Oncogenes in Radioresistant, Noncancerous Skin Fibroblasts from a Cancer-Prone Family

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Li-Fraumeni syndrome is manifested in a variety of neoplasms that are transmitted in a dominantly inherited pattern. The noncancerous skin fibroblasts of family members exhibit a unique characteristic of being resistant to the killing effect of ionizing radiation. A three- to eightfold elevation in expression of c-*myc* and an apparent activation of c-*raf*-1 gene have been observed in these noncancerous skin fibroblasts. These results may provide insight into the heritable defect underlying the familial predisposition to a variety of cancers.

THE ROLE OF GENETIC FACTORS IN carcinogenesis may be clarified by molecular studies of cancer-prone families. In the cancer family syndrome originally described by Li and Fraumeni, a constellation of cancers (sarcomas, breast and other carcinomas, brain tumors, and leukemias) are found in a dominantly inherited pattern (*1*). The diverse neoplasms tend to arise in young people and as multiple primaries in the same individual, and sometimes they appear related to carcinogenic exposures including ionizing radiation. One such cancer-prone family with some radiogenic tumors was found to have an unusual radiation-resistant (RR) phenotype in noncancerous skin fibroblasts (*2, 3*). This study independently confirms the RR phenotype and documents altered expression of specific human oncogenes in the skin fibroblasts of cancer-prone individuals. A partial pedigree of the cancer-prone family under study is shown in Fig. 1A. The malignancies in this family appeared across six generations in a pattern that suggests incomplete penetrance of an autosomal dominant genetic defect with pleiotropic effects (*2*). No karyotypic abnormalities have been identified. As previously reported (*3*), skin fibroblasts from normal forearm skin biopsies of several family members were resistant to the lethal effects of γ irradiations (RR). The skin fibroblast line (2800T) of family member IV-19, who has polycythemia vera, has shown the highest level of in vitro radiation resistance among the affected family members. A skin fibroblast line (1872T) derived from a spouse (V-7) served as a control. To independently assess the level of RR in cell lines 2800T, 1872T, and NIH/3T3 in our laboratory, we determined survival (measured as colony-forming ability) after exposure to γ radiation (*3, 4*). The average D_{10} values (radiation dose required to reduce survival to 10%) of cell lines 2800T (affected), 1872T (unaffected spouse), and NIH/3T3 were determined in three separate assays to be 524, 438, and 429 rads, respectively (Fig. 1B). The first two values are comparable to those obtained by Bech-Hansen *et al.* (*3*). The D_{10} value of NIH/3T3 cells is approximately the same as that of the normal human controls. The difference in D_{10} values between 2800T and 1872T or NIH/3T3 is about 80 to 90 rad, which is a statistically significant difference ($P < 0.001$ by t test) (Fig. 1B).

To detect abnormal oncogene expression in skin fibroblasts of family members, cytoplasmic RNAs were isolated and analyzed by Northern blot and dot blot hybridization. Ten oncogene probes—H-*ras* (*5*), K-*ras* (*5*), N-*ras* (*6*), *sis* (*7*), *erb*B (*8*), *myb* (*9*), *fos* (*10*), *fes* (*11*), *raf* (*12*), and c-*myc* (*13*)—were used in this study. No transcripts of abnormal size were detected with these probes in the RNA from fibroblasts of clinically affected family members (2800T, 2824T, and 2675T), a normal spouse (1872T), or unrelated normal controls (4032T and 3064T). However, a quantitative difference in the expression of the transcripts specific for c-*myc* was apparent when cytoplasmic RNA from skin fibroblasts of family members was compared with that from spousal or unrelated normal human fibroblasts (Fig. 2A). Quantitative dot blot analysis revealed a three- to eightfold in-

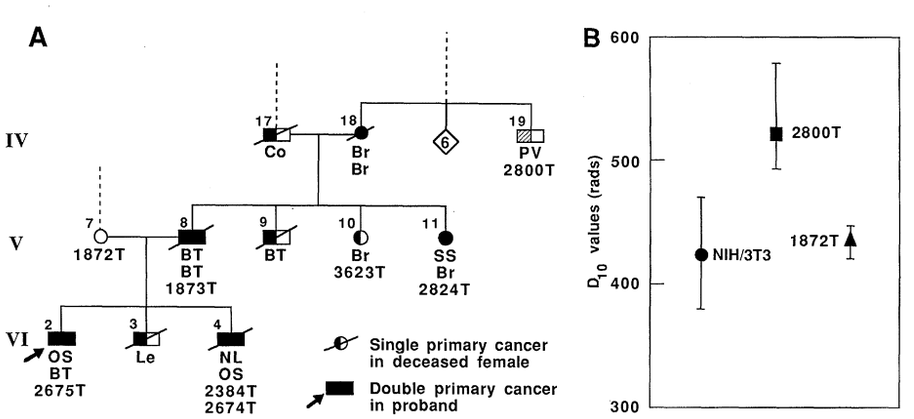

Fig. 1. (**A**) Partial pedigree of cancer-prone family. The family studied has the Li-Fraumeni syndrome characterized by tumors of diverse sites including sarcomas, leukemias, brain tumors, and some epithelial sites (particularly breast, lung, and larynx). Shown here is a branch of a much larger pedigree that traces cancers over five generations in three separate lineages from a woman who died with breast cancer in 1865 (*2*). New cancers that have developed in this branch of the family, since last reported in 1981 (*3*), include a new primary glioma in the proband VI-2 who had survived osteosarcoma for 12 years, a second primary fatal brain tumor in the father of the proband (V-8), a fatal left-side adenocarcinoma of the colon in the paternal grandfather of the proband, a primary breast adenocarcinoma in a paternal aunt of the proband (V-10), and a new primary breast carcinoma in another aunt (V-11) who had survived leiomyosarcoma of the leg for 9 years. Normal skin fibroblast cell strain numbers from single or multiple separate biopsies are shown as "T" numbers. Hatched area of IV-19 indicates that polycythemia vera (PV) is a premalignant neoplasm. Abbreviations: Co, colon; Br, breast cancer; BT, brain tumor; SS, soft tissue sarcoma; OS, osteosarcoma; Le, leukemia; and NL, bilateral malignant neurilemoma. (**B**) Scattergram of the D_{10} values of fibroblast cells after graded doses of γ radiation. Monolayer cultures of each cell line were harvested and suspended in fresh medium (Ham's F12 supplemented with 10% fetal bovine serum) at 1×10^5 cells per 10 ml and irradiated with graded doses of ^{60}Co γ rays at room temperature; cells were diluted and plated at a concentration of 1,000 to 16,000 cells per 75-cm^2 flask. The resulting colonies were stained and counted approximately 21 days later. The scattergram of D_{10} values represents mean values from three experiments for 2800T and NIH/3T3 and from one experiment for 1872T. Error bars represent the minimum and maximum values.

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crease in expression of c-*myc* when cytoplasmic RNA from affected family members was compared to corresponding controls (Fig. 2C). No significant differences were detected in transcript levels with the probes for the other nine oncogenes. Hybridizations of the same blots were also performed with a probe for human actin; the results suggest that the amounts of cytoplasmic RNA samples in each lane were comparable (Fig. 2B). On the basis of limited genomic Southern blots of the high molecular weight DNA from family member fibroblasts, there appeared to be no amplification or obvious rearrangement for any of the genes probed.

A possible connection between the RR phenotype and c-*myc* amplification has been provided by studies on variant lines of "oat cell" lung carcinoma that display in vitro a marked resistance to x-irradiation (*14*). These cells also appear to have elevated levels of c-*myc* RNA and a highly amplified c-*myc* gene (20- to 76-fold) (*15*).

The induction, maintenance, and progression of malignancy may require the action of more than one oncogene. While cancer-prone family members may exhibit an alteration in certain oncogenes, a further alteration in target tissue may be required for the actual development of cancer. Since there is no obvious cytogenetic abnormality associated with the familial susceptibility, we examined the transforming potential of the DNA isolated from noncancerous skin fibroblasts derived from the most radioresistant family member and a normal spouse. The high molecular weight DNAs of 2800T and 1872T were cotransfected with pSV2neo plasmid. Cells resistant to G418 were propagated and injected into nude mice (*16*). Morphological transformation of the fibroblast cells was not a selection criterion before inoculation into nude mice. Thus, tumor development in nude mice was the first indication of the possible existence of human transforming sequences. The presence of human repetitive sequences in a significant proportion of primary nude mouse tumor (2800T-1°) DNAs was confirmed with a human Alu probe (*17*). The primary transfectant NIH/3T3 tumor DNA was isolated and used in a second round of transfection. The resultant tumor DNA exhibited a simplified, human Alu banding pattern after Eco RI–Hind III digestion (Fig. 3A). Two secondary mouse tumors displayed similar human Alu patterns, which suggests that the transformants may have an identical portion of the human genome. High molecular weight DNA from 1872T did not produce any Alu$^+$ nude mouse tumors.

Southern blots that contained Eco RI–Hind III–digested DNA from the primary and secondary mouse tumors were examined with several ^{32}P-labeled probes for oncogenes. The first seven oncogenes tested (H-*ras*, K-*ras*, N-*ras, sis, src, fos*, and c-*myc*) hybridized to mouse endogenous bands but not to additional bands and were thus considered negative in hybridization. However, strong homology was detected between one of the human Alu bands (3.5 kb) and the *raf* probe (*12, 18*) (Fig. 3B). This Alu$^+$ *raf*$^+$ band was seen in DNA from three primary and two secondary mouse tumors (Table 1). In addition, a less prominent band of 1.8 kb was seen with the *raf* probe. In a separate experiment, both of these bands were observed to be present in human DNA of the parental skin fibroblasts and an unrelated normal control (Fig. 4). A third *raf* band (2.8 kb) observed in the Hind III–digested human DNA corresponds to c-*raf*-2, a pseudogene (*12*) (Fig. 4). In this experiment, the mouse endogenous *raf* band is around 18 kb in size. Furthermore, in a preliminary experiment, a cell line derived from one of these *raf*$^+$ tumors displayed an increase in radiation resistance relative to that of NIH/3T3 cells. The D_{10} value of this cell line was elevated to approximately that of the parental 2800T cell line (*19*). These results suggest that an activated human c-*raf*-1 gene arising from normal skin fibroblast DNA of the RR, but not from the normal spouse control DNA, is present in the primary and secondary nude mice tumors. The human c-*raf*-1 gene is most likely to be present in the activated form in its parental cell line 2800T. A 5′ truncation has been proposed to be the mechanism for activating c-*raf*-1 (*20*). It is conceivable that a mutation leading to an increased tendency toward c-*raf* truncation may be an inherited defect in the family under study.

It has been reported that the concomitant expression of *raf* and *myc* oncogenes in hematopoietic and epithelial cells alters their respective transforming activities. The synergistic effect involved in tumor development has been demonstrated in vivo and in vitro (*20, 21*). Cleveland *et al.* have speculat-

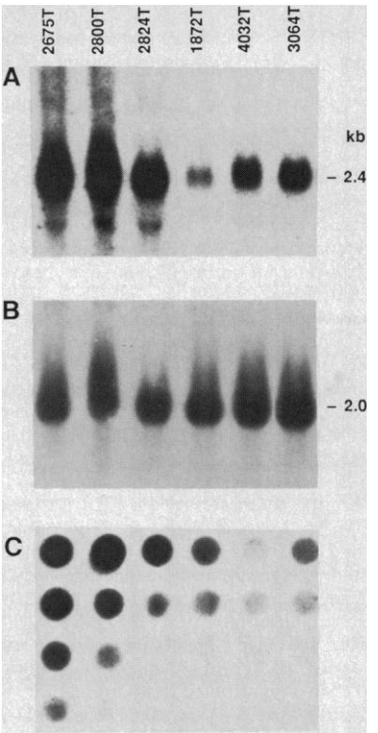
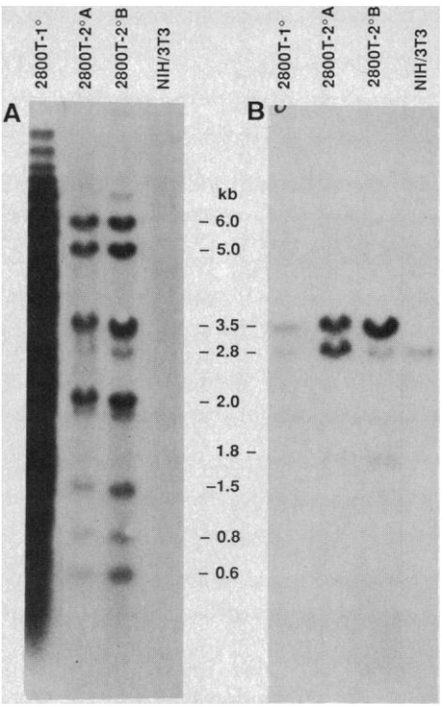


Fig. 2. Northern and dot blot analysis of cytoplasmic RNA from skin fibroblasts of family members and controls. (**A**) Cytoplasmic RNAs (30 μg per lane) were separated by electrophoresis in a 1% formaldehyde–agarose gel (*30*), transferred to nitrocellulose membrane (*16*), and hybridized to the cloned third exon of human c-*myc* labeled with ^{32}P-dCTP and d-TTP by nick translation (*31*). Affected family members are 2800T, 2675T, and 2824T; normal controls are 1872T, 3064T, and 4032T. (**B**) The same blot hybridized with ^{32}P-labeled (*31*) human pseudo β actin probe after removal of the c-*myc* probe, to demonstrate that approximately equal amounts of RNA were applied and transferred in each lane. (**C**) Samples (10, 5, 2.5, or 1.25 μg) were spotted onto nitrocellulose and hybridized to the radiolabeled human c-*myc* third exon probe. Cytoplasmic RNA was isolated by the Triton-phenol-chloroform method (*32*). Hybridizations were performed at 40°C in a solution of 50% formamide, 0.1% Denhardt's solution (*33*), 5× SSC, 0.0*1M* tris (*p*H 7.5), salmon sperm DNA (0.25 mg/ml), and 10% dextran sulfate for 16 to 18 hours. Blots were washed with 2× SSC–0.1% SDS three times at room temperature followed by one or two washes at 50°C in 0.1× SSC–0.1% SDS, and then autoradiographed at −70°C.

Table 1. Summary of transfection–nude mouse experiments with high molecular weight DNA from noncancerous skin fibroblast cell lines 2800T and 1872T. A total of 1000 μg of each DNA was cotransfected with pSV2neo plasmid into NIH/3T3 cells, selected with G418, and the survivors were injected into 6- to 8-week-old female athymic nude mice. Tumor-bearing animals were killed and high molecular weight DNA was obtained from the tumors and analyzed for the presence of human sequences. DNA from Alu$^+$ mouse primary tumors were used in a second round of transfection, injection, and analysis. All Alu$^+$ and primary and secondary tumors were probed with the oncogenes listed in the text.

Source of DNA	Primary nude mice tumors			Secondary nude mice tumors		
	Total	Human Alu$^+$	Human *raf*$^+$	Total	Human Alu$^+$	Human *raf*$^+$
2800T	20	12	3	4	2	2
1872T	7	0	0			

Fig. 3. Southern blot analysis of high molecular weight DNA from primary and secondary mouse tumors and NIH/3T3 cells. (**A**) Samples (2 μg of 2800T-1° and 4 μg of each secondary and NIH/3T3 DNA) were digested with Eco RI and Hind III, separated by electrophoresis on a 0.6% agarose gel, transferred to nylon membrane (*34*), and hybridized to a ^{32}P-labeled (*31*) Blur 8 human Alu probe (*17*). Sizes of Alu$^+$ bands in kilobases in secondary mouse tumors are shown. (**B**) Identical blot as in (A) hybridized to a ^{32}P-labeled (*31*) v-*raf* probe (Oncor) after removal of the Alu signal. The label 2800T-1° indicates primary mouse tumor DNA; 2800T-2°A and 2800T-2°B indicate two separate secondary mouse tumors. High molecular weight DNA, obtained by the procedure of Gross-Bellard *et al.* (*35*) from skin fibroblast line 2800T was cotransfected with pSV2neo plasmid (*16*) and selected with G418. Surviving colonies were injected into 6- to 8-week-old female athymic nude mice. Tumor-bearing animals were killed when the tumor was 0.5 to 1 cm in diameter. High molecular weight DNA was obtained from the tumor and digested with the appropriate restriction enzyme, according to suppliers' directions, then separated by electrophoresis on a 0.6% agarose gel, and transferred to nylon membrane (*34*). Hybridization with a ^{32}P-labeled (*31*) probe was performed at 40°C in a solution of 50% formamide, 0.1% Denhardt's (*33*), 5× SSC, 0.01*M* tris (*p*H 7.5), salmon sperm DNA (0.25 mg/ml), and 10% dextran sulfate for 16 to 18 hours. The blot was washed with 2× SSC–0.1% SDS three times at room temperature followed by one or two washes at 50°C in 0.1× SSC–0.1% SDS, then autoradiographed at −70°C. DNA from Alu$^+$ tumors was then used in a second round of transfection and injection into mice.



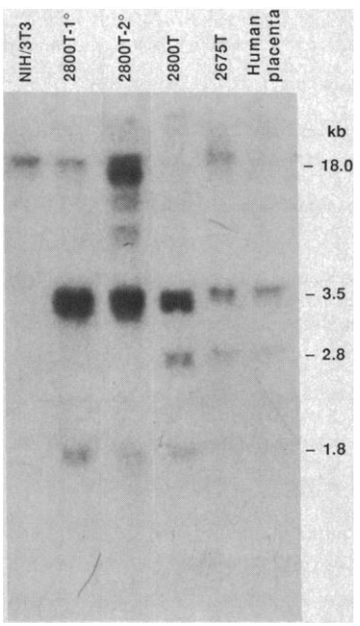


Fig. 4. Detection of human specific *raf* sequences in primary and secondary mouse tumors derived from transfection of high molecular weight DNA of 2800T and 2800T-1° and injection into nude mice. A 4-μg sample of each DNA was digested with Hind III and separated by electrophoresis on a 0.6% agarose gel, transferred to nylon membrane (*34*), and hybridized to a ^{32}P-labeled (*31*) v-*raf* probe. Sizes of *raf*$^+$ bands in kilobases are indicated. Labels: 2800T and 2675T are noncancerous skin fibroblast cell lines from affected family members, 2800T-1° is a primary mouse tumor, and 2800T-2° is a secondary mouse tumor.

ed that *myc* provides competence signals and *raf* progression signals, which are required for cell growth regulation (*21*). Although in our study *raf* expression is not elevated as is *myc* in family member skin fibroblasts (*19*), a presumed qualitative change in *raf* may contribute to derangement in the progression signal pathway. The relationship, if any, between the elevation of *myc* expression and the activation of *raf* is yet to be established.

The unusual diversity of malignancies in the family points to a fundamental defect in the regulation of cell growth of epithelial and mesenchymal tissue (Fig. 1A). A molecular basis for this phenotype is suggested by the elevation of c-*myc* expression and the activation of *raf* found in the normal skin fibroblasts of an affected family member. Furthermore, recent studies by Kasid *et al.* (*22*) suggest a role of activated *raf* in tumor DNAs from RR head and neck tumors in NIH/3T3 transfectants. Whereas studies of radioresistance in both the oat cell carcinoma of the lung and tumors of the head and neck were conducted on RR tumor samples whose cells were potentially selected by in vivo radiation exposure, we used cells from normal skin fibroblasts. Needleman *et al.* (*23*) found no evidence of transforming activity with NIH/3T3 cells in normal fibroblast lines derived from individuals with familial genetic cancer syndromes including familial site-specific carcinomas, Gardner's syndrome, xeroderma pigmentosum, and von Recklinghausen's syndrome. In contrast to their results, however, we found that the skin fibroblasts from individuals with the familial multiple cancer Li-Fraumeni syndrome displayed a transforming phenotype that may be related to the susceptibility state.

In hereditary cancer, it is hypothesized that the presence of inborn genetic defects lessens the requirement for subsequent somatic events as compared to nonhereditary forms of cancer (*24–29*). When a susceptibility gene supplies a mutational event to a variety of tissues, the array of neoplasms as seen in the Li-Fraumeni cancer family syndrome may be influenced by the prevailing carcinogenic exposures or oncogenic events affecting particular somatic cells. The inherited mutation in such cases may involve the set of genes related to cellular regulation or tissue differentiation. Our findings suggest that the inborn defect predisposing to diverse cancers is expressed by at least two abnormalities, elevated c-*myc* expression and activated *raf* gene in normal skin fibroblasts of family members.

REFERENCES AND NOTES

1. F. P. Li and J. F. Fraumeni, Jr., *Ann. Intern. Med.* **71**, 747 (1969).
2. W. A. Blattner *et al.*, *J. Am. Med. Assoc.* **241**, 259 (1979).
3. N. T. Bech-Hansen *et al.*, *Lancet* **1981-I**, 1335 (1981).
4. M. C. Paterson, A. K. Anderson, B. P. Smita, P. T. Smith, *Cancer Res.* **39**, 3725 (1979).
5. E. H. Chang, M. Gonda, R. Ellis, E. Scolnick, D. Lowy, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 4848 (1982).
6. E. Taparowoky, K. Shimzu, M. Goldfarb, M. Wigler, *Cell* **34**, 581 (1983).
7. S. G. Devare, E. P. Reddy, J. D. Law, K. C. Robbins, S. A. Aaronson, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 731 (1983).
8. T. Yamamoto *et al.*, *Cell* **35**, 71 (1983).
9. K. H. Klempnauer, T. J. Gonda, J. N. Bishop, *ibid.* **31**, 453 (1982).
10. F. Van Straaten, R. Muller, T. Curran, C. Van Beveren, I. M Verma, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3183 (1983).
11. M. Barbacid, A. V. Lauver, S. G. Devare, *J. Virol.* **33**, 196 (1980).
12. T. Bonner *et al.*, *Science* **223**, 71 (1984).
13. P. L. Rothberg, M. D. Erisman, R. E. Diehl, U. G. Rovigatti, S. M. Astrin, *Mol. Cell. Biol.* **4**, 1096 (1984).
14. D. N. Carney, J. B. Mitchell, T. J. Kinsella, *Cancer Res.* **43**, 2806 (1983).
15. C. D. Little, M. M. Nau, D. N. Carney, A. F. Gazdar, J. D. Minna, *Nature (London)* **306**, 194 (1983).
16. O. Fasano, D. Birnbaum, L. Edlund, J. Fogh, M. Wigler, *Mol. Cell. Biol.* **4**, 1695 (1984).
17. W. R. Jelinek *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 77, 1398 (1980).
18. T. Bonner *et al.*, *Mol. Cell. Biol.* **5**, 1400 (1985).
19. E. H. Chang and K. F. Pirollo, unpublished data.
20. U. R. Rapp, J. L. Cleveland, T. I. Bonner, in *Handbook of Oncogenes*, P. Reddy, T. T. Curren, A. Skalka, Eds. (Elsevier, New York, in press).
21. J. L. Cleveland *et al.*, *J. Cell. Biochem.* **30**, 195 (1986).
22. U. Kasid, A. Pfeifer, R. R. Weichselbaum, A. Dritschilo, G. E. Mark, *Science* **237**, 1039 (1987).
23. S. W. Needleman, Y. Yuasa, S. Srivastava, S. A. Aaronson, *ibid.* **222**, 173 (1983).

24. A. G. Knudson, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 820 (1971).
25. ———, *Semin. Oncol.* **5**, 57 (1978).
26. ———, L. C. Strong, D. E. Anderson, *Prog. Med. Genet.* **9**, 113 (1973).
27. J. F. Fraumeni, Jr., in *Cancer Medicine*, J. F. Holland and E. Frei, Eds. (Lea and Febiger, Philadelphia, 1982), pp. 5–12.
28. L. C. Strong, in *Cancer Epidemiology and Prevention*, D. Schottenfeld and J. F. Fraumeni, Jr., Eds. (Saunders, Philadelphia, 1982), pp. 506–516.
29. J. J. Mulvihill, in *Genetics of Human Cancer*, vol. 3 of *Progress in Cancer Research and Therapy*, J. J. Mulvihill, R. W. Miller, J. F. Fraumeni, Jr., Eds. (Raven, New York, 1977), pp. 137–143.
30. H. Lehrach, D. Diamond, J. M. Wozney, H. Boedtker, *Biochemistry* **16**, 4743 (1977).
31. P. W. J. Rigby, M. Diechmann, C. Rhodes, P. Berg, *J. Mol. Biol.* **113**, 237 (1977).
32. R. N. Bastos, Z. Volloch, H. Aviv, *ibid.* **110**, 191 (1977).
33. D. Denhardt, *Biochem. Biophys. Res. Commun.* **23**, 641 (1966).
34. E. M. Southern, *ibid.* **98**, 503 (1975).
35. M. Gross-Bellard, P. Oudet, P. Chambon, *Eur. J. Biochem.* **35**, 32 (1973).
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The *raf* Oncogene Is Associated with a Radiation-Resistant Human Laryngeal Cancer

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In order to identify the genetic factors associated with the radiation-resistant human laryngeal carcinoma cell line (SQ-20B), tumor cell DNA was transfected into NIH/3T3 cells. A high incidence (six out of six) of *raf* sequences was found in transfected NIH/3T3 clones and the tumorigenic potential of SQ-20B DNA could be linked to genomic fragments that represent most of the kinase domain of human c-*raf*-1. An apparently unaltered 3.5–kilobase pair (kb) human c-*raf* transcript was identified in SQ-20B cells but was not observed in the transfected NIH/3T3 cell clones. Two new transcripts (4.2 kb and 2.6 kb) were found in tumorigenic clones; the large transcript was missing in a very poorly tumorigenic clone. Cytogenetic analysis indicated that the normal autosomes of chromosome 3 were absent in SQ-20B karyotypes and had formed apparently stable marker chromosomes. Unlike the recipient NIH/3T3 cell line, 30 percent of the transformed clone-1 metaphases had minute and double-minute chromosomes representative of amplified DNA sequences. The frequency of the c-*raf*-1 identification by NIH/3T3 transfection of SQ-20B DNA suggests the presence of some genetic abnormality within this locus.

CANCERS OF HEAD AND NECK ORIgin have been screened for viruses (*1*) and for oncogenes with little success (*2*). The transforming properties of many oncogenes, of which *raf* is one example, have been identified through DNA-mediated gene transfer (*3–5*). The tumor cell line SQ-20B (*6*) was established from the squamous cell carcinoma of the larynx of a patient who suffered progression of malignancy while undergoing a course of radiation therapy; this cell line is also radiation-resistant in culture.

In order to identify the transforming gene in SQ-20B, SQ-20B DNA (in the presence of pSV2neo DNA as a coselectable marker) was transfected into NIH/3T3 cells by calcium phosphate precipitation (*7*). The efficiency of transfection was greater than 100 clones per microgram of marker DNA. Colonies resistant to gentamycin at 400 μg/ml ($G418^r$) were subcloned 3 to 4 weeks after transfection. DNAs from ten transfected clones were digested with either Eco RI or Hind III and examined by Southern blot hybridization with a ^{32}P-labeled human Alu probe (Blur 8, Oncor) for the presence of human sequences. Six of these clones were found to be Alu^+. DNAs from the Alu^+ transfectants were then analyzed for the presence of human sequences homologous to v-*myv*, N-*myc*, v-*myc*, v-K-*ras*, N-*ras*, v-H-*ras*, c-*myc*, and v-*raf* or human papilloma-viral DNA (HPV 16 and HPV 18). Mouse cellular homologs of various oncogenes were identifiable in the clones; v-*raf* was the only oncogene to hybridize to human DNA. Human papilloma viral sequences were not seen.

To characterize further the nature of the transfected sequences, we used probes derived from a complementary DNA (cDNA) clone (3.05-kb insert) of c-*raf*-1 (*8*) that was isolated from an Okayama-Berg human fibroblast cDNA library (*9*). The following fragments of this clone were used as probes in all the subsequent hybridization analyses: (i) a 620-bp Pst I–Hind III fragment containing 180 bp of the 5′ untranslated region and 442 bp of the translated exons 2 to 4 of c-*raf*-1, and (ii) a 1.09-kb Ava II fragment representing the entire kinase domain of c-*raf*-1.

All the NIH/3T3 transfectant clones that were Alu^+ were also observed to contain human *raf* sequences (Fig. 1). No significant hybridization to the NIH/3T3 murine c-*raf* sequences could be detected under our hybridization conditions. Furthermore, restriction patterns or differences in the inten-

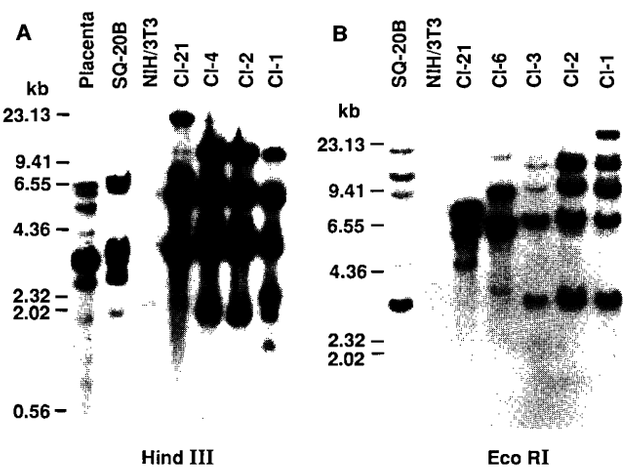

Fig. 1. Southern blot analysis of DNA isolated from various cell types (*19*). DNA samples (10 μg per lane) were digested to completion with Hind III (**A**) or Eco RI (**B**) and hybridized to a ^{32}P-labeled 1.09-kb Ava II fragment of c-*raf*-1 cDNA. Size markers are indicated on the left. DNA isolation, restriction endonuclease digestion, 0.8% agarose gel electrophoresis, Southern blotting onto nitrocellulose, and ^{32}P-labeling of probes were all performed as described (*17*). Hybridization conditions were 5× standard saline citrate (SSC), 1× Denhardt's solution, 50 m*M* sodium phosphate (*p*H 6.8), 0.1% SDS, 200 μg of sheared salmon testes DNA per milliliter of solution, and 10% dextran sulfate at 65°C. Washing was in 0.2× SSC at 65°C.

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