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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-

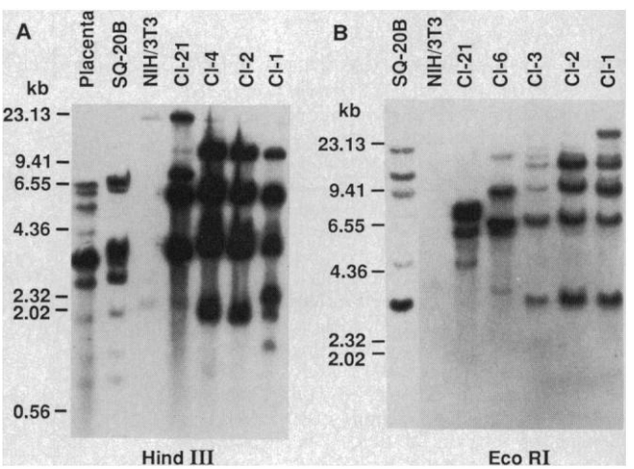

Fig. 1. Southern blot analysis of DNA isolated from various cell types (*19*). DNA samples (10 μg per lane) were digested to completion with Hind III (**A**) or Eco RI (**B**) and hybridized to a ^{32}P-labeled 1.09-kb Ava II fragment of c-*raf*-1 cDNA. Size markers are indicated on the left. DNA isolation, 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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sities of various bands (or both) confirmed that each NIH/3T3 clone was the result of an independent transfection event. None of the clones hybridized to probes that represented the Pst I–Hind III fragment.

There was also a conservation in all NIH/3T3 transfectants, except for clones 6 and 21, of the 8.4- and 3.0-kb Eco RI fragments attributable to the c-*raf*-1 locus of SQ-20B (Fig. 1B). These two bands represent almost all of the kinase domain (exons 11 through 17) of human c-*raf*-1 (*8*). Clone 6 lacked the 3.0-kb Eco RI fragment, and neither this fragment nor the 8.4-kb fragment were demonstrable in clone 21. None of the six clones revealed the 10.3-kb band, which represents exons 7 to 10 of c-*raf*-1. As would be expected if the 5′ recombinational event occurred near exon 10, Hind III–digested DNAs lacked the 7.5-kb fragment that would have represented exon 10 sequences (Fig. 1A). Eco RI, Hind III, Sst I, Bgl II, Eco RV, and Hpa I restriction maps of c-*raf*-1 sequences in normal human placental DNA and SQ-20B DNA were found to be similar as measured by Ava II and other fragments of c-*raf*-1 cDNA as probes (*10*). Therefore, we deduce that no grossly aberrant sequence changes had occurred within the c-*raf*-1 locus of the SQ-20B genome.

The transcriptional activity of the transfected *raf* sequences was subsequently investigated (Fig. 2). The Ava II cDNA fragment of c-*raf*-1 recognized a 3.5-kb transcript in SQ-20B and a 3.4-kb transcript in NIH/3T3 cells and in the clones, a mobility expected of normal human and murine c-*raf* transcripts, respectively. In addition, two new transcripts of 4.2 and 2.6 kb were observed in clones cl-1, cl-2, and cl-4. Only the 2.6-kb RNA band was found in cl-21 (*10*).

The above evidence linking the c-*raf*-1 protooncogene to a human laryngeal cancer may be further strengthened by the observation of a close relation between Eco RI restriction fragments, transcript sizes, and the extent of tumorigenicity of the original tumor and the transfected clones. Progressively growing tumors (1 to 3 cm) were observed as early as 10 days after inoculation of SQ-20B and cl-1, cl-2, cl-3, or cl-4 cells into athymic nude mice (*nu/nu*). The cl-6 was initially a slow-growing tumor (0.4 cm, day 10). Histopathologic sections of the SQ-20B–induced tumor in nude mouse identified it to be an invasive squamous cell carcinoma, whereas tumors formed by the individual clones were invasive sarcomas (*10*). No tumors were observed at 4 weeks in animals injected with either cl-21 cells or NIH/3T3 cells transfected with pSV2neo DNA alone.

The conservation of the 8.4-kb and 3.0-kb Eco RI fragments in tumorigenic clones (cl 1 through 4) and their absence, or alteration, in very poorly tumorigenic cl-21 strongly implied that these sequences obtained from the SQ-20B c-*raf*-1 protooncogene contain the necessary protein domains to effect NIH/3T3 transformation. Specifically, our data are consistent with the theory that the kinase domain represents a functionally significant region and removal of the amino-terminal ligand-binding region from the polypeptide results in significant "activation" of the c-*raf* protooncogene.

Giemsa-banding analysis of the karyotypes showed absence of normal autosome 3

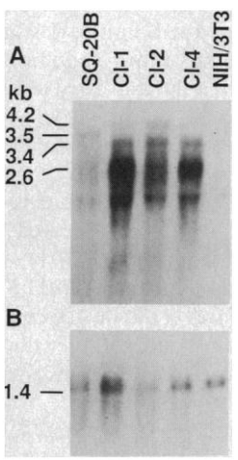

Fig. 2. Northern blot analysis of RNA isolated from SQ-20B and NIH/3T3 clones transfected with SQ-20B DNA. Hybridizations were performed with ^{32}P-labeled DNAs representing either a 1.09-kb Ava II–digested cDNA fragment of c-*raf*-1 (**A**) or GAPDH (**B**) obtained from clone pRGAPDH (*19, 20*), the gene for glyceraldehyde-phosphate dehydrogenase. Total cytoplasmic RNA was isolated by the guanidinium isothiocyanate extraction technique (*21*); 10 μg of each sample was separated by electrophoresis through a 1% agarose-formaldehyde gel, transferred to GeneScreen Plus (New England Nuclear), and hybridized in 1% SDS, 1.5*M* NaCl, 10% dextran sulfate, 50 m*M* tris HCl (*p*H 7.4), and 250 μg of denatured salmon sperm DNA per milliliter of solution, at 65°C. The hybridized blots were washed twice with 2× SSC at room temperature for 5 minutes, twice with 2× SSC containing 1% SDS at 60°C for 30 minutes, and twice with 0.1× SSC at room temperature for 30 minutes.

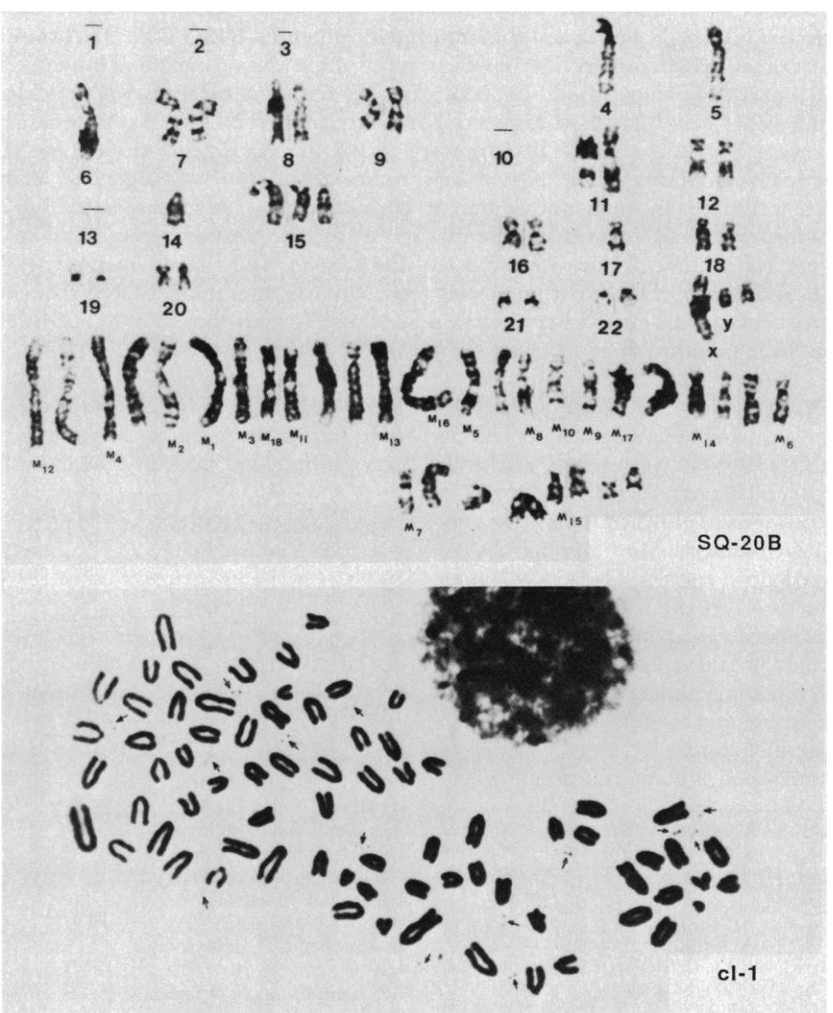

Fig. 3. Giemsa banding karyotype of human laryngeal carcinoma cell line (SQ-20B) and metaphase spread of NIH/3T3 cl-1. Arrows indicate double-minute chromosomes.

in all examined metaphases of SQ-20B. These chromosomes had apparently formed stable markers represented by M3, M11, M16, and M18 (Fig. 3). Human c-*raf*-1 has been localized on chromosome 3 [3p25 (*11*)]. Double-minute chromosomes representative of amplified DNA sequences (*12*) were found in NIH/3T3 cl-1 (Fig. 3) and may account for the amplified *raf* hybridization apparent in this and other transfected clones.

Similar transfection-mediated identifications of the c-*raf* protooncogene have been noted for DNAs obtained from carcinomas of the stomach (*3*) and liver (*5*), as well as from a glioblastoma (*4*). It seems likely that a truncated form of c-*raf* is responsible for transformation of transfected NIH/3T3 cells. In those instances where the recombinational limits can be approximated, removal of at least the first five or six exons has led to activation. Analysis of the predicted amino acid sequence of c-*raf*-1 (*8*) reveals that these exons encode domains that exhibit strong primary and secondary structural homology to the ligand-binding region of the protein kinase C family (*13, 14*). The region of highest homology encompasses the two cysteine "fingers" of protein kinase C and the related sequences of c-*raf*-1 (exons 3, 4, and 5). By analogy to protein kinase C, events associated with ligand binding may result in alterations in protein conformation so as to allow adenosine 5′-triphosphate (ATP) binding and subsequent phosphorylation mediated by the catalytic (kinase) domain. Thus, one might surmise that removal of regions capable of directly or indirectly blocking ATP binding may constitutively activate the kinase. The v-*raf* (*15*) and v-*mil* (*16*) fusion proteins would thus be examples of activated kinases, whose expression in infected cells is also unrestrained.

We believe the c-*raf*-1 product is related to the tumor phenotype for three reasons. First, the frequency of its identification by NIH/3T3 transfection of SQ-20B DNA suggests the presence of some genetic abnormality within the c-*raf*-1 locus, perhaps related to that described by Chang *et al.* (*17*). The nature of this abnormality remains to be elucidated. Second, since we have found that NIH/3T3 cells in which unrestrained normal c-*raf*-1 expression occurs are malignant in nude mice (*18*), similar unregulated expression in another competent cell may also contribute to its transformed phenotype. Finally, by means of the same approaches described in this report, two additional squamous cell carcinomas of head and neck origin (JSQ-3 and SCC-35) have yielded DNAs that produced NIH/3T3 foci in which truncated human c-*raf*-1 loci were frequently found. As with the SQ-20B–derived transformants, the majority of these transfectants were tumorigenic in athymic nude mice (*10*).

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A Stereospecific Cyclization Catalyzed by an Antibody

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A monoclonal antibody elicited by a transition-state analog that is representative of an intramolecular six-membered ring cyclization reaction acted as a stereospecific, enzyme-like catalyst for the appropriate substrate. Formation of a single enantiomer of a δ-lactone from the corresponding racemic δ-hydroxyester was accelerated by the antibody by about a factor of 170, which permitted isolation of the lactone in an enantiomeric excess of about 94 percent. This finding demonstrates the feasibility of catalytic-antibody generation for chemical transformations that require stereochemical control.

RECENT EXPERIMENTS HAVE BEGUN to exploit the repertoire of receptor molecules that can be generated by the immune system for chemical transformations through strategies designed to elicit antibodies capable of selective chemical catalysis (*1, 2*). Since antibodies can be produced against virtually any antigen with precise structural specificity (*3*), the possibility exists for a general catalytic method for the synthesis and functionalization of mole-

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Table 1. Kinetic parameters for the cyclization of ester **1** by monoclonal antibody 24B11. Velocities were determined spectrophotometrically by measuring the initial linear absorbance change at 271 nm. The antibody (2 μ*M*, from Lowry assay, with a molecular weight of 150,000 for immunoglobulin G) was first incubated at 25°C in 100 m*M* KCl in 25 m*M* phosphate buffer at *p*H 7.0. Reactions were initiated by addition of varying amounts of a stock solution of ester **1** to give a substrate concentration of 20 to 100 μ*M*. Ester **1** was obtained by deprotection of the trimethylsilyl derivative with 5% citric acid in methanol followed by dilution with 25 m*M* phosphate buffer, *p*H 7.0, to give the desired concentration of stock solution. The concentrate was stored frozen at −80°C until immediately prior to use. The first-order rate constant (k_{uncat}) for the cyclization in the absence of antibody was measured similarly and used to correct the initial rate data, which were used to obtain kinetic parameters from the Lineweaver-Burk plots shown in Fig. 4.

K_M* (μ*M*)	K_i (μ*M*)	V_{max} (μ*M* min^{-1})	k_{cat} (minute^{-1})	k_{uncat} (minute^{-1})	k_{cat}/k_{uncat}
76	0.25	0.99	0.50	0.003	167

*In the absence of binding by the unreactive enantiomer, $K_M \approx 38$ μ*M* for the reactive substrate.