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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 *ras*^{Ha} 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 (ψ-2) v-*ras*^{Ha} and v-*fos* viruses in culture. When tested in nude mice within several days of infection, v-*fos*/v-*ras*^{Ha}-coinfected keratinocytes produced squamous cell carcinomas. Introduction of v-*fos* alone resulted in normal or hyperplastic skin, whereas v-*ras*^{Ha} 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-*ras*^{Ha} 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-*ras*^{Ha} into normal skin cells (2–5) indicate that activation of c-*ras*^{Ha} is one type of early genetic change in mouse skin carcinogenesis associated with benign tumor formation. Further changes in c-*ras*^{Ha} 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 *E1A* oncogenes did not (8). Since the papilloma cell lines contained an activated c-*ras*^{Ha} gene (9), the results implied that *ras*^{Ha} 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 *ras*^{Ha} gene mutation defined at codon 61.

We now demonstrate that coinfection of primary murine keratinocytes with helper-free (ψ-2) Finkel–Biskis–Reilly (FBR) or Finkel–Biskis–Jinkins (FBJ) v-*fos* and v-*ras*^{Ha} retroviruses resulted in carcinomas when recipient cells were tested in nude mice. Introduction of only v-*fos* produced normal or hyperplastic skin, whereas v-*ras*^{Ha} 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 ψ-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 ψ-2 cell lines was titered using the NIH 3T3 focus formation assay. A replication-defective variant of Ha MSV that comprised a v-*ras*^{Ha} gene possessing mutations at codons 12 and 59 was generated as described (5). In addition, as a negative control, a ψ-2 *neo* virus was generated by transfecting cloned pZIP*neo* DNA (14) into ψ-2 cells and subsequent G418 selection.

Primary newborn BALB/c keratinocytes were prepared as described (15) and plated at 1×10^7 cells per 175-cm^2 flask. Cultures were maintained in fibroblast-conditioned Eagle's minimal essential medium containing 0.05 mM Ca^{2+}, 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 ψ-2 v-*ras*^{Ha} or ψ-2 v-*fos*, both viruses, or ψ-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 ψ-2 virus did not transform NIH 3T3 cells. Five days after infection, primary keratinocytes were isolated by trypsinization, and 5×10^6 cells were combined with 8×10^6 primary dermal fibroblasts for grafting onto each nude mouse (5). Infected cells (5×10^6 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.

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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 (kb) *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 tumor 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^{Ha} 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^{Ha} benign tumors observed previously (21). Subcutaneous injection of ψ-2 v-ras^{Ha}-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^{Ha} 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. 2*D*). Histologically, the malignant tumors ranged from poorly differentiated to a moderately well-differentiated phenotype, but all were typical epidermal carcinomas.

Table 1. Tumorigenicity of infected newborn primary keratinocytes

Viral construct	No. of animals with histotype *in vivo*		
	Normal or hyperplastic skin	Papilloma	Carcinoma
ψ-2 *neo*	28	0	0
ψ-2 v-*fos* (FBR or FBJ)	31	0	0
FBJ or FBR MSV	21	0	0
ψ-2 v-ras^{Ha}	3*	31	3
ψ-2 v-ras^{Ha}/ψ-2 v-*fos*†	6*	3	29

Analysis of tissue phenotype from a total of eight experiments that encompassed tissue grafts or subcutaneous injections of primary keratinocytes infected with replication-defective retroviruses or infectious virus and helper virus (FBJ or FBR MSV). Results include cells derived from experiments utilizing six independent viral preparations and at least five animals per injection or graft group. Only histologically verified tumors are included. Animals were excluded where a graft take clearly failed (usually from infection) or a subcutaneous site was negative for any exogenous tissue.

*Animals may represent normal host tissue in sites where tumors failed to take as noted (5).

†Coinfection.

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. 1*B*); 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^{Ha} construct expressed in epidermal tumors (4). A c-ras^{Ha} 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^{Ha} tumors and normal skin did not have a transcript that hybridized to the v-*fos* probe. The absence of detectable cellular ras^{Ha} 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^{Ha} 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^{Ha} 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 these two 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^{Ha} carcinomas (16/16) were devoid of detectable γ-glutamyl transpeptidase activity, as were v-ras^{Ha}-induced papillomas (9/9). As in our previous study (8), the γ-glutamyl

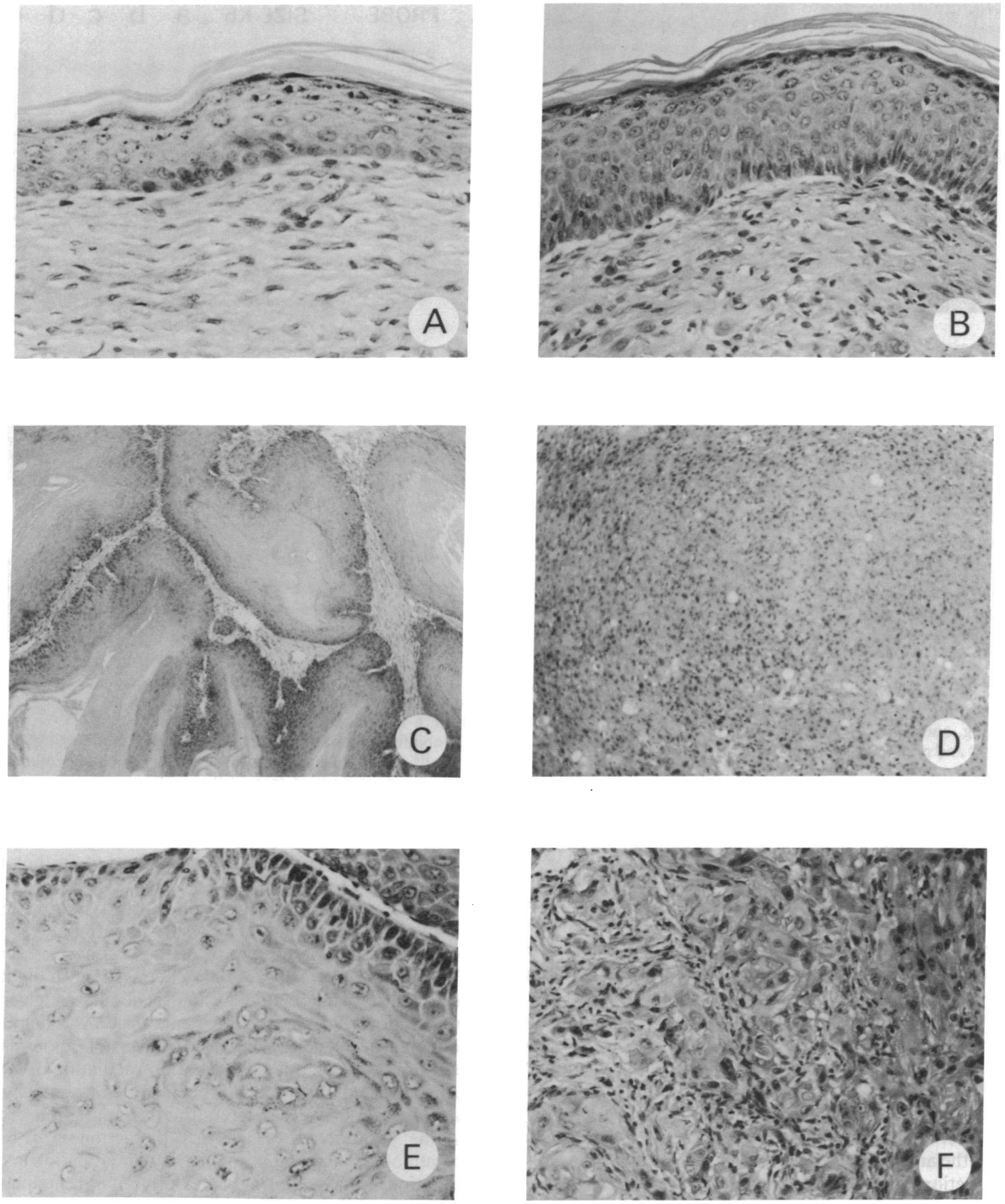

FIG. 1. Histological analysis of nude mouse grafts from infected primary murine keratinocytes. (*A*) Normal skin from cells infected with ψ-2 *neo* virus. (×230.) (*B*) Hyperplastic skin produced by ψ-2 v-*fos* virus infection. (×230.) (*C* and *E*) Papilloma histotype produced by grafts of ψ-2 v-*ras*^{Ha}-infected keratinocytes. (*C*, ×90; *E*, ×230.) (*D* and *F*) Squamous cell carcinoma histotype produced by coinfection of keratinocytes with ψ-2 v-*ras*^{Ha} and ψ-2 v-*fos*. (*D*, ×90; *F*, × 230.)

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-

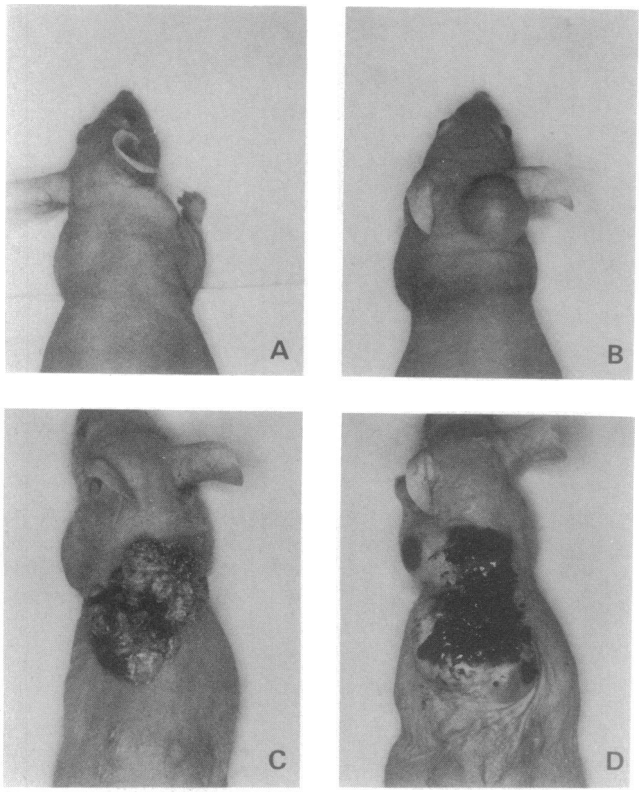

FIG. 2. Tumor formation in nude mice from infected primary murine keratinocytes. Four weeks after subcutaneous injection or skin grafting, animals were sacrificed and photographed. (*A* and *C*) Injected (*A*) and grafted (*C*) cells infected with ψ-2 v-*ras*^{Ha}. (*B* and *D*) Injected (*B*) and grafted (*D*) cells infected with ψ-2 v-*ras*^{Ha} and ψ-2 v-*fos*.

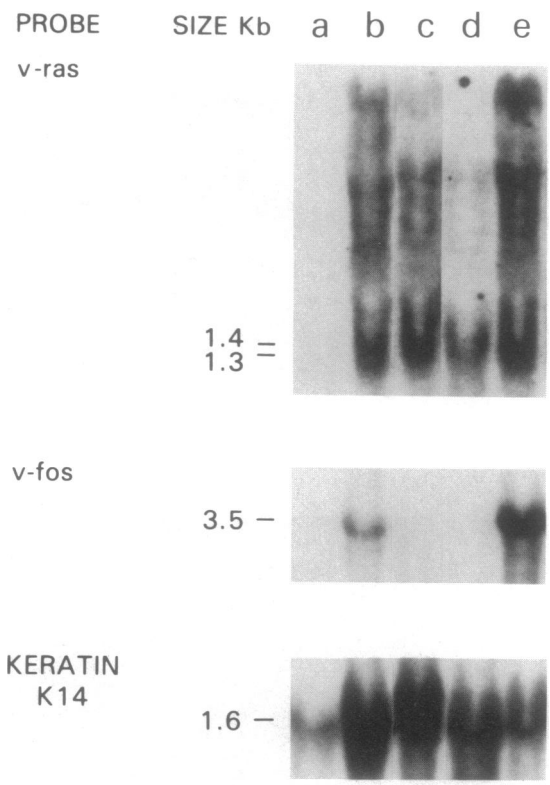

FIG. 3. Northern analysis of RNA extracted from grafted and subcutaneous tumors. Lanes: a, normal mouse skin RNA; b, RNA from a carcinoma graft arising from ψ-2 v-*ras*^{Ha}- and ψ-2 v-*fos*-coinfected keratinocytes; c, RNA from papilloma graft from ψ-2 v-*ras*^{Ha}-infected keratinocytes; d, RNA from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

tion of transforming *ras*^{Ha} p21 protein into NIH 3T3 cells, which produced a transient increase in c-*fos* expression (25). Similarly, c-*fos* expression and expression of a c-*fos* promoter-CAT construct were stimulated >10-fold by introduction of a *ras* protooncogene or *ras* oncogene into 3T3 cells (26). However, we did not see a c-*fos* signal when RNA from v-ras-induced papillomas was probed with v-*fos*, suggesting that the endogenous transcript is not elevated. This is consistent with our previous analysis of chemically induced mouse skin tumors (22) and tumor cell lines (8). The *fos* gene is known to be a regulator of transcription, forming a complex with p39, the c-*jun*/AP1 protooncogene product, and this modulates gene expression by binding to the AP1 regulatory site (27, 28). Thus, the converting functions of v-*fos* could center on the anomalous expression of AP1-controlled genes. These functions are likely to be specific since the *E1A* gene of adenovirus type 5, another transcriptional regulator, failed to cooperate with *ras*^{Ha} in the conversion of papilloma cell lines to the carcinoma phenotype (8). Preliminary evidence suggests that novel expression of AP1-controlled protease molecules such as transin (stromelysin) (29) could be important in this model system. This gene has been closely associated with skin malignancies (29) and is not expressed in v-*ras*^{Ha}-induced papillomas, but transcripts are elevated in *ras*^{Ha}/*fos* carcinomas (D.A.G., unpublished observations). Phenotypic conversion of papillomas to carcinomas could be mediated by activation of protease expression by means of the *fos* gene or other mechanisms. Increased protease secretion by papilloma cells would disrupt the basement membrane and disturb other extrinsic regulatory molecules. This could cause the disorganization in structure and altered differentiation associated with the malignant and invasive phenotype. Experiments are necessary to further test this hypothesis.

The failure of ψ-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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