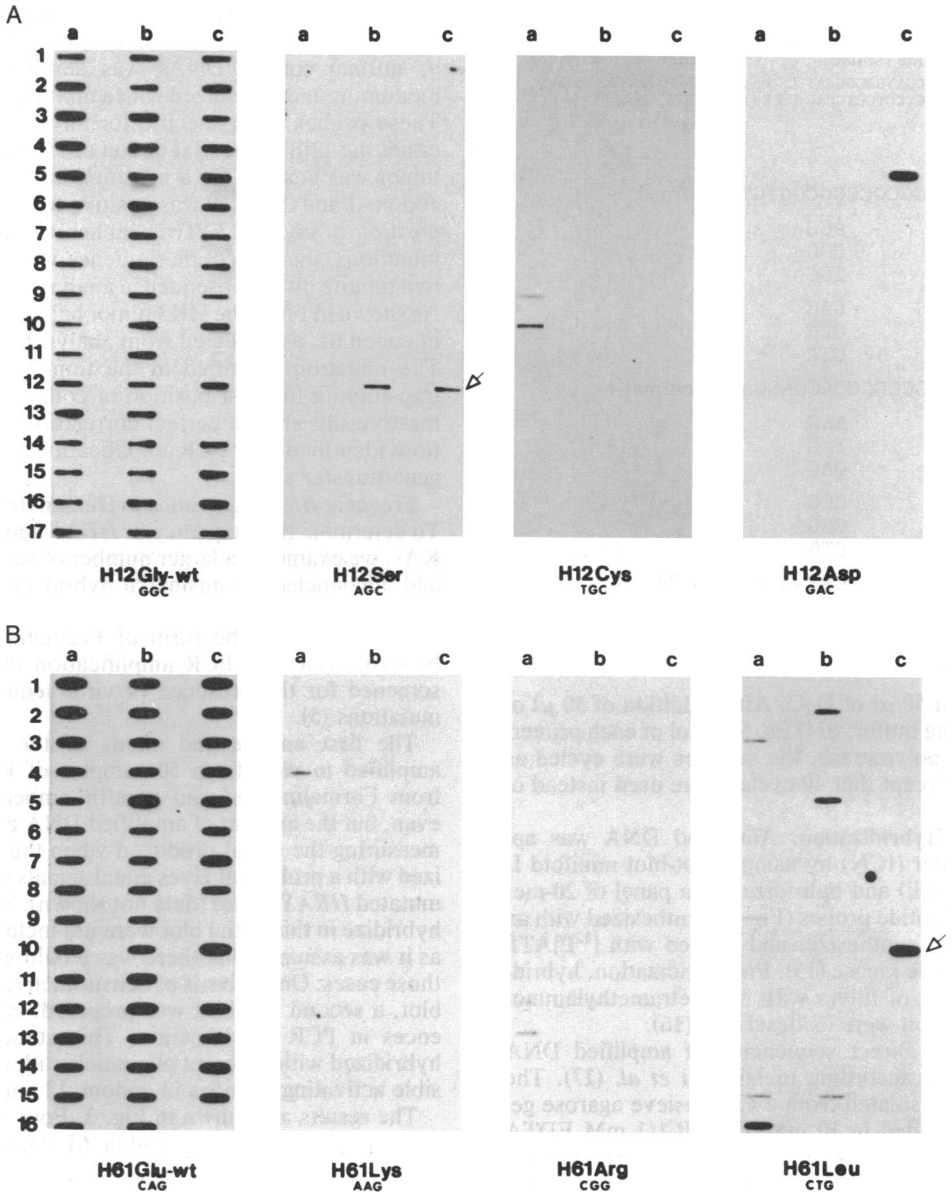

FIG. 3. Detection of *HRAS* mutations in codons 12 (*A*) and 61 (*B*) from human Formalin-fixed and paraffin-embedded KAs. First and second exons of *HRAS* were amplified *in vitro* after extraction of paraffin with xylene, spotted onto a nylon filter, and hybridized with specific mutated probes and the normal oligonucleotide (H12Gly-wt) (Fig. 1). The mutated probes are named by the codons they contain and the amino acids they encode. Slots 14c, 15c, 16c, and 17c in *A* and 12c, 13c, 14c, and 15c in *B* represent samples from normal skin. Slot 12c in *A* corresponds to HK9 and slot 10c in *B* corresponds to HK3; these were used as positive controls and are pointed out with arrows.

results, combined with those obtained with the fresh samples, represent an incidence of 30% of *HRAS* mutations among the KAs analyzed. Since positive controls were not available for all mutations and only a portion of the amplification reaction products was used in these experiments, samples were slot blotted again and hybridized with the set of oligonucleotides to confirm the different mutations. Therefore tumors giving faint bands such as 6a, 9a, and 13a in Fig 3*B* are consistently positive. The variable intensity of the signal among the tumors is most likely due to the different quantities of the mutated allele in the samples.

Although the use of the same filter for the different probes is in itself a demonstration of the selectivity of washing conditions used, to validate further the data obtained in the PCR–oligonucleotide hybridization analysis we sequenced some of the DNA fragments amplified from paraffin blocks. Fig. 4 shows the nucleotide sequence of the region around codon 12 of normal skin and the HK9a tumor, confirming the G-to-T transversion in the first position previously identified in the slot blot (Fig. 3*A*). This result demonstrates that one 6-μm section of Formalin-fixed and paraffin-embedded tissue could provide sufficient material of appropriate quality for direct DNA sequencing.

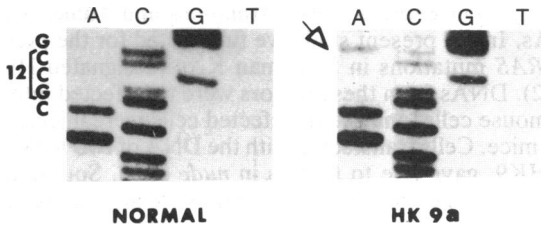

FIG. 4. Direct sequencing of amplified *HRAS* first exon from human paraffin-embedded tissues. Region around codon 12 from normal skin and human KA 9a (Fig. 3*A*) is shown. The antisense primer used for the *in vitro* amplification was the sequencing primer. Arrow indicates the mutated band.

SCCs Have a Lower Frequency of *HRAS* Mutations Than KAs Do. KAs histologically resemble SCCs with respect to an initial infiltrative pattern of growth and cytological atypia. Because of their different biological behaviors, we wanted to compare the frequency of *HRAS* mutations in the two types of tumors.

The analysis was done as described above. The first and second exons of the *HRAS* gene were amplified *in vitro* from 50 samples of Formalin-fixed and paraffin-embedded SCCs. Mutations in codon 12 and 61 were identified by oligonucleotide mismatch hybridization. The results are shown in Fig. 5. Samples giving faint bands were slot blotted again to confirm the mutations. Three mutations in codon 12 and 3 in codon 61 were identified, representing an incidence of 13% in these malignant skin tumors. There are thus 16 mutations among 56 KAs, and 6 mutations among 50 SCCs. Testing these data as a 2 × 2 contingency table yielded a χ^2 value of 4.4, $P < 0.05$, indicating that the frequency of *HRAS* activation in SCCs is significantly lower than in KAs.

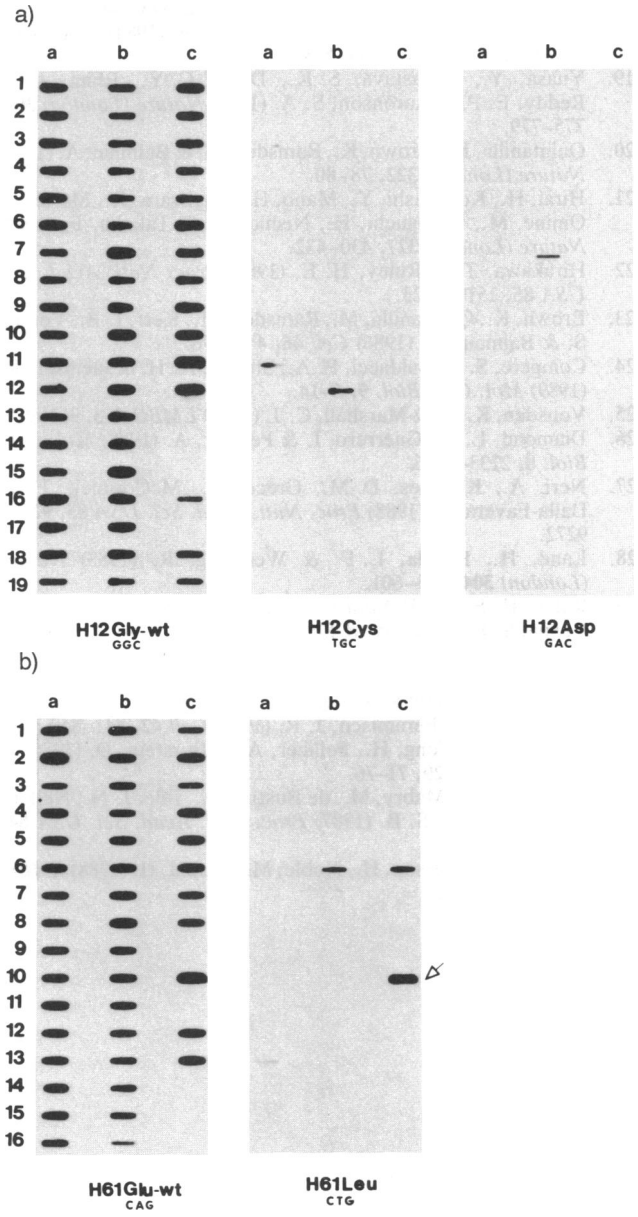

FIG. 5. Detection of *HRAS* mutations in codons 12 (*A*) and 61 (*B*) from Formalin-fixed and paraffin-embedded human SCCs. Amplification and slot-blot hybridization were as described in Fig. 3. Slots 18c and 19c in *A* and 12c and 13c in *B* represent normal skin. Slot 10c in *B* corresponds to HK3 used as a positive control and pointed out with an arrow.

DISCUSSION

Taking advantage of PCR-mediated amplification of DNA sequences from paraffin-embedded tissues, we have analyzed the presence of activated *HRAS* in the benign skin tumor KA and in its malignant counterpart, SCC.

Several conclusions can be drawn from this study: (*i*) Activated *HRAS* oncogenes are present in a substantial percentage (30%) of the human benign, self-regressing KA. (*ii*) Activation of the *HRAS* oncogene in the skin malignancy SCC is significantly lower. (*iii*) The most prevalent mutation identified in both tumors is the A·T-to-T·A transversion in the second position of codon 61, changing the amino acid glutamine to leucine (Figs. 3 and 5). (*iv*) *RAS* activation detected by PCR and oligonucleotide mismatch hybridization correlates with its biological activity in the *nude* mice tumorigenicity assay. (*v*) Direct DNA sequencing of material obtained from Formalin-fixed and paraffin-embedded tissue is feasible in many instances.

The transversion A·T-to-T·A in codon 61 of *hras* genes has been previously identified in tumors induced with polycyclic aromatic hydrocarbons (10, 20) as well as in DMBA-induced KAs in rabbits (unpublished results). The high prevalence of this specific mutation would suggest a similar etiologic factor or a particular high potency of that mutation in the skin.

The relatively high frequency of *HRAS* mutations in benign skin tumors that spontaneously regress, such as KAs, supports the involvement of this particular oncogene in the early stages of human neoplasia. Additionally, it indicates that activated *RAS* genes are not sufficient to induce a malignant phenotype or even to maintain the growth of a benign tumor. These results are consistent with our previous findings (8) as well as other studies in preleukemic disorders in humans (16, 21). High frequencies of mutated *RAS* genes have also been found in human colonic adenomas, a lesion considered premalignant, from which most colorectal carcinomas appear to arise (3, 4, 11). With respect to animal models, H-*ras* activation in benign tumors has been reported in the mouse skin (7, 9) and rabbit KAs (8). In particular, there is evidence which indicates a specific role of H-*ras* activation in the initiation of mouse skin carcinogenesis (7, 9, 23). It has been shown recently that retroviral transduction of the human *HRAS* oncogene into midgestation mouse embryos promotes epithelial hyperplasia. The late appearance of the lesions, which are mostly benign, also suggests that *ras* alone is not sufficient to induce a malignant phenotype (24). Other reports, however, indicate that in other systems, *ras* activation may be a late event, as in the spontaneous activation of the c-K-*ras* gene in cultured mouse lymphoma cells (25) or in the case of N-*ras* activation during the development of some mouse lymphomas (26) or human myeloma cases (27).

Malignant transformation by *ras* genes in primary cells is dependent on cooperating oncogenes (28, 29). The results presented here are consistent with the hypothesis that in KA the early *RAS* mutation could induce an increased rate of proliferation in a germinative cell that is not immortalized. In the absence of a complementing oncogene alteration, the epithelial cells with an activated *HRAS* would grow faster, creating a benign tumor. However, when these cells reach the end of their regular life span, they would differentiate and regress. Although we have studied only *HRAS*, since it is the predominant oncogene found to be activated in animal tumors of epidermal origin (9, 10), activation of *KRAS* or *NRAS* cannot be ruled out.

Another more speculative hypothesis would suggest a role of *HRAS* in the regression phase of the KA. Although *in vitro* studies have suggested that expression of oncogenic *ras*

increases the proliferative capacity and blocks the Ca^{2+}-induced terminal differentiation of primary keratinocytes (30), the conditions in the Petri dish are very different than those in the tumor, with epithelial cells interacting with other cell types and the influence of a host of growth factors. Moreover, there are many reports indicating that activated *ras* genes induce differentiation of certain cell types. Sarcoma viruses carrying K- or H-*ras* oncogenes induced differentiation-associated properties in the rat pheochromocytoma cell line PC12 (31). This differentiation process is also seen in PC12 cells after microinjection of activated H-*ras* p21 (32) or transfection with an activated mouse N-*ras* gene (26, 33). In a similar manner, introduction of the *HRAS* oncogene induces differentiation of cultured human medullary thyroid carcinoma cells (34). Other studies indicate that activated *ras* genes induce growth arrest, as in murine Schwann cells (35) and rat embryo fibroblasts (22). Therefore it is conceivable that an activated H-*ras* gene could also play a role in the spontaneous regression of KAs that is associated with differentiation of hyperplastic epithelium.

Analysis of different stages of DMBA-induced rabbit KAs and SCCs should produce information indicating whether the activated H-*ras* oncogene present in those tumors is equally expressed through the different phases of tumor evolution or regression of KAs is accompanied by changes of H-*ras* oncogene expression. This should shed more light on the role of *ras* genes during tumorigenesis in skin.

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