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Retroviral Transduction of the Human c-Ha-*ras*-1 Oncogene into Midgestation Mouse Embryos Promotes Rapid Epithelial Hyperplasia

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Infection of mouse embryos at 8 days of gestation with a replication-defective retrovirus carrying the human c-Ha-*ras*-1 oncogene led to efficient and rapid induction of hyperplastic lesions. Twenty-four percent of viable offspring developed abnormal growths after infection with purified virus. The lesions contained a single integrated provirus and produced viral RNA and the Ha-*ras* oncogene product (p21). The latency period between the time of infection and appearance of the lesions suggested that secondary alterations in addition to activated *ras* were necessary for neoplasms to develop. The earliest and most abundant growths were cutaneous and appeared from 4 to 36 weeks of age, with a median of 4 weeks of age. A number of subcutaneous lesions also developed over the same time span but at a median of 18 weeks of age. The rapid development of cutaneous lesions in response to transduction of the *ras* oncogene contrasts with other studies in which adult skin required secondary treatment with promoters prior to *ras* induction of epithelial hyperplasia. These results demonstrate that infection of midgestation mouse embryos allows rapid analysis of oncogene potency in skin.

Alterations of the structural or regulatory components of numerous genes have been associated with the pathogenesis of cancer. In human tumors, alterations of the *myc* and *ras* families of genes are most frequently associated with malignancy (4, 13, 36). The *ras* genes encode a 21-kilodalton (kDa) protein (p21) localized in the cytoplasm and associated with the plasma membrane. The oncogenic p21 is frequently generated by a point mutation in the structural gene, resulting in a substitution at amino acid 12 or 61 (6, 9, 23). In most previous studies, oncogenic transformation was assessed in cells growing in culture by studying changes in morphology, ability to grow in soft agar, and tumor-forming ability after subcutaneous injection into nude mice. However, such in vitro analyses are limited by the relatively few cell types that can be adapted to growth in tissue culture and do not reflect the normal environment of a cell progressing to a cancerous state in vivo.

In this study, we analyzed the effects of the *ras* oncogene on cells in their normal environment by introducing the oncogene into developing embryos. Infection of postimplantation mouse embryos with replication-competent retroviruses allows efficient spread of the virus, resulting in animals that are viremic at birth (15). As postimplantation embryos are permissive for retrovirus expression, they provide a useful system for studying the effects of specific genes on tissue formation and embryogenesis. Because oncogenes are believed to be important regulators of cellular differentiation in developing organisms (for review, see reference 1), we wished to determine whether retroviral vectors carrying oncogenes could be introduced into midgestation embryos and result in viable offspring. We constructed a replication-defective retrovirus containing the human c-Ha-*ras*-1 oncogene for our initial study because *ras* is present in many tumor types and low levels of expression elicit a transformed phenotype. Furthermore, since *ras* is one of the most potent oncogenes, it provides a test of the feasibility of this approach for studying oncogenes in developing embryos.

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MATERIALS AND METHODS

Materials. All cell culture reagents were purchased from Gibco. Polybrene and mitomycin C were purchased from Sigma Chemical Co. The drug Geneticin (G418 sulfate) was purchased from Gibco. All NIH 3T3-derived cell lines were grown in monolayer culture in Dulbecco modified Eagle medium with 10% newborn calf serum (Hyclone). Restriction endonucleases were purchased from New England Biolabs (the manufacturer's assay conditions were used).

Construction of a replication-defective retrovirus containing the human c-Ha-*ras*-1 oncogene. We constructed a replication-defective retrovirus carrying the human c-Ha-*ras*-1 oncogene (DOEJ; Fig. 1A). The c-Ha-*ras*-1 oncogene, isolated from the EJ bladder carcinoma cell line (kindly provided by R. Weinberg), was subcloned into pUC13 by using the endogenous *Sst*I restriction sites. A *Bgl*II linker was inserted into a *Sma*I restriction site residing 13 base pairs (bp) upstream of the first AUG of the human c-Ha-*ras*-1 gene (6). The 2.3-kilobase (kb) fragment from the *Bgl*II restriction site to a *Bam*HI site in pUC13 was cloned into a *Bam*HI site in the retrovirus vector DOL (kindly provided by R. Mulligan [17]). In this vector, the transcription of the *ras* gene is directed by the Moloney murine leukemia virus (Mo-MuLV) long terminal repeat. The neomycin resistance gene from transposon Tn*10* (which confers resistance to G418 sulfate upon expression in mammalian cells) is present in the vector and is transcribed from the simian virus 40 (SV40) early-region promoter (17).

Transfection of ψ2 packaging cell lines. The methods for transfection have been described by Wigler et al. (39). Vector DNA (200 ng) was mixed with 20 μg of carrier NIH 3T3 cell DNA in 250 μM $CaCl_2$–1 mM Tris (pH 7.9)–0.1 mM EDTA and slowly added to an equal volume of 2× HEPES-buffered saline (280 mM NaCl, 50 mM HEPES [*N*-2-hy-

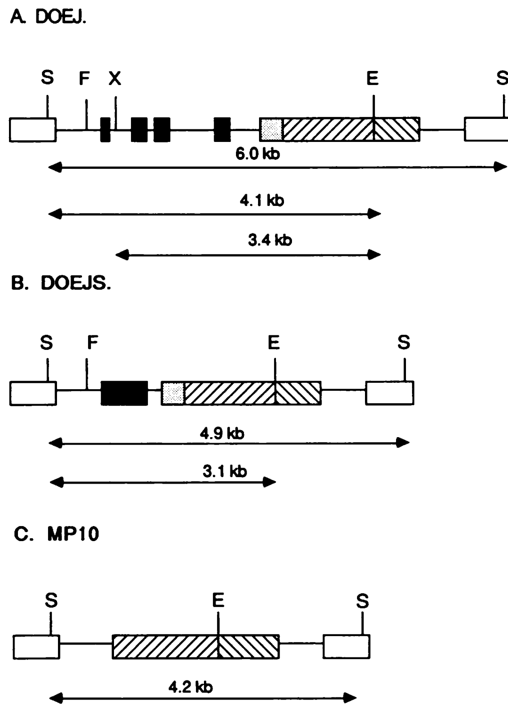

FIG. 1. Structure of recombinant viruses. Key: □, SV40 origin of replication; ▨, *neo*; ▧, pBR322 origin of replication; ■, human Ha-*ras* exons. Designated restriction endonuclease sites: X, *Xba*I; S, *Sst*I; B, *Bam*HI; E, *Eco*RI; F, *Bgl*II-*Bam*HI fusion sequence generated on insertion of EJ genomic sequences. (A) Structure of the DOEJ virus, a replication-defective Mo-MuLV carrying the human c-Ha-*ras*-1 oncogene. (B) Structure of the DOEJS virus, a spliced version of the DOEJ virus. (C) Structure of the MP10 virus, a replication-defective Mo-MuLV carrying the neomycin resistance marker of Tn*10*.

droxyethylpiperazine-*N*′-2-ethanesulfonic acid], 1.5 mM Na_2PHO_4 [pH 7.1]). The precipitate was allowed to form for 30 min at room temperature and was added to a 100-mm dish containing 5×10^5 ψ2 cells (20) in 10 ml of medium. After 4 h, the precipitate was removed and a solution of 15% glycerol and 1× HEPES-buffered saline was added for 5 min at 37°C. Twenty-four hours after transfection, cells were placed in selective medium containing 0.5 mg of G418 sulfate per ml. Selective medium was changed every 3 to 4 days. Individual colonies were cloned in glass cylinders and expanded in selective medium.

Concentration of virus supernatants. Virus supernatants were harvested from 90% confluent ψ2 producer (20) cells 8 to 12 h after the medium had been changed. The supernatants were centrifuged at 10,000 × *g* for 10 min to remove cellular material and were then centrifuged at 10,000 × *g* for 8 to 16 h at 4°C. The viral pellets were suspended in 10 mM HEPES containing 0.5% dimethyl sulfoxide and stored at −70°C or in liquid nitrogen.

Titration of virus supernatants. NIH 3T3 cells (seeded at 5×10^5 per 10-cm dish 12 h prior to each experiment) were incubated with 1 ml of serial dilutions of filtered virus supernatants or concentrated viral stocks containing 4 μg of Polybrene per ml for 2 to 4 h at 37°C in a 5% CO_2 atmosphere. The cells were incubated with fresh medium for 24 h and then passaged into selective medium containing 0.5 μg of G418 sulfate per ml. Colonies of resistant cells were scored for growth 10 to 14 days later.

Injection of mouse embryos in utero. Females of three inbred strains (C57BL/6J, BALB/c, and FVB) were mated with males of the same strain, and the day that the vaginal plug appeared was counted as day 0 of gestation. The C57BL/6 and BALB/c strains were originally obtained from Jackson Laboratories, and the FVB strain was originally obtained from the National Institutes of Health. All three of these strains are maintained in our breeding colony. Pregnant females were anesthesized with metomidate hydrochloride (Hypnodil) at 20 μg/g of body weight prior to injection of the embryos on embryo day 8.5. The developing embryos were infected by microinjection of either ψ2 cells producing virus or concentrated viral stocks directly through the uterine wall (15). Approximately 1 μl was injected into each conceptus. The virus producer cells were pretreated with mitomycin C at 20 μg/ml for 2 h in order to prevent cell division while permitting virus production.

Histology. Tissues were fixed in 10% buffered Formalin, then dehydrated in graded alcohols and xylenes, and embedded in paraplast. Sections 4 μm thick were prepared and stained with Harris hematoxylin and eosin (32).

Electron microscopy. Samples were prepared for examination by electron microscopy by first being fixed in 2% glutaraldehyde–2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 4 h. The fixative was rinsed out with three changes of 0.1 M sodium cacodylate (pH 7.2) over a 2-h period and then postfixed in 1% OsO_4 in 0.1 M sodium cacodylate for 1 h at 4°C. After rinses in distilled water, the tissue was treated with 1% uranyl acetate, rinsed again in distilled H_2O, and then dehydrated in graded ethanols and propylene oxide. Tissue was then infiltrated with propylene oxide-resin (Polybed 812-Araldite 502; Polysciences). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 410 LS electron microscope at 80 kV.

Immunohistochemistry. Paraffin sections (4 μm thick) were placed on glass slides that had been lightly coated with glue (LePage Bond Fast white glue; LePage Limited, Bramalea, Ontario, Canada), permitted to dry thoroughly, placed in an oven at 56°C for 45 min, and allowed to cool. Slides were then deparaffinized, placed in methanolic peroxide (5:1, vol/vol) for 30 min, washed thoroughly, and placed in 0.05 M Tris, pH 7.6, supplemented with 2% normal swine serum. Slides that were processed for keratin studies were incubated with trypsin (25 mg/dl in 0.134-g/dl $CaCl_2$ dihydrate in 0.05 M Tris buffer [pH 7.8]) for 20 min at 37°C according to Pinkus et al. (24). Slides were then incubated with antibodies as described previously (8). Briefly, slides were initially incubated with a solution of 2.5% egg albumin and 5% swine serum to minimize background staining and then sequentially incubated with rabbit antibodies to bovine muzzle keratins (1:80 dilution; Dako Corporation, Santa Barbara, Calif.), myoglobin (1:500 dilution; Dako), S100 (1:150 dilution; Dako), or desmin (1:500 dilution [Dako] and 1:80 dilution [Sigma]) for 1 h, followed by swine anti-rabbit immunoglobulin (1:30 dilution; Dako) and horseradish peroxidase-rabbit anti-horseradish peroxidase immune complexes (PAP, 1:200 dilution; Dako) with 30-min incubations for the latter two reagents. Antibody localization was visualized by using a peroxidase reaction with 3,3′-diaminobenzidine tetrahydrochloride (Aldrich) as the chromagen (1.5 mg/ml, with 0.1 ml of 3% H_2O_2). Slides were counterstained with methyl green, dehydrated, and mounted with Permount.

For immunoperoxidase studies with goat primary antibodies, the procedure was the same except that a swine anti-goat immunoglobulin–peroxidase conjugate (Tago; 1:50 dilution)

was used instead of swine anti-rabbit immunoglobulins and PAP. Goat primary antibodies to vimentin (1:80 dilution [Polysciences] and 1:60 dilution [Sigma]) were used in these studies.

Detection of viral DNA sequences. Total nucleic acids were isolated from tumor tissue by digestion of homogenized tissue in SET buffer (1% sodium dodecyl sulfate [SDS], 5 mM EDTA, 10 mM Tris chloride [pH 7.5]), extraction with phenol-chloroform, and ethanol precipitation. DNA was purified from total nucleic acids by treatment with RNase A (25 μg/ml) for 30 min at 37°C, followed by proteinase K digestion, phenol-chloroform extraction, and precipitation with ethanol. DNA was digested with restriction endonucleases, separated by electrophoresis on 0.7% agarose gels, transferred to nylon filters, and hybridized to radiolabeled probe (12). Lambda DNA was digested with *Hin*dIII restriction endonuclease and electrophoresed in one lane of each gel to provide size markers.

Detection of viral RNA sequences. Total cellular RNA was isolated from tissues with lithium chloride and urea according to Auffray and Rougeon (3). RNA was extracted from cell lines by the hot phenol technique described by Scherrer (30). Twenty micrograms of total RNA was applied to nitrocellulose by using a manifold (37) and hybridized to radiolabeled probes (12). Northern (RNA) blot analysis was performed as described by Thomas (37).

Detection of *ras* proteins by Western blotting (immunoblotting) analysis. Tissues were homogenized in 0.2 M LiCl–0.02 M Tris–1 mM EDTA, pH 8, and Nonidet P-40 was added to a final concentration of 5%. Nuclei and cell debris were removed by centrifugation at 15,600 × *g* for 5 min. The protein content of the cellular lysates was assayed according to McKnight (21). Polyclonal antibodies (Cetus) immunoreactive with the *ras* protein were used to immunoprecipitate the p21 proteins present in 0.5 to 1 mg of protein of the cellular lysates of each lesion analyzed. Detailed protocols for this Western blotting procedure are provided by Cetus Corporation, Emeryville, Calif. The immunoprecipitates were electrophoresed on a 12% polyacrylamide–SDS gel and electrophoretically transferred to nitrocellulose. The samples were analyzed in duplicate sets, and the nitrocellulose sheets were probed with either polyclonal rabbit antibodies with broad cross-reactivity to all *ras* proteins (Cetus) or polyclonal antibodies immunoreactive with the oncogenic p21 protein containing a valine at amino acid 12 (for best results we used twice the recommended amount of this antibody and 1 mg of protein lysate).

RESULTS

Construction of retroviral vector transducing the human c-Ha-*ras*-1 oncogene. To prepare a recombinant virus transducing the *ras* oncogene, the DOEJ construct was transfected into ψ2 packaging cells and selected for resistance to the drug G418 sulfate. A producer line was selected for an intact virus structure by Southern blotting analysis (Fig. 2) and a high titer based on the ability of the virus to confer G418 sulfate resistance with a transformed morphology to NIH 3T3 cells. This ψ2 cell line (DOEJ) produced a recombinant virus carrying both spliced and unspliced versions of *ras* (data not shown). Since aberrant splicing could result in nonfunctional *ras* transcripts, we prepared a DOEJ-infected ψ2 line producing a homogeneous population of spliced virus by infecting tunicamycin-treated ψ2 cells with virus supernatant from DOEJ at a multiplicity of infection of 1 (tunicamycin reduces the gp70 block to reinfection in these cells [7]). G418 sulfate-resistant ψ2 colonies with a transformed morphology were clonally isolated and screened for production of viruses which carried a spliced *ras* gene, conferred G418 sulfate resistance, and conferred a transformed morphology to NIH 3T3 cells. The titer of the DOEJS producer cell line supernatant was approximately 5 × 10^5 virus infectious units per ml of medium, and this could be further concentrated by centrifugation to obtain virus stocks of 5 × 10^6 to 10 × 10^6 virus infectious units per ml (as described in Materials and Methods). Southern blotting analysis of the virus producer cell line (DOEJS) demonstrated that the size of the provirus (3.1-kb *Sst*I-*Eco*RI fragment) and the absence of an *Xba*I site in the first intron were consistent with an appropriately spliced *ras* virus (Fig. 2). This spliced structure was stably transmitted in subsequent infections of NIH 3T3 cells. Furthermore, Western blotting analysis demonstrated that the ψ2 producer cell line produced p21 which was recognized by both an antibody with broad cross-reactivity to normal and abnormal p21 proteins (Fig. 3A) and a polyclonal antibody which recognized only the oncogenic p21 with a valine substituted at amino acid 12 (Fig. 3B).

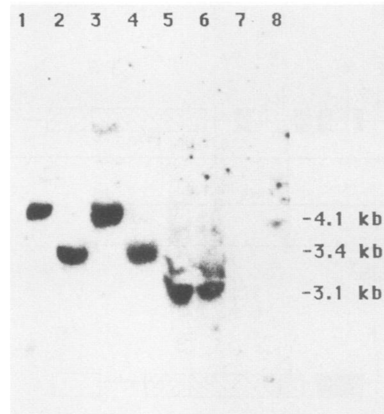

FIG. 2. Characterization of the virus producer cell lines. DNA from the DOEJ plasmid (lanes 1 and 2), DOEJ ψ2 producer cells (lanes 3 and 4), DOEJS ψ2 producer cells (lanes 5 and 6), or ψ2 producer line (lanes 7 and 8) were digested with either *Sst*I and *Eco*RI (lanes 1, 3, 5, 7) or *Sst*I, *Eco*RI, and *Xba*I (lanes 2, 4, 6, 8) restriction endonucleases and subjected to electrophoresis on a 0.7% agarose gel, blotted onto Genescreen nylon membrane, and hybridized to ^{32}P-radiolabeled *Bam*HI-*Eco*RI *neo* probe (Fig. 1) as described in the text.

Infection with DOEJS virus results in cutaneous and subcutaneous lesions. Midgestation mouse embryos were injected with either mitomycin c-treated producer cells or concentrated virus to assess the best means of introducing the virus. Previous studies had demonstrated that cells injected into midgestation embryos can survive and produce infectious virus (31, 35), and we speculated that the producer cells might facilitate virus infection through cell-cell contact.

Infected animals were born without signs of disease or developmental anomaly. Approximately 50% of the embryos injected with the *ras* virus survived to birth, which is comparable to the survival obtained with a virus carrying the *neo* gene (MP10 neo) (Fig. 1, Table 1) and to previous studies with injection of Mo-MuLV (15, 34, 35). Thus, the *ras* virus does not affect embryonic survival to birth. After birth, the animals were monitored for signs of disease or the development of abnormal growths for a period of 6 months. The results of these experiments are summarized in Table 1. Both methods of administering the virus were effective in

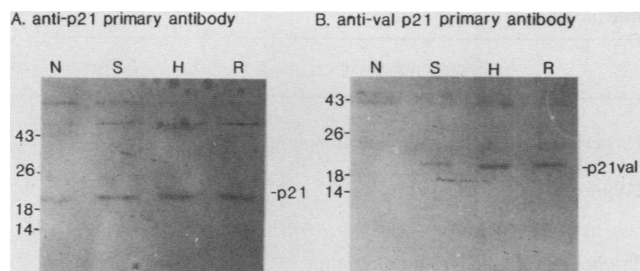

FIG. 3. Western blotting analysis of p21 expression in lesions. Western blotting analysis of normal skin (N), a subcutaneous tumor (S), a hamartomatous skin lesion (H), and the DOEJS producer cell line (R) was performed as described in Materials and Methods. The samples were prepared in duplicate, and separate duplicate nitrocellulose filters were differentially probed with either (A) an antibody broadly immunoreactive with most p21 proteins or (B) an antibody immunoreactive only with oncogenic p21 containing a valine substitution at amino acid 12.

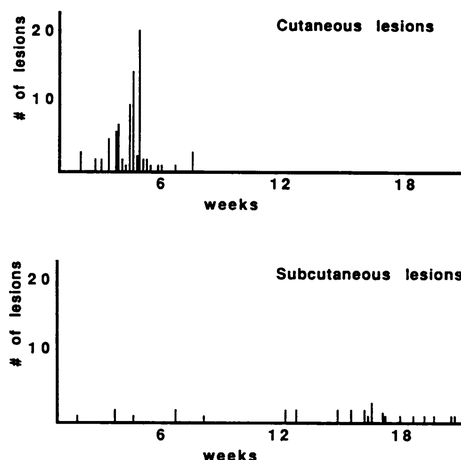

FIG. 4. Age of the animals when Ha-*ras*-induced cutaneous lesions and subcutaneous lesions appeared.

eliciting lesions. To exclude the possibility that recombination generated a helper virus, which could lead to spread subsequent to infection, 50 animals were tested for viremia by radioimmunoassay for p30 and were found to be negative (22). Because this is a sensitive assay for detection of infectious virus, our results indicate that no helper virus was generated.

The efficiency with which lesions were induced depended on the method of infection. While injection of concentrated virus promoted formation of lesions in 24% of the animals injected, only 14% of animals injected with producer cells developed lesions. Furthermore, multiple growths (with as many as six lesions observed on one mouse) were seen in 26% of the animals injected with concentrated virus stocks, in contrast to animals infected by the alternative method, in which only single lesions were detected. These results demonstrate that injection of concentrated virus into midgestation embryos is more effective in inducing lesions than injection of producer cells.

A total of 116 lesions were detected on 57 animals; 88 (76%) were cutaneous, and 28 (24%) were subcutaneous. Cutaneous lesions appeared as firm, raised, hairless areas on the skin of the head, back, limbs, and tail. Subcutaneous lesions, comprising firm, white tissue, most often appeared directly below the skin; however, three lesions were discovered in the peritoneal cavity. The first cutaneous lesions appeared at 1 week of age, with a median of 4 weeks, while the subcutaneous lesions appeared later, with a median of 16 to 19 weeks (Fig. 4). The median latency of lesions induced by mitomycin-treated producer cells was longer. This was possibly due to the less efficient method of infection, which resulted in only single hyperplasias and longer survival of the animals and therefore allowed the detection of late-appearing lesions. Alternatively, infection of embryo cells with virus produced by microinjected cells may actually occur later than the time of injection because the injected cells may survive for longer periods of time and continue to produce virus. This may influence the time that lesions appear in the animal. In either case, the long latency period observed from the time of infection of the embryos and the appearance of lesions with both methods of infection suggests that expression of *ras* alone is not sufficient to induce transformation; additional events are necessary to induce neoplastic growth in the animal.

Histologic analysis. Light microscopic analysis of the lesions revealed that the majority consisted of benign neoplasms (88 of 108 examined), while the rest were malignant (20 of 108). The histologic classification of the lesions is summarized in Table 2, and representative lesions are shown in Figure 5.

The benign lesions could be divided into two major categories. The most common type (47%) were tumors of the epidermal appendages. The majority of these appendage tumors exhibited differentiation of hair follicle elements and were composed of horn cysts, consisting of a fully keratinized center surrounded by flattened layers of basophilic cells, as well as islands composed of basophilic cells arranged in solid aggregates and intricate lacelike networks (Fig. 5b). In some cases, a high degree of differentiation was observed with abortive hair papillae formation. In a few cases tumors exhibited differentiation toward eccrine sweat glands or sebaceous glands or had mixed features of differentiation toward hair and sebaceous and eccrine glands. Some of the latter appeared as abnormal proliferations (hamartomatous) of hair follicle elements (Fig. 5d).

The next most common type of lesion (36%) involved hyperplasia of the surface epithelium (Fig. 5c). Approximately half of these lesions exhibited benign proliferation of

TABLE 1. Effect of injecting midgestation embryos with DOEJS or *neo* retrovirus

Virus injected	Gene introduced	Infection level	No. of embryos injected	No. (%) of animals		
				Alive after 1 wk	With lesions	With multiple lesions
DOEJS	Ha-*ras*/*neo*	1,000 cells	258	118	17 (14)	0
		5,000 CFU	421	234	57 (24)	26 (11)
MP10	*neo*	1,000 cells	302	148	0	0
		5,000 CFU	88	56	0	0

TABLE 2. Frequency of different hyperplasias induced by the *ras* virus[a]

Type of lesion	Tumor type	Specific tumor(s)	No. observed
Benign	Tumors of the surface epithelium		
	Epidermal hyperplasia	Hyperkeratotic seborrheic keratosis, epidermal nevus	10
	Atypical keratinocyte proliferation, premalignant	Hypertrophic actinic keratosis	15
	Epidermal cysts		4
	Tumors of epidermal appendages	Trichoepithelioma, tricholemmoma	
	Tumors with hair follicle element differentiation		26
	Tumors with eccrine sweat gland differentiation		3
	Tumors with sebaceous gland differentiation		1
	Mixed tumors containing hair and eccrine and sebaceous gland elements		14
	Other		
	Mesenchymal cell proliferation		6
	Nevus cell proliferation	Nevocellular nevi	4
	Endothelial cell proliferation	Hemangioma	1
Malignant	Spindle cell neoplasms	High-grade sarcomas or spindle cell squamous cell carcinomas	20

[a] Of the 125 lesions, 108 were analyzed: 88 of the cutaneous lesions and 20 of the subcutaneous lesions.

the epithelium with marked laminated hyperkeratosis, papillary epidermal hyperplasia, proliferation of basaloid cells, and horn cyst formation. Other lesions showed atypical keratinocyte proliferation and were remarkable for pleomorphism of large irregular hyperchromatic nuclei in the basal layers of the epidermis and loss of polarity of cells in the basal layer, indicating a premalignant phenotype. In some lesions both hyperplasia of surface epithelium and epidermal appendage elements were seen, while others showed benign proliferation of mesenchymal cells, nevus cells (which are of neuroectodermal origin; Fig. 5e), or endothelial cells.

All 20 malignant lesions were subcutaneous and contained abundant mitotic figures, including atypical mitoses (Fig. 5f). They all had remarkably similar histology, which consisted primarily of elongated spindle-shaped cells with elongated nuclei, arranged in clusters, whorls, and cartwheel formations. In addition, nodular areas of epithelioid polygonal cells were also present. No keratin or collagen production was observed, making it difficult to determine whether these tumors were spindle-celled squamous cell carcinomas, amelanotic melanomas, or high-grade sarcomas. Immunohistochemical analyses were negative for myoglobin and desmin, excluding a smooth or skeletal muscle cell origin, and for S100, excluding a neuroectodermal cell origin. Similarly, the tumors did not stain with antibodies that recognized keratins in more differentiated epithelial cells in mouse skin, but stained positively for vimentin. Thus, with the antibodies available, immunohistochemical studies were not conclusive. Electron microscopic analyses of the lesions revealed no melanosomes but in one case clearly showed desmosome structures, indicating an epithelial origin for this lesion. Because tissue was not available to analyze the other lesions by electron microscopy, it was not possible to determine whether they were of epithelial or fibroblastic origin.

In summary, our results indicate that the *ras* oncogene, when introduced into midgestation mouse embryos, induces proliferation of skin cells. Detailed histologic analyses suggested that in most cases cells of a single sublineage were induced to proliferate in a single lesion. Some tumors, however, contained elements of several sublineages, suggesting that a multipotential stem cell had been transformed. Alternatively, *ras*-transformed cells may induce the proliferation of other noninfected cells, giving rise to mixed tumors.

Detection of viral DNA. To correlate tumor formation with virus infection, DNA was isolated from 37 lesions, and the presence of the provirus was detected by Southern blotting analysis (31) (Fig. 6). Double digestion of the DNA samples with *Bam*HI and *Xba*I generated an internal fragment of 3.2 kb (as it does in the virus producer cells), indicating no gross rearrangement of the proviral structure (Fig. 6, lanes 2, 4, 6, 8, 10, 12, 14). To determine whether the lesions contained one or multiple proviral copies, the DNAs were digested with *Bam*HI alone, which cleaves the provirus once, generating a flanking sequence when probed with the *neo* gene (lanes 1, 3, 5, 7, 9, 11, 13). The results demonstrated that each lesion carried a single provirus. Moreover, in all cases in which multiple lesions from the same animal were analyzed, each lesion had a unique integration site, indicating that each growth was derived from independent infections (Fig. 6; e.g., the unique flanking fragments visualized in lanes 7 and 9 are from two lesions which developed on the same animal). In addition, the internal fragment generated by double digestion of the DNA with *Bam*HI and *Xba*I had an intensity roughly comparable to the intensity of the internal fragment of the DOEJS producer cell line, which contains a single integrated provirus.

Detection of viral RNA. RNAs isolated from 17 tumors were tested for the presence of viral transcripts by using dot blot analysis or Northern (RNA blot) hybridization techniques with a *neo* probe. Thirteen tumors were positive by dot blot analysis, 11 of which were analyzed by Northern analysis and showed the presence of viral RNA. RNA from normal livers of the same animals contained no detectable viral transcripts. The integrity of the RNA samples was assessed by hybridization to a collagen or actin probe. Northern blotting analysis of the strongly hybridizing samples detected viral RNA of approximately 5 kb, as expected from the DOEJS virus (Fig. 7).

Detection of mutant *ras* protein. To demonstrate that the

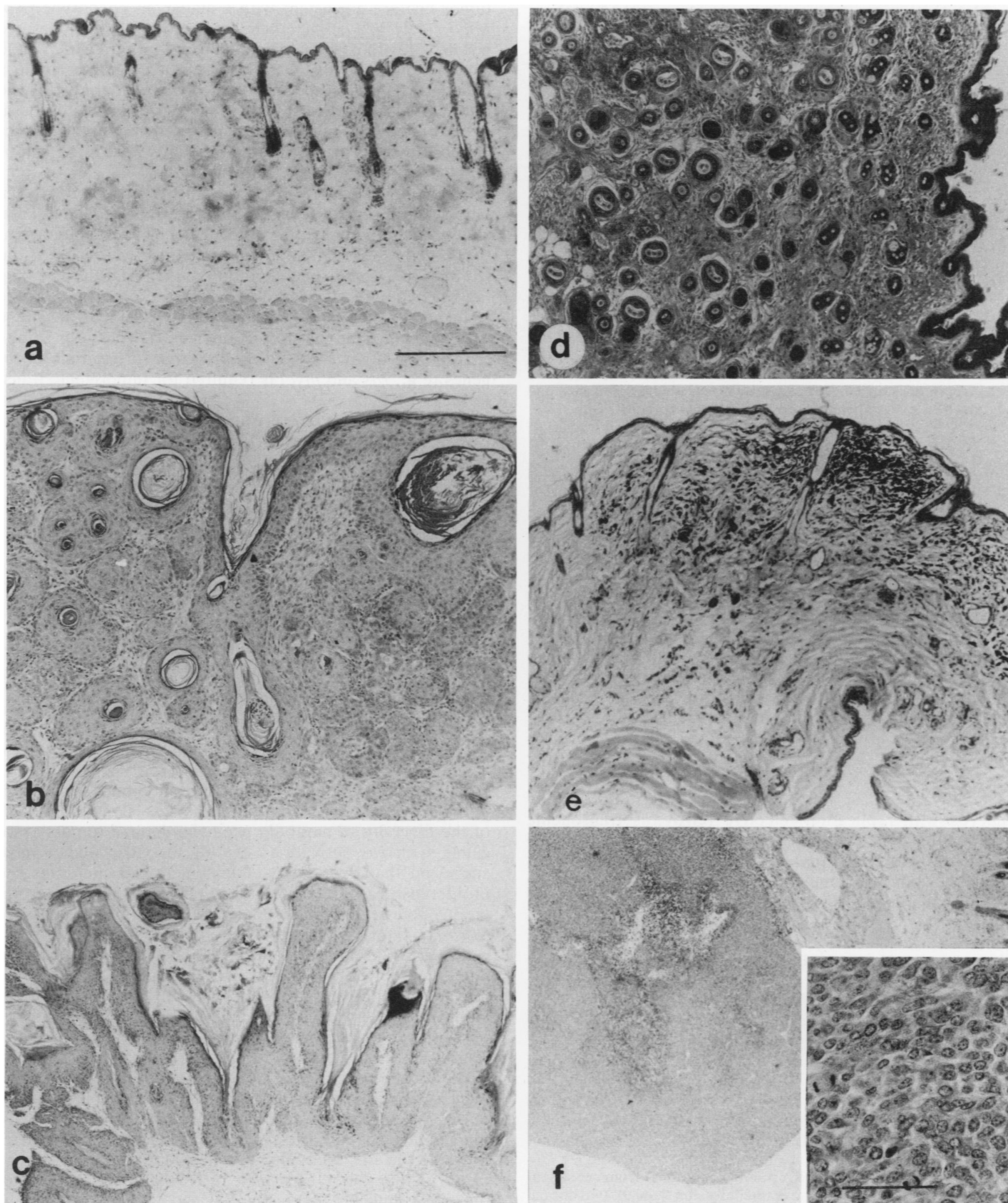

FIG. 5. Histology of representative lesions obtained from infected mice. (a) normal mouse skin; note thin surface epithelial layer. (b) Benign epidermal appendage tumor exhibiting hair follicle element differentiation. (c) Benign tumor of surface epithelium, showing epidermal hyperplasia and hyperkeratosis. (d) Benign epidermal appendage tumor exhibiting abnormal (hamartomatous) proliferation of hair follicle elements. (e) Benign tumor exhibiting nevus cell proliferations. (f) Malignant subcutaneous tumor. Inset shows cell morphology and mitotic figures at higher magnification. (A–F) Bar, 500 μm; (F inset) bar, 50 μm.

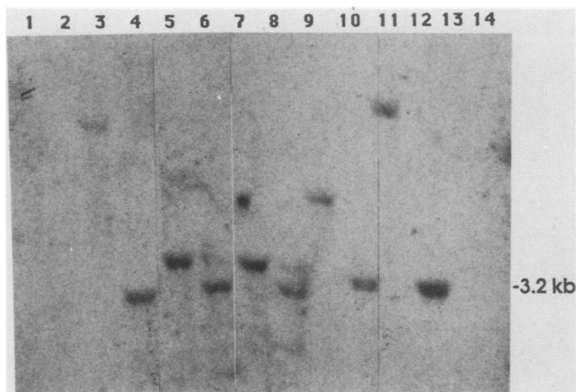

FIG. 6. Detection of proviral sequences in lesions. DNA from the kidney (lanes 1 and 2), several skin lesions (lanes 3–10), the DOEJS producer cell line (lanes 11 and 12), or ψ2 cells (lanes 13 and 14) were digested with *Bam*HI (lanes 1, 3, 5, 7, 9, 11, 13) or *Bam*HI and *Xba*I (lanes 2, 4, 6, 8, 10, 12, 14). Digested DNA was subjected to electrophoresis on a 0.7% agarose gel, blotted to nylon filters, and hybridized to a ^{32}P-labeled *Bam*HI-*Eco*RI *neo* fragment (Fig. 1).

viral transcripts produced the expected gene product p21, cell lysates prepared from two lesions large enough for biochemical analysis were assayed by Western blotting. Figure 3A illustrates that an antibody cross-reactive with normal and abnormal p21 proteins detected p21 in a cutaneous lesion (the hamartomatous appendage tumor shown in Fig. 5d) and a subcutaneous lesion (Fig. 5f) as well as in normal skin and the DOEJS virus producer cells. However, when an antibody directed against the oncogenic p21 (specifically immunoreactive with a valine substitution at amino acid 12) was used, only the cutaneous lesion, the subcutaneous lesion, and the DOEJS virus producer cells had the expected band corresponding to p21 (Fig. 3B).

DISCUSSION

Our study demonstrates that infection of postimplantation embryos with replication-defective retrovirus transducing the *ras* oncogene resulted in efficient induction of skin lesions. None of the cutaneous lesions but all of the subcutaneous lesions were malignant, as judged from their histologic appearance. The successful culture of several subcutaneous lesions and subsequent tumor induction of one tumor line are consistent with a malignant phenotype. Approximately 20% of the cutaneous lesions exhibited features consistent with a premalignant state (pleomorphism of large irregular nuclei in the lower layers of the epidermis and loss of cellular polarity, especially in the lower epidermis). In one out of four attempts, we were able to transplant the basal cells of a skin lesion and obtain regrowth of the lesion. Overall, approximately 32% of the lesions were malignant or premalignant.

The skin lesions in our experiments appeared after a latency of several weeks. Because retroviruses have been shown to be actively transcribed in postimplantation embryos, cells infected by the virus are expected to express the *ras* oncogene following integration of the provirus. The long latency period of several weeks before the appearance of the lesions suggested that *ras* alone is not sufficient to induce even premalignant transformation in vivo. Thus, our results corroborate other reports that activation of multiple oncogenes (e.g., *ras* and *myc*) is required for tumorigenic transformation (18, 28, 33).

Southern analysis of individual lesions indicated a monoclonal origin in all cases. In particular, multiple lesions arising in one animal carried different proviruses, indicating that separate infections with virus initiated the cellular proliferation leading to each lesion. Because infectious retroviruses are unstable at 37°C, it is likely that provirus integration occurred soon after the time of in utero injection and not at a later stage in embryonic development. A later infection would be expected to occur if replication-competent helper virus had been formed by recombination, as has been seen with ψ2 cell-derived vectors in several instances (20). All sera from the infected animals were negative for viremia, as assessed by radioimmunoassay for p30, which is a sensitive test that detects replication-competent helper virus in the animal (22). Our results therefore indicate that infection of a presumptive epidermal cell at midgestation with a single *ras* virus can result in a hyperplastic response in the postnatal animal.

The predominance of skin transformation upon transduction of the *ras* oncogene into the embryos is unusual in light of the diversity of naturally occurring human tumors associated with *ras* activation (13, 36). In vitro studies have suggested that expression of oncogenic *ras* increases the proliferative capacity of keratinocytes (40, 41). One interpretation of our results is that epidermal cells are more sensitive to *ras* transformation in the developing embryo than other cell types. Alternatively, our results could reflect a technical limitation of the assay system, in which skin cells are infected at a greater frequency due to the physical constraints of the injection procedure. We favor the former possibility because other studies have demonstrated that retroviral vectors can transduce genes into cells of many tissues of the developing embryo (29, 35). Furthermore, the high incidence of skin tumors early in the experiment reduces the number of animals which are maintained for 6 months and thereby causes rapid tumor types to be overrepresented in our final analysis. In addition, recent experiments in our laboratory with retroviruses carrying other oncogenes indicate that neoplasias can arise in many organs following injection of various vectors into midgestation embryos. Our results therefore suggest that while cells other than those of the skin are infected and presumably express the oncogene, the skin cells are the most susceptible target cells for *ras*-induced transformation in midgestation embryos.

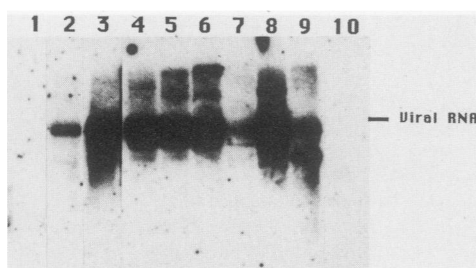

FIG. 7. Detection of viral RNA in lesions. RNA from six subcutaneous tumors (lanes 3–8), one epithelial lesion (lane 9), two normal liver samples (lanes 1 and 10), and the DOEJS producer cell line (lane 2) were analyzed by Northern blotting analysis for viral RNA. A radiolabeled *neo* probe was used to detect viral sequences. A composite of two gels (lanes 1–3 and lanes 4–10) which were electrophoresed, transferred to nitrocellulose, and probed in parallel is shown. The expected viral transcript of 4.9 kb was detected (compare Fig. 1).

The *ras* oncogene has been expressed from a variety of tissue-specific promoters in transgenic mice (2, 14, 25, 33). In these studies, the promoter controlling the oncogene is activated in many or all cells of a given tissue, and the rapid premalignant hyperplasia observed may not be due to clonal expansion of a single transformed cell but rather to abnormal growth of a group of cells. In contrast, after injection of *ras* virus into midgestation embryos, only single cells are infected, always resulting in monoclonal premalignant lesions. As human cancers are frequently clonal outgrowths of cells (4, 11), our model recreates the normal situation in which a single infected cell has to undergo transformation and clonal outgrowth while surrounded by normal cells. Normal cells have in fact been shown to repress the transformed phenotype of cells expressing oncogenes (10a). Transduction of oncogenes into midgestation embryos may provide a model which mimics the pathogenesis of naturally occurring tumors, in which a cell with a spontaneous mutation expands clonally to form a tumor in the presence of normal tissue.

The importance of the *ras* oncogene for malignant transformation of skin cells in the adult animal has been demonstrated by mutagenesis as well as transduction experiments. Both mutational conversion of c-*ras* to the activated oncogene and the introduction of an activated *ras* into skin cells were shown to be essential events early in the process of epidermal cell transformation (5, 27, 40, 41). This initiation step, however, required additional treatment with tumor promoters for realization of the premalignant and continued progression to the malignant phenotype. In contrast, transgenic animals carrying the bovine papillomavirus genome (18) developed benign skin lesions without promoter treatment after a long latency period. Likewise, the introduction of the *ras* oncogene into midgestation embryos induces skin tumor formation without subsequent treatment with tumor promoters. This may be due to the rapid proliferation and differentiation of the fetal tissue expressing the introduced gene, which renders the cells more vulnerable to the subsequent spontaneous events resulting in neoplastic growth, while the lower proliferative rate of adult skin may necessitate tumor promoters. However, because 84% of the skin lesions analyzed were benign, clearly other alterations are needed to elicit a malignant phenotype. We believe that the fetal transformation system described in this paper will be useful as a rapid model for studying the multiple and complex interactions of oncogenes in the developing embryo.

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