Two oncogenes, v-fos and v-ras, cooperate to convert normal keratinocytes to squamous cell carcinoma

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**ABSTRACT** Previous studies have implicated the rasHa oncogene in the initiation of skin carcinogenesis and the fos oncogene in malignant progression of premalignant skin cell lines. To determine if these two oncogenes are sufficient to convert normal keratinocytes to cancer cells, freshly isolated mouse keratinocytes were coinfected with replication-defective (v-2) v-rasHa and v-fos viruses in culture. When tested in nude mice within several days of infection, v-fos/v-rasHa-coinfected keratinocytes produced squamous cell carcinomas. Introduction of v-fos alone resulted in normal or hyperplastic skin, whereas v-rasHa alone produced squamous papillomas. These results indicate that two oncogenes are sufficient to produce the malignant phenotype in epidermal cells. Furthermore, they clearly link the fos oncogene with malignant conversion. Since fos acts as a transcriptional regulator of other genes, malignant conversion may be an indirect consequence of the overexpression of the fos-encoded protein leading to a change in the expression of fos-controlled cellular genes.

Experimental tumor induction is a multistep process that often proceeds through the formation of a benign precursor lesion prior to overt malignancy. In mouse skin carcinogenesis, the evolution of squamous carcinomas requires several genetic changes in the target cells since benign lesions (papillomas) formed in response to a single carcinogen exposure progress to malignancy at an accelerated rate if the tumor-bearing host is exposed to additional mutagenic carcinogens (1). Isolation of a c-rasHa oncogene from 7,12-dimethylbenz[a]anthracene and certain other polycyclic hydrocarbon-initiated skin papillomas and the formation of papillomas following the introduction of v-rasHa into normal skin cells (2-5) indicate that activation of c-rasHa is one type of early genetic change in mouse skin carcinogenesis associated with benign tumor formation. Further changes in c-rasHa genes, such as overexpression, amplification, and homozygosity, have been associated with malignant progression (6, 7). Changes in the fos gene have recently been implicated in skin carcinogenesis since transfection of oncogenic fos constructs converted established papilloma cell lines in culture to the carcinoma phenotype, whereas myc and EIA oncogenes did not (8). Since the papilloma cell lines contained an activated c-rasHa gene (9), the results imply that rasHa and fos genes could cooperate in malignant conversion of keratinocyte neoplasms. However, the prolonged cell culture maintenance of papilloma cell lines (9) may have produced other genetic changes in addition to the activating rasHa gene mutation defined at codon 61.

We now demonstrate that coinfection of primary murine keratinocytes with helper-free (v-2) Finkel–Biskis–Reilly (FBR) or Finkel–Biskis–Jinkins (FBJ) v-fos and v-rasHa retroviruses resulted in carcinomas when recipient cells were tested in nude mice. Introduction of only v-fos produced normal or hyperplastic skin, whereas v-rasHa produced papillomas. Thus, two oncogene events are sufficient to produce the malignant phenotype in epidermal cells.

**MATERIALS AND METHODS**

Cell Culture and Generation of Replication-Defective Virus. To obtain a FBR or FBJ murine sarcoma virus (MSV) devoid of helper virus, cloned FBR v-fos (10) or FBJ v-fos (11) DNA was transfected into a variant of v-2 cells (12) that contain a packaging defective Moloney murine leukemia virus engineered to minimize recombination and resultant packaging competence (13). Morphological transformants were cloned and conditioned medium from these cells was filtered using the NIH 3T3 focus formation assay. A replication-defective variant of Ha MSV that comprised a v-rasHa gene possessing mutations at codons 12 and 59 was generated as described (5).

In addition, as a negative control, a v-2 neo virus was generated by transfecting cloned pZIPneo DNA (14) into v-2 cells and subsequent G418 selection.

Primary newborn BALB/c keratinocytes were prepared as described (15) and plated at 1 × 10⁶ cells per 175-cm² flask. Cultures were maintained in fibroblast-conditioned Eagle’s minimal essential medium containing 0.05 mM Ca²⁺, a calcium concentration that selects for basal cells (15). The medium had been previously conditioned by primary dermal fibroblasts to enhance cell growth (16).

**Viral Infection and in Vivo Tumor Assay.** Three days after plating, primary keratinocytes were infected with v-2 v-rasHa or v-2 v-fos, both viruses, or v-2 neo as a control, using a variety of infection protocols in eight separate experiments. The ratios of fos:ras viruses ranged from 1:60 to 10:1 in separate experiments depending on the titer of the fos virus preparations. Multiplicities of infection ranged from 0.15 to 2.0 for single or double virus exposures. Results were consistent with all protocols, but the small number of animals in any group in each experiment makes a clear determination of the effects of multiplicities of infection or virus ratios difficult. As expected for these defective virus preparations, conditioned medium from keratinocytes infected with v-2 virus did not transform NIH 3T3 cells. Five days after infection, primary keratinocytes were isolated by trypsinization, and 5 × 10⁶ cells were combined with 8 × 10⁶ primary dermal fibroblasts for grafting onto nude mouse (5).

Infected cells (5 × 10⁶ per mouse) were also injected subcutaneously into the interscapular area of nude mice. Tumor-bearing animals were sacrificed at 4 weeks. Control and non-tumor-bearing animals (including graft failures) were scored at 10–12 weeks. Graft sites and subcutaneous nodules were processed for routine histological staining by hematoxylin/eosin as well as for special analysis as described below.

Abbreviations: MSV, murine sarcoma virus; K14, keratin 14; K1, keratin 1; FBJ, Finkel–Biskis–Jinkins; FBR, Finkel–Biskis–Reilly.

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Tumor diagnoses were determined grossly by the extent of local invasion and ulceration and confirmed histologically by the standard criteria of cellular atypia, tissue organization, invasion, and metastasis.

**Northern Analysis, Immunofluorescence, and Histochemistry.** Four weeks after in vivo application of cells, tumor-bearing mice were sacrificed, and the tumors were excised and frozen in liquid nitrogen. Total RNA was prepared according to the method of Chirgwin et al. (17), separated by agarose gel electrophoresis, and transferred to nitrocellulose. Filters were probed with (i) a v-ras-specific probe isolated from plasmid BS9 (18); (ii) a v-fos-specific probe isolated as a 1.8-kilobase Bgl II fragment from plasmid pFBJR2 (11); or (iii) a keratin 14 (K14)-specific probe to account for loading or transfer discrepancies (19). Filters were washed to a final stringency of 15 mM NaCl/1.5 mM sodium citrate/0.1% sodium dodecyl sulfate (SDS) at 68°C prior to autoradiography using Kodak X-Omat film with intensifying screen at −70°C.

At the time of sacrifice, a portion of the excised tumors was placed in embedding medium (OCT, Miles Scientific), frozen in dry ice, and later sectioned onto glass slides. For double-staining immunofluorescence, frozen sections were incubated with affinity-purified monospecific rabbit antibody to mouse keratin 1 (K1) (AF109, diluted 1:500) and guinea pig antibody to mouse K14 (diluted 1:2000) for 20 hr as described (20). For K14 visualization, biotin anti-guinea pig IgG was used in conjunction with streptavidin Texas red. For K1 visualization, a secondary fluorescein isothiocyanate-labeled anti-rabbit IgG was employed. γ-Glutamyl transpeptidase staining was performed on frozen sections as described (8).

## RESULTS

Grafts of mock-infected or ψ-2 neo-infected keratinocytes produced a normal epidermis, and no lesions developed at subcutaneous injection sites (Table 1; Fig. 1). Grafted keratinocytes infected with ψ-2 v-ras<sup>Ha</sup> produced squamous papillomas (Figs. 1 C and E and 2; Table 1) in almost all cases where grafts were successful (5). These papillomas were larger but histologically indistinguishable from those produced in chemical carcinogenesis experiments (1). The small number of carcinomas observed in this group is consistent with the rate of spontaneous in vivo conversion of v-ras<sup>Ha</sup> benign tumors observed previously (21). Subcutaneous injection of ψ-2 v-ras<sup>Ha</sup>-infected keratinocytes produced papillomatous cysts in which a well-organized, slightly papillary stratified epidermis surrounded a homogeneously stained, acellular keratin plug. Primary keratinocytes coinfected with ψ-2 v-ras<sup>Ha</sup> together with either type of ψ-2 v-fos virus produced predominantly squamous cell carcinomas on grafting or subcutaneous injection (Figs. 1 D and F and 2; Table 1). Malignancies were diagnosed grossly at the first clinical observation (14 days) by a characteristic ulcerated surface and undermined margins. Carcinomas did not appear to arise from a papilloma stage. The large size of malignant tumors developing within a few weeks of grafting suggests that they originated from the proliferation of many grafted cells, as expected from the infection and in vivo transfer protocols. A similar conclusion, based on genetic analysis, had been previously made concerning papillomas arising from v-ras keratinocyte tumor grafts (5). Later tumors (28 days) were very invasive and some were metastatic (see Fig. 2D).

Histologically, the malignant tumors ranged from poorly differentiated to a moderately well-differentiated phenotype, but all were typical epidermal carcinomas.

Grafts of primary keratinocytes infected with either type of ψ-2 v-fos virus produced normal or, in certain cases, a hyperplastic donor skin (Fig. 1B); v-fos keratinocytes produced no lesions or small keratinous cysts on subcutaneous injection (Table 1) even after 3 months of observation. This result was confirmed by infection of primary keratinocytes with FBR or FBJ MSV infectious virus with helper virus at a multiplicity of infection of 0.5 (10, 11). In two separate experiments, the graft histotype was normal donor skin, and subcutaneous injections failed to produce tumors (Table 1) after 10–12 weeks.

Northern analysis, performed on RNA extracted from grafted and subcutaneous tumors, confirmed the uptake and expression of exogenous viral genes (Fig. 3). All of the neoplasms examined (28/28) expressed the 1- to 5-kb bands characteristic of the v-ras<sup>Ha</sup> construct expressed in epidermal tumors (4). A c-ras<sup>Ha</sup> transcript (1.4 kb) was not detected in normal mouse skin with this probe. The carcinomas from combined exposures (15/15) in addition showed the 3.5-kb v-fos transcript, whereas benign v-ras<sup>Ha</sup> tumors and normal skin did not have a transcript that hybridized to the v-fos probe. The absence of detectable cellular ras<sup>Ha</sup> and fos protooncogenes in normal skin could in part be related to the lower amount of RNA loaded in lane a as detected by the K14 probe in the lower panel. However, these transcripts were noted to be in very low abundance in normal skin (22).

The histological diagnosis of many of the keratinocyte-derived tumors was confirmed by characteristic markers that can distinguish malignant tumors from benign tumors and normal skin. K1 is a differentiation-specific keratin expressed in normal skin and papillomas but not in carcinomas, whereas K14 is a basal cell keratin expressed in normal skin and both tumor types (20, 23). Specific antisera in two species allow for the analysis of these markers in tissue sections (20) by indirect immunofluorescence techniques. All ψ-2 v-fos/v-ras<sup>Ha</sup> tumors examined (16/16) were negative for K1 expression but positive for K14 expression, which confirmed their epithelial origin and malignant phenotype. ψ-2 v-ras<sup>Ha</sup> tumors (9/9) retained K1 and K14 expression characteristic of squamous papillomas, although K1 expression was not uniform. ψ-2 v-fos graft sites (4/4) and ψ-2 neo graft sites (4/4) exhibited normal patterns of expression for keratins. Histochemically, detectable γ-glutamyl transpeptidase activity is commonly observed in 7,12-dimethylbenz[a]anthracene-initiated skin carcinomas and absent from interfollicular epidermis and papillomas (24). However, ψ-2 v-fos/v-ras<sup>Ha</sup> carcinomas (16/16) were devoid of detectable γ-glutamyl transpeptidase activity, as were v-ras<sup>Ha</sup>-induced papillomas (9/9). As in our previous study (8), the γ-glutamyl

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<th>Viral construct</th>
<th>No. of animals with histotype in vivo</th>
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<tr>
<td></td>
<td>Normal or hyperplastic skin</td>
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<tr>
<td>ψ-2 neo</td>
<td>28</td>
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<tr>
<td>ψ-2 v-fos (FBR or FBJ)</td>
<td>31</td>
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<td>FBJ or FBR MSV</td>
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<td>ψ-2 v-ras&lt;sup&gt;Ha&lt;/sup&gt;</td>
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<td>ψ-2 v-ras&lt;sup&gt;Ha&lt;/sup&gt;/ψ-2 v-fos&lt;sup&gt;†&lt;/sup&gt;</td>
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*In vivo* indicates normal host tissue in sites where tumors failed to take as noted (5).

†Coinfection.

Table 1. Tumorigenicity of infected newborn primary keratinocytes
transpeptidase results suggest that tumors induced by a combination of the ras^{Ha} and fos oncogenes are phenotypically different from spontaneous converted papillomas induced by 7,12-dimethylbenz[a]anthracene and tumor promoters, even though c-ras^{Ha} is presumably activated at the initiation stage in both systems. Alternatively, the target cell for chemical carcinogenesis in vivo could be different (i.e., hair follicle) than the cells transformed in our in vitro study (i.e., interfollicular) since γ-glutamyl transpeptidase is detectable in normal hair follicles.

**DISCUSSION**

These studies clearly indicate that v-fos can cooperate with v-ras^{Ha} in skin carcinogenesis. The possibility that other changes occurred in the infected keratinocytes while in culture seems unlikely as the lag time between infection and in vivo analysis was short, and time-matched and virus controls produced normal skin. The cooperation between fos and ras is particularly interesting since v-fos alone produced no reproducible pathology in keratinocytes. Previously, a link between fos and ras^{Ha} was demonstrated by microinjec-
Fig. 2. Tumor formation in nude mice from infected primary murine keratinocytes. Four weeks after subcutaneous injection or skin grafting, animals were sacrificed and photographed. (A and C) Injected (A) and grafted (C) cells infected with \( \psi^- 2\) v-ras\(^{Ha}\) (B and D) Injected (B) and grafted (D) cells infected with \( \psi^- 2\) v-ras\(^{Ha}\) and \( \psi^- 2\) v-fos.

Fig. 3. Northern analysis of RNA extracted from grafted and subcutaneous tumors. Lanes: a, normal mouse skin RNA; b, RNA from a carcinoma graft arising from \( \psi^- 2\) v-ras\(^{Ha}\); c, RNA from papilloma graft from \( \psi^- 2\) v-ras\(^{Ha}\)-infected keratinocytes; d, RNA from a subcutaneous papillomatous cyst derived from \( \psi^- 2\) v-ras\(^{Ha}\)-infected keratinocytes; e, RNA from subcutaneous carcinoma derived from \( \psi^- 2\) v-ras\(^{Ha}\) and v-fos-coinfected keratinocytes.

The failure of \( \psi^- 2\) v-fos or either wild-type fos MSV virus to alter the phenotype of newborn keratinocytes contrasts with the transforming nature of these genes in some other systems (10, 11). This result was consistent in eight experiments comprising 55 animals and six separate high-titer virus preparations. Previously, c-fos expression was demonstrated to increase transiently under conditions of epidermal hyperproliferation (30), but permanent up-regulation of fos has not been reported in skin. It has been shown previously that the introduction of v-ras\(^{Ha}\) alters the differentiation program of normal keratinocytes (31). Thus, the action of fos on normal keratinocytes may be abrogated by an intact intrinsic differentiation program but becomes effective in neoplastic cells where the differentiation program is altered.

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